Cytopathic Effects of Aurones in a Mouse Cell Line L929 Cells

By

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ABSTRACT

Cancer has been one of the major concerns in healthcare. The disease is most lethal when it becomes metastatic, Fibroblast aids in this process through microenvironment formation. The extracellular matrix around the cancer site is remodeled, enhancing cancer progression. Therefore, there has been research ongoing to find compounds with medicinal implications by targeting the fibroblast and restraining cancer site progression. A potential compound is aurones, that are naturally found in the pigmentation of yellow rocket snapdragon flowers. Due to the versatility of aurones for many treatments, the compound has been chemically modified and screened on TNF-induced L929 cells for their effects on cytotoxicity. A cell viability test was used to gather data on the effect of aurones and the ANOVA and posthoc Dunnett test the following three groups were categorized: death inhibitory, death enhancing, and neutral. The aurones 1 & 2 were death inhibitory, while 4, 5, & 8 were found to be neutral, and the rest had death-enhancing effects. Based on the categories further studies will be done on the specific pathway affected by the aurones.

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CHAPTER I: INTRODUCTION

Cancer is the leading cause of death globally, with nearly 10 million deaths in 2020 alone, as reported by the World Health Organization (Cancer). Cancer cells lose control over gene regulation, leading to uncontrolled cell growth that becomes invasive and can be fatal, if not treated promptly. The most significant challenge with cancer is the uncertainty of its origin. It is a combination of both innate and acquired health problems. The causes of cancer can be attributed to genetic mutations, exposure to unknown carcinogenic factors, an unhealthy lifestyle, or environmental stress affecting gene regulation. Unfortunately, treatment options for cancer are often associated with severe side effects. Treatment typically involves surgery or radiation therapy to remove cancerous tissue and chemotherapy after surgery. However, the high toxicity of these chemotherapy drugs on humans, non-specificity, and the development of resistance over time against it, limit their effectiveness, particularly in treating advanced stages of cancer.

After much research molecular target therapy have shown promising results for future applications. The types of treatments on which researchers are currently working include target therapy for growth factors, signaling molecules, hormone receptors, gene expression regulators, modulators of apoptosis, cell-cycle protein, and immunotherapies (Lee et al., 2018). As part of these therapies, researchers aim to extract naturally occurring compounds and organically modify or biologically build multiple replicates of those compounds to use later to artificially regulate certain cell processes to halt the cancer growth within the patient. The researcher finds a potential process that needs to be artificially regulated for treatment; the researcher then tries to develop a compound that affects any molecule in the biological cascade. Then transporting that compound near the

chosen molecule, a chemical bond forms that affects the function of that molecule leading to a stop of the biochemical process causing cancer. The goal of this therapy is low toxicity, high accuracy, fewer side effects, and lower treatment costs (Alsayari, 2019).

One recent milestone in molecular target therapy is the discovery of aurones [2-benzylidenebenzofuran-3(2H)-ones] (Figure 2) (Zwergel et al. 2012). Aurones possess a simple structure, they are stable and naturally occurring compounds. Research on this compound has shown its importance for anti-inflammatory (Hassan et al. 2014), anti-microbial (Jardosh and Patel. 2017), anti-fungal (Alqahtani et al. 2019), anti-malarial (Carrasco et al. 2014), anti-Alzheimer (Li et al. 2017), and importantly anti-cancer properties. For this project, modified derivatives of the aurones found naturally in yellow rocket snapdragon flowers were used (Asen et al. 1972). The major goal was to evaluate and determine the most bioactive aurones on murine fibroblast cells L929 cytotoxicity, following the treatment with tumor necrosis factor-alpha (TNF-α) to trigger cell death pathways. The effectiveness of the aurones was screened to determine any inhibitory or enhancing effect on the TNF-alpha-induced pathway in L929 cells. Previous research has shown that fibroblast cells are vital in the microenvironment formation of cancers, which was well explained by the seed and soil hypothesis of researcher Stephen Paget (Maia and Wiemann. 2021). Using this microenvironment cancer cells communicate with fibroblasts and other cancer cells using extracellular signaling to make them secrete the cytokine dosage for destroying the extracellular matrix (ECM) and then this co-existing fibroblast uses that space to rebuild ECM compatible with the cancer cells allowing growth of the tumor; as fibroblasts are responsible for secreting and modeling the ECM components like collagen, fibronectins, hyaluronic acid, etc. (Maia and Wiemann. 2021).

Therefore, the aim was to evaluate the 11 aurones synthesized in the Dr. Scott Handy laboratory at the Middle Tennessee State University Chemistry Department. Our analysis focused on how these aurones affect murine fibroblast cells in the TNF-alpha-induced cell death pathway. Then the data was used to classify death inhibitory, death enhancing, and not interacting aurones on TNF-induced signaling pathways (Figure 1). During the whole screening process, the Presto Blue cell viability assay (ThermoFisher) was used (Table 1).

