Impact of Synthesized Aurones on Adipocyte Differentiation

by
Destiny Donald

A thesis presented to the Honors College of Middle Tennessee State University in partial fulfillment of the requirements for graduation from the University Honors College

Spring 2017

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Destiny Donald

APPROVED:

_____
Dr. William Stewart
Department of Biology

_____
Dr. Lynn Boyd
Department of Biology

_____
Dr. John Dubois
Department of Biology
Honors Council Representative

_____
Dr. John Vile
Dean, University Honors College

Abstract

The ability of thirty-one aurones to initiate adipogenesis were tested by the Oil Red O staining method. This was done to examine these compounds as a possible precursor to future drug therapies for type 2 diabetes mellitus. Of that group, five aurones showed potential for further testing. These five aurones underwent a glycerol assay to examine their ability to inhibit lipolysis and a Western Blot Analysis to observe their ability to enhance adiponectin expression and secretion. After analysis and comparison, five aurones were found to be candidates for further testing and possibilities in future medicinal developments for type 2 diabetes mellitus.

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Introduction

From 2010 to 2012, the number of Americans who were estimated to have diabetes rose from 25.8 million to 29.1 million people (American Diabetes 2016). The CDC found that of the 29.1 million, 28.9 million were adults and about 90-95% of the diagnosed cases were type 2 diabetes mellitus (CDC 2014). This would mean that, in 2012, it is probable that 26 million to 27.5 million adults were thought to suffer from type 2 diabetes mellitus, or T2DM. This is not only alarming because diabetes is seventh on the list of leading causes of death in the United States; it is also concerning because diabetes has been linked to increased risk in acquiring other causes of death located in the top ten, including the number one cause, heart disease (CDC 2017). Also, per the CDC, in 2012, it was recognized that those with diabetes consisted of about 9.3% of the American population, with that prevalence rising to 25.9% for those sixty-five years or older (American Diabetes 2016). The CDC's report also estimated that in 2012 eighty-six million Americans twenty-years-old or older were prediabetic (American Diabetes 2016). Analyzing these statistics, the widespread reach of this problem becomes conspicuous and daunting. To combat the effects of diabetes on the ever-growing percentage of the American population, there is a constant push for better treatment and preventative methods.

There are two forms of diabetes mellitus: type 1 and type 2. Type 1 diabetes mellitus results from a limited or nonexistent production of insulin release from pancreatic beta cells and is typically associated with an autoimmune disorder, and it typically occurs in youth (National Institute 2016). In a healthy individual, insulin is released by beta cells in response to high blood sugar levels following a meal; it operates

to decrease heightened sugar levels to normal blood values. Type 2 diabetes mellitus is characterized by a defect in insulin receptor function resulting in elevated insulin levels without the compensatory reduction in blood sugar levels. The pathologically elevated levels of insulin are greatly correlated to accelerated insulin resistance.

In a study conducted by Dr. FHJ van Tienen, in patients previously diagnosed with T2DM, it was found that "genes involved in insulin signaling and lipid metabolism were downregulated and inflammation/apoptosis was upregulated" in the preadipoctyes (Tienen, et al. 2011). This supports a pathology involving an insufficient insulin signaling pathway. Lipid droplets are essential to the physiology of the mature fat cell, and a functioning fat cell characteristically contains a large amount of lipid. Lipid droplets act as a storage unit for triglycerides. In times of insufficient caloric intake, the triglycerides are broken down into glycerol and fatty acids, which are used as energy substrates. An additional function of fat cells is the secretion of a protein called adiponectin. Adiponectin is a protein hormone that is secreted by mature fat cells. It has been shown to function as an insulin sensitizer and an "anti-atherosclerotic hormone" (Balasubramanyam). In patients with T2DM, adiponectin secretion has been shown to be minimal. This is one of the factors in the development of secondary complications due to T2DM, such as heart disease and atherosclerosis.

Another correlation found in the study by Dr. FHJ van Tienen is the gene expression for necessary components of transcription pertaining to adipogenesis, as well as other aspects of the division of preadipocytes, were also not as prevalent in patients with T2DM (Tienen, et al. 2011). These occurrences indicate an overall decreased differentiation of preadipocytes into mature adipocytes. As with all cells, the process of

differentiation is essential to their function and viability, according to one of the core statements seen in Cell Theory. Therefore, the data of this study show that T2DM affects multiple core functions of mature fat cells.