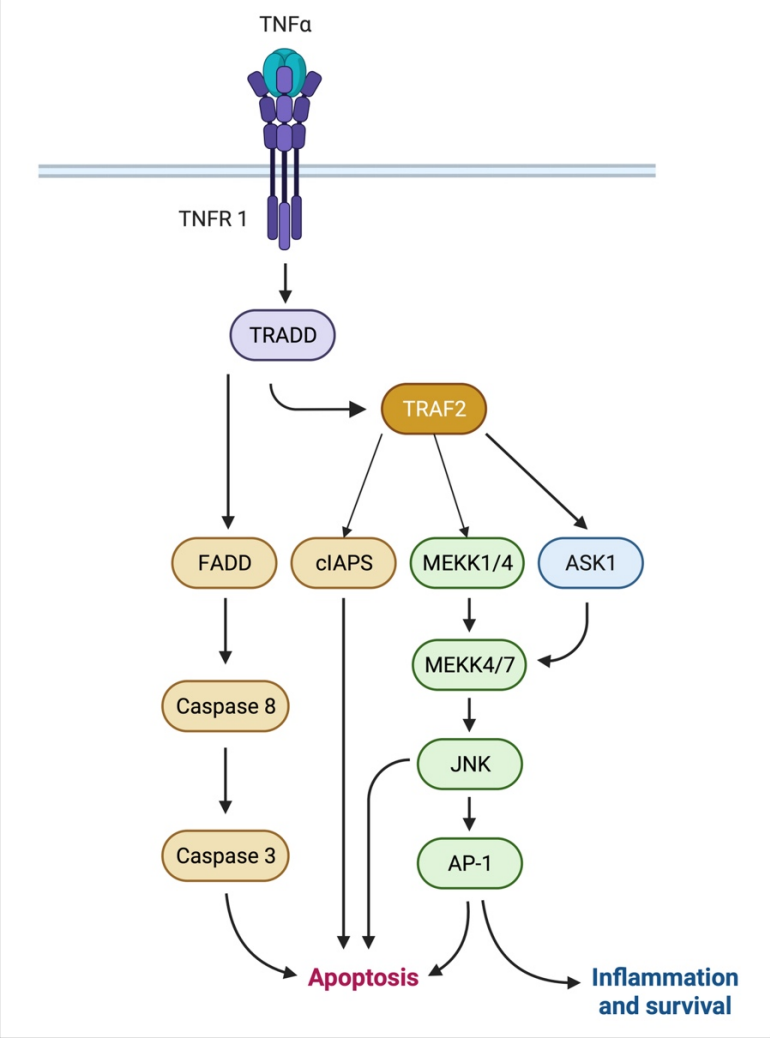


Figure 1: Signaling pathway of Tumor Necrosis Factor-alpha binding to Tumor Necrosis Factor Receptor 1.

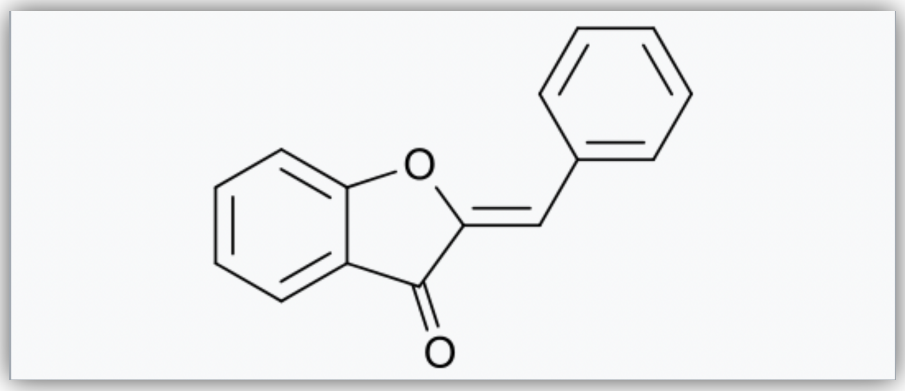
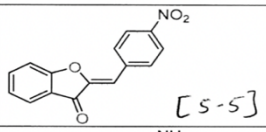
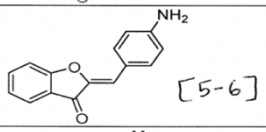
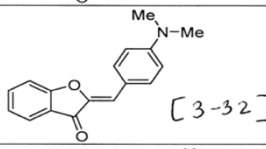
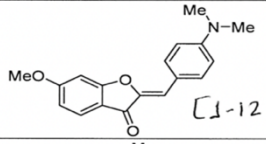
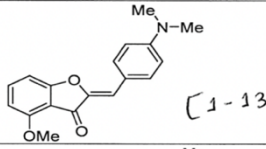
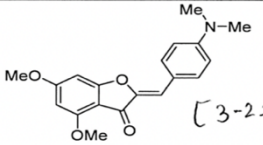
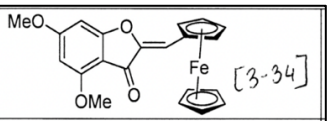
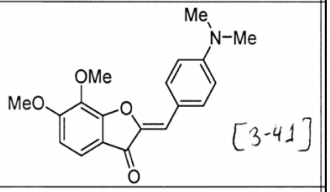
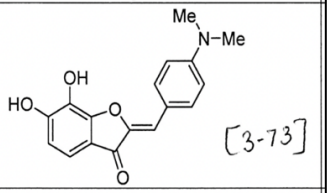
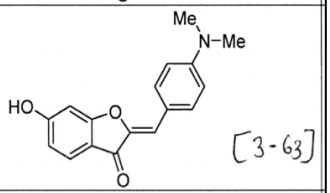
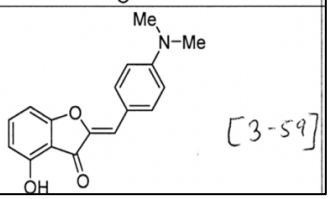


Figure 2: Chemical Structure of an Aurone (Mazziotti et al. 2021*)*.

Table 1: The 11 novel aurones being tested in this project. They are synthesized in the laboratory of Dr. Scott Handy in the Middle Tennessee State University Chemistry Department.

No.	Structure of compounds
1	 [5-5]
2	 [5-6]
3	 [3-32]
4	 [1-126]
5	 [1-132]
6	 [3-21]
7	 [3-34]
8	 [3-41]
9	 [3-73]
10	 [3-63]
11	 [3-59]

CHAPTER II: MATERIALS AND METHODS

2.1 Cell Culture

L929 murine fibroblast were cultured in Dulbecco's Modified Eagle's medium (DMEM) (ThermoFisher), containing 4.5 g/L glucose, L- glutamine, sodium pyruvate, 5% fetal bovine serum (FBS), and 1% penicillin-streptomycin (ThermoFisher)**.** For optimal growth and consistency purposes, the cells were placed in an incubator with 5% CO_2 and 37°C humid environment. Every 3-5 days the cells were passaged by applying trypsin (ThermoFisher) with one-fifth the volume of the cell flask to dislocate the cell from the bottom of the flask. Then one-tenth of the culture volume is transferred into a new flask containing fresh medium.

2.2 Treatments and Cell Viability Assay

During the testing, consistency of process, technique, and time points were maintained to minimize human error or bias in the results. Additionally, each test with aurone treatments was replicated three independent times with three technical replicates (N=9) in each plate which can be visualized in Figure 3. Tissue cultures were seeded in a plate by following the same process as passaging, except the cells were precisely plated in 96-well plates. After dislodging, the cells were then counted on a hemocytometer using 0.4% trypan blue as a cell viability stain. After counting, the cells were diluted in the medium until there were 1×10^5 cells in 100 uL per well in the 96-well plate. Following seeding, the cells were incubated for 16 hours before the treatment phase.

On day 2, the medium in the wells was discarded and the wells were washed twice with phosphate-buffered saline (ThermoFisher) before the addition of treatments. Treatments consist of co-treatment of 100 uM aurone in DMEM with 5pg/mL of TNF-

alpha, except for the three controls: TNF-alpha control, DMSO control, and medium-only wells. TNF-alpha control wells consisted of 5pg/mL TNF-alpha in DMEM. The DMSO control well consisted of 0.1% DMSO in DMEM. Lastly, the untreated well only contained DMEM. Following the treatment, the plate was incubated for another 24 hours before conducting the cell viability assay.

On day 3, the steps were repeated as in the treatment protocol and 100 uL of Presto Blue (ThermoFisher) was added in every well, including three additional empty wells with no cells to collect blank absorbance of the Presto Blue. After the addition of the Presto Blue, the plate was incubated for 3 h before collecting the absorbance data using the BMG Labtech spectrophotometer and MARS software. The Presto Blue reagent is a resazurin-based compound, during the incubation period, in a metabolically active cell the resazurin will be reduced in cytosol-producing resorufin. Due to the change in the compound, the Presto molecule excitation wavelength is 570nm and the emission wavelength is 600nm, these were used to quantify the data visually seen in the wells using the above-mentioned instrument and software.

To determine the co-treatment concentration of TNF-alpha, the three-day long test was done in triplicate with the dose curve as seen in Figures 4 & 5, and 5pg/mL concentration was determined to be used for all aurone screening. Lastly, for the DMSO control, 0.1% was determined based on the 1:1000 dilution from 100,000 uM stock to 100 uM working concentration.

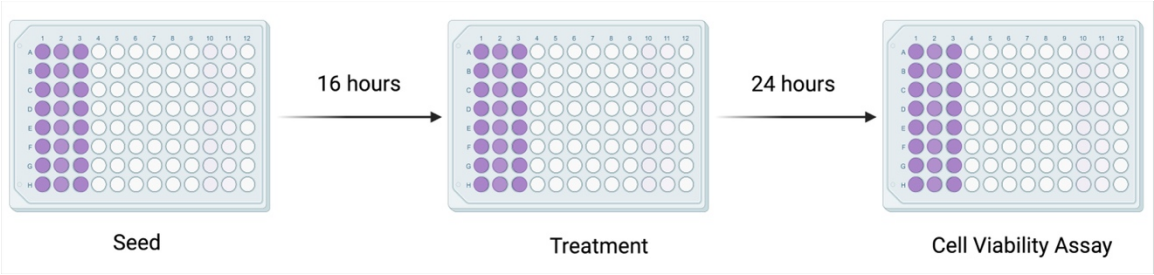


*Figure 3:*The three steps in conducting all the tissue culture tests. The three columns are technical replicates of each other in one test, and three individual test replicates will be performed a total of n=9 for aurone screening. Figure created using BioRender.com.

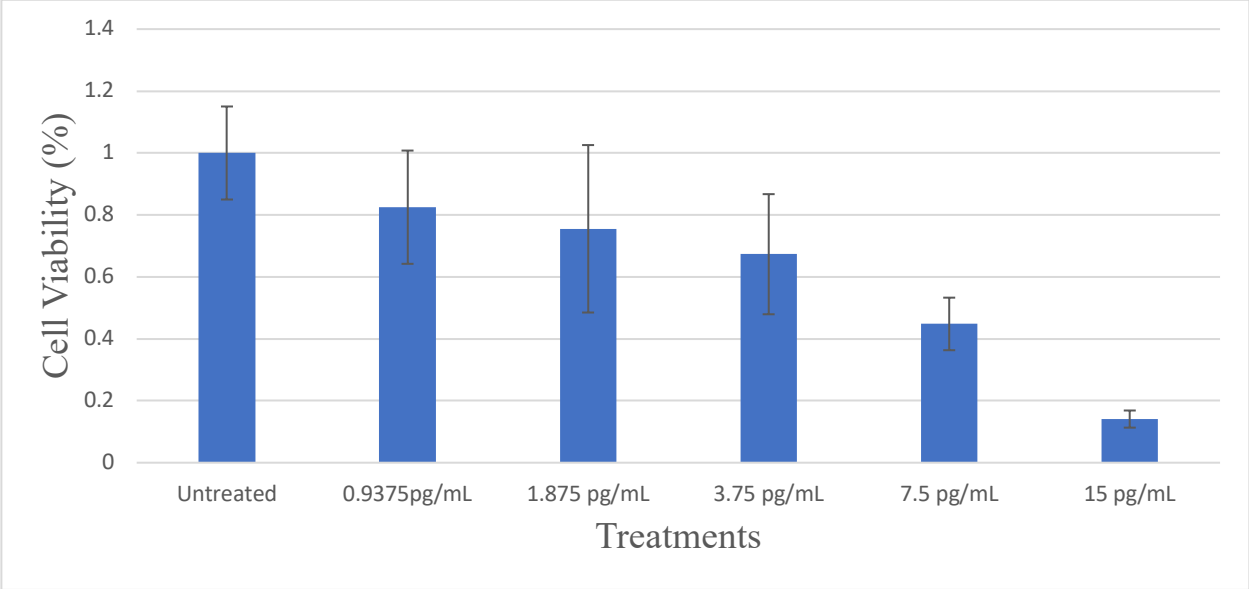


Figure 4: Data for TNF-alpha control testing for L929 cells at 1x105 cells/mL density. Cells were seeded, treated, and tested in the 30-h process depicted in Figure 2, except for any aurone treatments. The standard error is due to three technical replicates for each concentration and the bar is the average of all replicates.

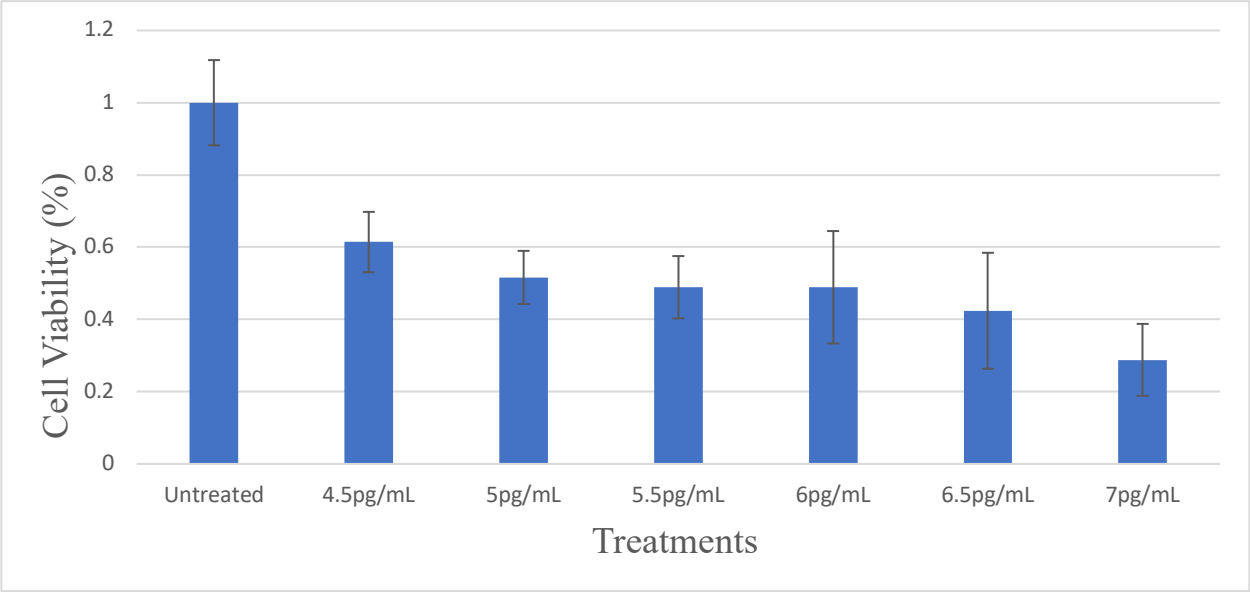


Figure 5: Data for TNF-alpha control testing for L929 cells at 1x105 cells/mL density. Cells were seeded, treated, and tested in the 30-hour process mentioned above, except for any aurone treatments. The standard error is due to three technical replicates for each concentration and the bar is the average of all replicates.

2.3 Mathematical analysis

There were three levels of statistical tests conducted on the collected data. First, a basic visual representation of the MARS data was made in Microsoft Excel Software by normalizing all the treatments and controls to the untreated wells to see the proportion of cells viable after each treatment.

Secondly, a one-way analysis of variance (ANOVA) was run on the data to determine if there was any significance within the data. ANOVA is a test that compares the group means within different groups to determine the significant difference in the aurones treatments. To conduct the ANOVA test, our data did satisfy all four assumptions of ANOVA: same sample size, normality of data sets, sample independence, and variance equality. Lastly, after finding differences in ANOVA, a post hoc Dunnett test was run on the data to statistically determine and categorize the significant aurones.

CHAPTER III: RESULTS

3.1 Absorbance spectrum normalized to Untreated

Initial screening of the 11 Aurones is shown in Figures 6, 7, and 8. Each figure is an independent test with three technical replicates, all tests equaling N=9. Each test followed the same trend for each aurone, but it was difficult to make any conclusion based on this normalized graph as the standard error between some of the aurone's technical replicates was high, which required the use of advanced statistical tests: ANOVA and Dunnett Test.

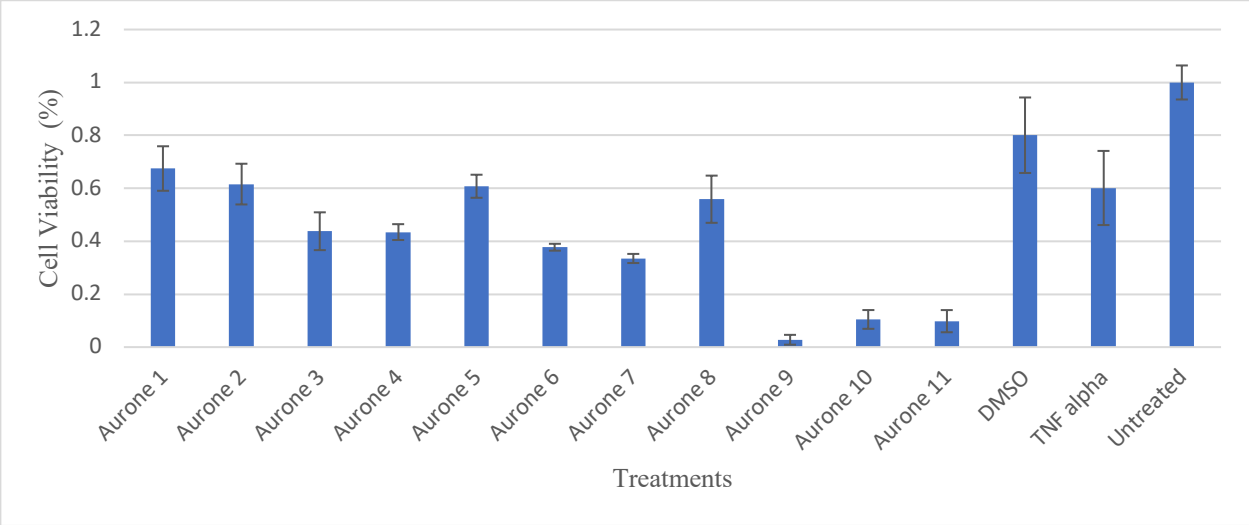


*Figure 6:*Cell Viability 24 h post-treatment and results are normalized to the untreated wells. All aurones were co-treated at 100 uM with 5 pg/mL TNF-a concentration, while DMSO control was at 0.1% concentration.

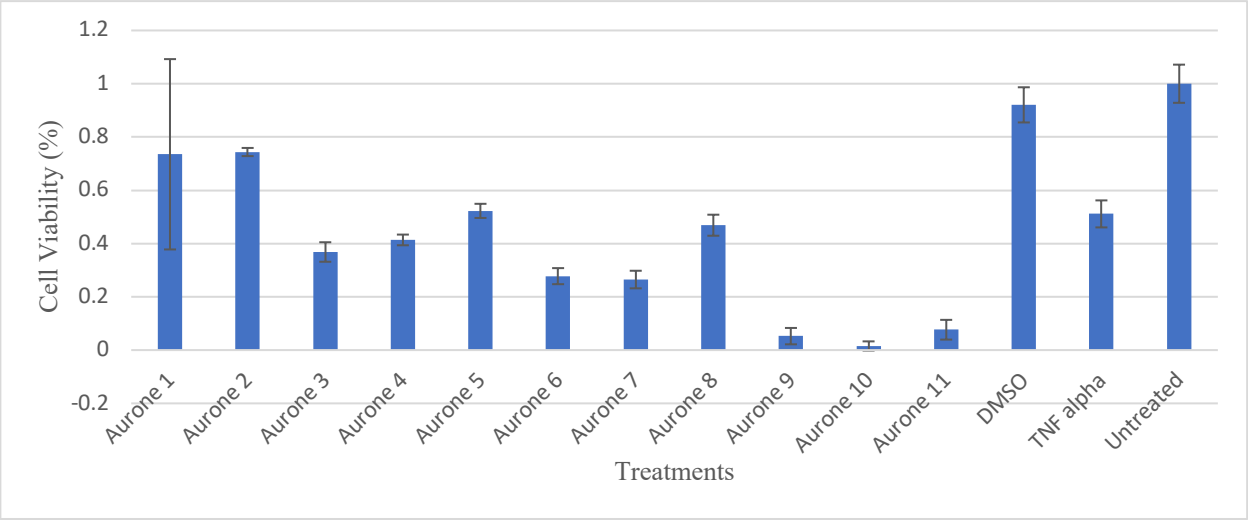


*Figure 7:*Cell Viability 24 h post-treatment and results are normalized to the untreated wells. All aurones were co-treated at 100 uM with 5 pg/mL TNF-a concentration, while DMSO control was at 0.1% concentration.

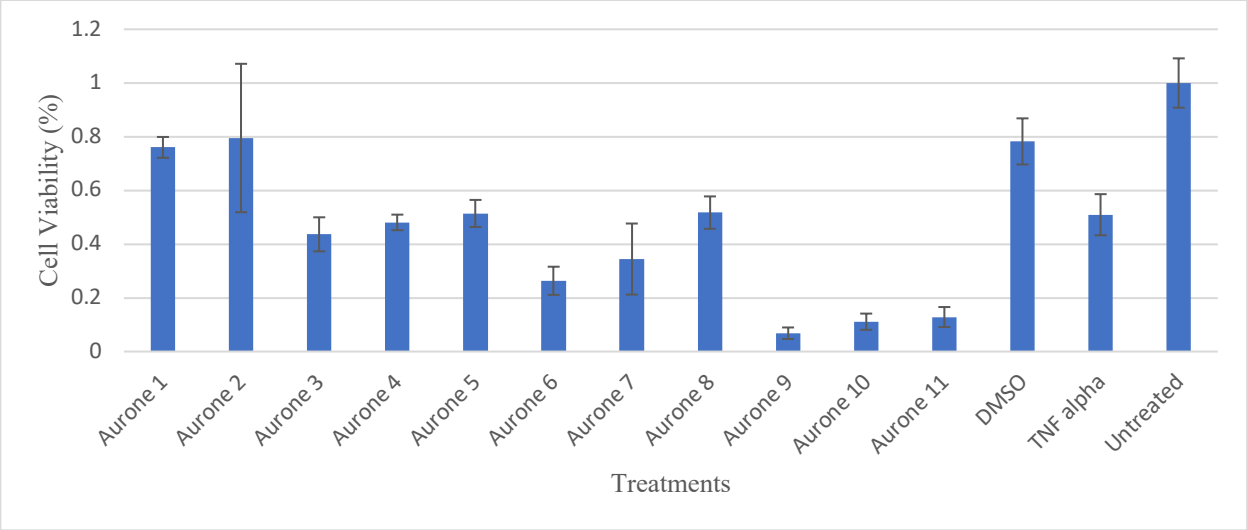


*Figure 8:*Cell Viability 24 h post-treatment and results are normalized to the untreated wells. All aurones were co-treated at 100 uM with 5 pg/mL TNF-a concentration, while DMSO control was at 0.1% concentration.

3.2 ANOVA and Posthoc Dunnett test

In Table 2, the ANOVA calculations revealed that there is a significant difference between group means. Therefore, with the assistance of the software Minitab, the Dunnett test was concluded as the most accurate statistical tool due to its ability to independently

compare each group with the TNF-alpha control group. Table 3, compares each group of treatment with the control mean, eventually grouping the insignificant aurones with the control treatment. Table 4 takes the analysis further by numerically showing the difference values between means and computationally forcing the overall confidence value to be at 95% confidence with individual confidence levels at 99.32% for the individual aurone significance compared to TNF-alpha control.

Finally, for better visualization Figures 9 & 10 are produced using the software Minitab, illustrating that aurones 4, 5, and 8 are insignificant due to no difference, while aurones 3, 6, 7, 10, and 11 cause enhanced death. The only aurones inhibiting death are Aurones 1, and 2.

Table 2: ANOVA test for determination of any significance within Data.

Analysis of Variance

Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Factor	10	4.1797	0.417972	46.30	0.000
Error	88	0.7945	0.009028		
Total	98	4.9742			

Table 3: Dunnett Multiple Comparisons with the TNF-alpha control.

Grouping Information Using the Dunnett Method and 95% Confidence

Factor	N	Mean	Grouping
TNFalpha (control)	9	0.5407	A
Aurone 1	9	0.7234	
Aurone 2	9	0.7183	
Aurone 5	9	0.5483	A
Aurone 8	9	0.5150	A
Aurone 4	9	0.4431	A
Aurone 3	9	0.4142	
Aurone 7	9	0.3148	
Aurone 6	9	0.3062	
Aurone 11	9	0.1010	
Aurone 10	9	0.0771	

Means not labeled with the letter A is significantly different from the control level mean.

Table 4: Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
Aurone 1 - TNFalpha	0.1827	0.0448	(0.0585, 0.3069)	4.08	0.001
Aurone 2 - TNFalpha	0.1776	0.0448	(0.0533, 0.3018)	3.96	0.001
Aurone 3 - TNFalpha	-0.1265	0.0448	(-0.2507, -0.0022)	-2.82	0.044
Aurone 4 - TNFalpha	-0.0977	0.0448	(-0.2219, 0.0266)	-2.18	0.195
Aurone 5 - TNFalpha	0.0075	0.0448	(-0.1167, 0.1318)	0.17	1.000
Aurone 6 - TNFalpha	-0.2345	0.0448	(-0.3588, -0.1103)	-5.24	0.000
Aurone 7 - TNFalpha	-0.2260	0.0448	(-0.3502, -0.1017)	-5.04	0.000
Aurone 8 - TNFalpha	-0.0257	0.0448	(-0.1499, 0.0986)	-0.57	0.998
Aurone 10 - TNFalpha	-0.4636	0.0448	(-0.5879, -0.3394)	-10.35	0.000
Aurone 11 - TNFalpha	-0.4397	0.0448	(-0.5639, -0.3154)	-9.82	0.000

Individual confidence level = 99.32%

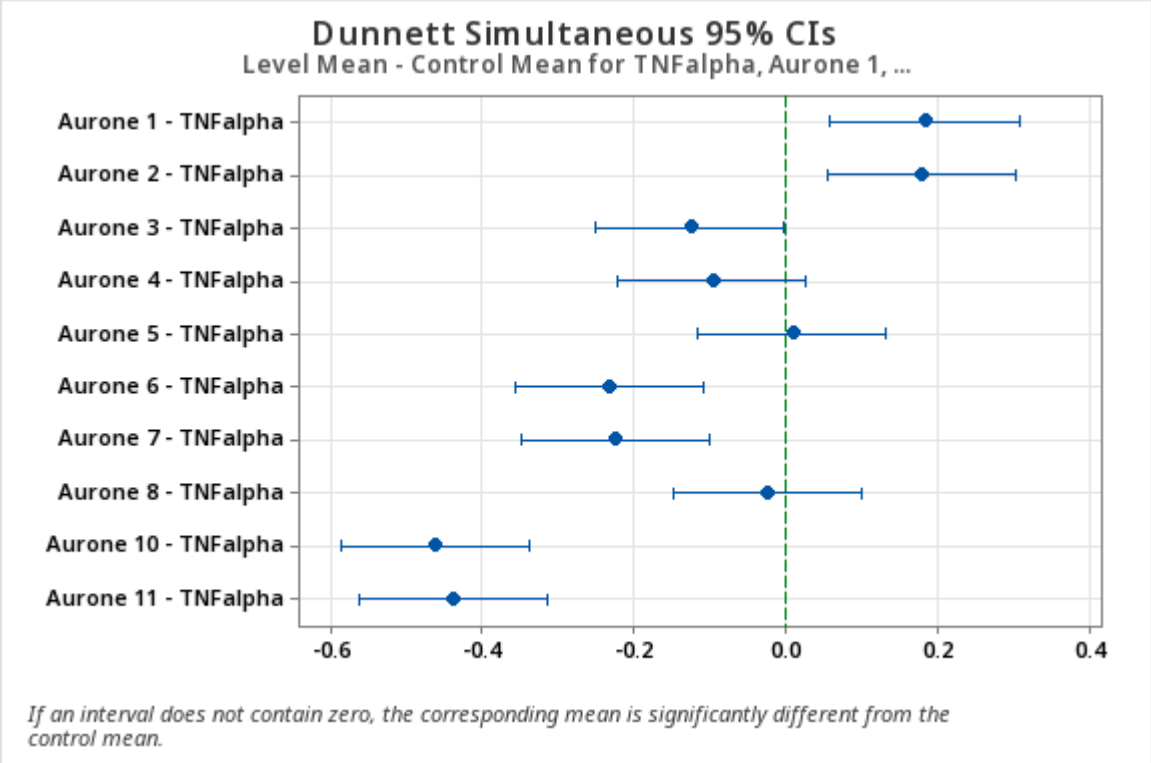


Figure 9: Confidence interval drawn proportional to the mean of the TNF-alpha control.

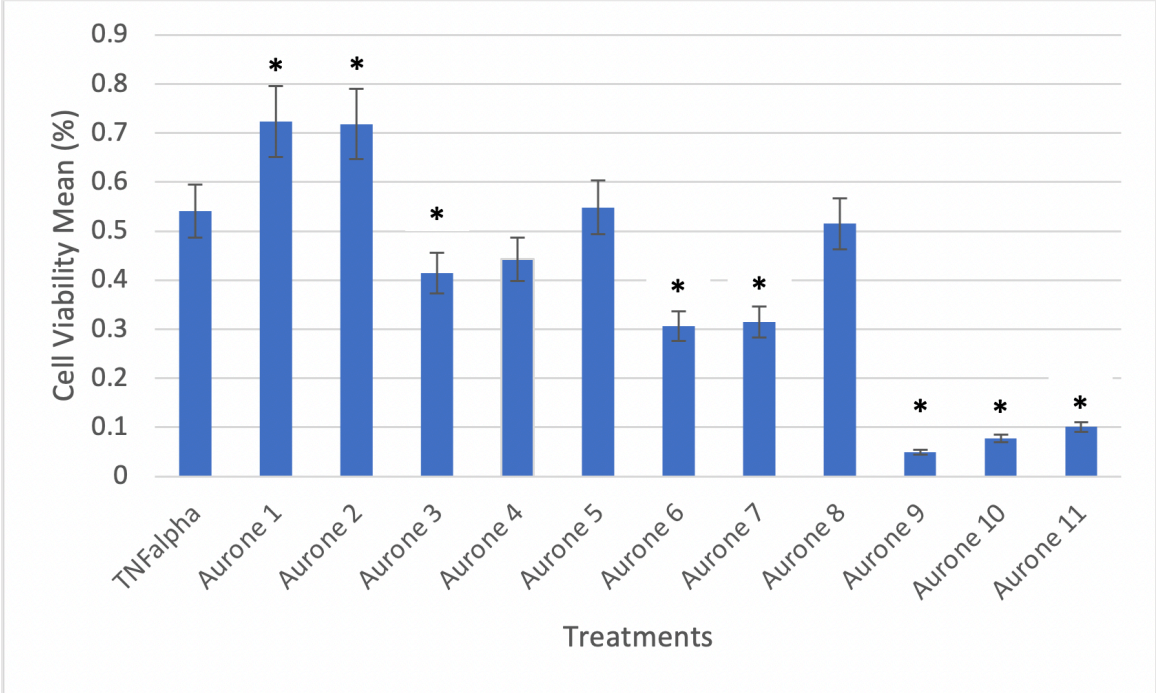


Figure 10: A bar graph indicating the significant aurones with an asterisk mark.

CHAPTER IV: DISCUSSION

Through this study, the experimentation and statistical analysis of the aurone screening revealed the three groups of aurones initially expected from this project. From the Dunnett simultaneous test on the viability assay results, there was overall 95% confidence in the grouping with an individual analysis confidence level of each aurone with TNF-alpha at 99.32%. The death inhibitory group contains, aurones 1 and 2. While the aurones 3, 6, 7, 9, 10, and 11 have a synergistic effect in the TNF-alpha-induced cell death pathway, grouping them into death-enhancing groups. Lastly, the aurones 4, 5, and 8 have a neutral effect on the TNF-alpha pathway, making them insignificant for future studies.

However, during the statistical analysis, aurone 3 was difficult to group in neutral or death enhancing due to statistical test restriction. As can be noticed in Figure 9, aurone 9 was not included in the Dunnett simultaneous test due to its variance inequality from the rest of the data points. Therefore, when the test ran including aurone 9, aurone 3 was grouped with neutral. Additionally, when different non-parametric post-hoc Games-Howell tests were used for analysis aurone 3 was also grouped into neutral effects. However, when tested concerning all the assumptions for the parametric test, aurone 3 was unequivocal in the death-enhancing group, due to this aurone 3 can’t be confidently placed in either group.

For future testing, the significantly active aurones interaction will be analyzed with known cell components in the TNF-alpha pathway. Using the chemical analysis software, the attached R functional groups can be matched with active sites for proteins within the TNF-alpha pathway to determine aurones' involvement at specific positions

throughout the pathway. Previous studies have done similar analyses for other aurones and have concluded some possible options such as the donation of hydrogen from the R functional group of aurones and changing protein role due to the protonation (Mazziotti et al. 2021). After donation aurones may also act as reactive oxygen species (ROS) within cells affecting the functioning of specific pathways, and eventually leading to the death of cells (Mazziotti et al. 2021). This type of activity may also be found in the future from analysis of our aurones 9, 10, and 11; as these aurones were also very cytotoxic which might be due to the hydroxyl functional group on the benzene ring of the benzofuran part of the aurone.

Another trend analyzed in the previous studies is focused on the methoxy functional group of the aurones. The study stated that some genetic enzymes like topoisomerase are sensitive to certain positioning of methoxy functional groups on the benzene rings of the benzofuran part of the aurones (Priyadarshani et al. 2016). Therefore, further studies on aurones 6, and 7 shed light on the reason aurones 4 & 5 are neutral while 6 & 7 were significantly toxic in L929 cells.

Lastly, the two novel aurones with no previous research findings are the aurones 1 & 2. For these aurone functional groups, there hasn't been many studies or implication in tumor cells, therefore currently there is no data on these aurones, and can only be further understood from literature and future studies.

Overall, the previous trends for the significantly active aurones will make further studies more guided for the aurones 1 & 2. In conclusion, after conducting more testing there could be a potential lead for aurones-based treatments from this screen.

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