When insulin resistance is fully realized within cells, it hampers the cell's ability to translocate a glucose receptor type termed GLUT4 to its surface for glucose uptake from the blood. GLUT4 is the glucose transporter protein that is insulin-sensitive and has been found to be the primary means of glucose uptake in muscle, hepatic, and fat cells (Konstantopoulos, Molero-Navajas 2009). During these conditions, instead of travelling to the cell's surface, GLUT4 receptors remain in GLUT4 storage vesicles (Konstantopoulos, Molero-Navajas 2009). This occurs because, without insulin, a signaling cascade that involves SNARE proteins and Rab proteins is not activated (Konstantopoulos, Molero-Navajas 2009). SNARE proteins are proteins that are involved in the merging of membranes between vesicles found within a cell and the plasma membrane of a cell (Dunman, Forte 2003). Rab proteins regulate numerous transport functions within a cell (Hutagalung, Novick 2011). Because they are the main mode of regulation for so many pathways, their dysfunction has also been linked to many other diseases including cancer and various neurological diseases, i.e. Huntington's Disease and Parkinson's Disease (Hutagalung, Novick 2011). For a better picture of the impact of each of these protein categories on diabetes, it is necessary to discuss each of these proteins in greater detail.

Individual proteins within each of these categories, such as: Rab proteins: 8A, 10, 13, and 14 (Ishikura, Bilan, Klip 2007; Bhatnager, et al. 2011) and SNARE proteins: syntaxin-1A and syntaxin-4 (Chen et al., 2012) (Sun, et al. 2010), have been observed to

play possible roles in initiating and sustaining diabetes. For example, in a study focusing on the functionality of Rab and SNARE proteins within a dysfunctional lacrimal gland in a diabetic mouse model, it was found that the expression of syntaxin 1, a SNARE protein, and Rab proteins 27b and 3d were decreased, and there was a notable reduction in the release of secretory vesicles (Dias 2015). In more recent studies, Rab proteins were found to contribute to lipid droplet accumulation and lipolysis regulation, which are major problems for those with diabetes (Li, Yu 2016). Syntaxin 1 has also been found to affect the release of adiponectin though its required binding to Munc18c, a SNARE protein regulator (Jewell, et al. 2011). Despite each of these protein types having their own independent role, dysfunctions manifested in these proteins compound to contribute to the symptomology of T2DM.

As stated before, at the macroscopic level, the prevalence of type 2 diabetes mellitus is a large concern in the United States, as well as in other developed nations. This issue also has an upward trend for an even more astounding influence on the overall health of the population. To combat this disease, there is a renewed interest in treatment with botanical medicine. Botanical medicine is the use of "a plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes" (Univ. of Maryland 2015). While the use of plants and plant extracts as a means of combating disease is not a new concept by any means, modern techniques of discerning the effectiveness of plant extracts based on their overall dimensional structure is a relatively recent approach to the subject. This method applies the knowledge of their dimensional makeup and how they may react with certain chemicals or proteins utilized within the body. The combination of molecular knowledge with traditional techniques has proven to be useful in the field of development of new

medicines and medicinal treatment. In 2015, the Nobel Prize in Physiology or Medicine was awarded to a researcher by the name of Youyou Tu for her research into the use of botanicals against Malaria (Nobel 2015). Her discoveries led to a decrease in the mortality rate from Malaria "by more than 20% overall and by more than 30% in children" (Nobel 2015). Today, her contribution saves around 100,000 individuals in the continent of Africa every year (Nobel 2015). Her work, as well as the work of others, show and consistently proves that botanicals have the potential for widespread application.

Flavonoids are one of the most important families of compounds that have been found in botanical extracts. They are a part of a group called biophenols, which are compounds that are derived from plants. Biophenol research was initiated with the discovery "that dietary antioxidants can prolong the lifespan of terminally ill mice" by Denham Harman in the 1950s (Obied 2013). Flavonoids are a family of biological compounds that have been shown to be useful when applied to a number of vastly different diseases. Within this family, there are over 5000 known flavonoids (Oregon State c2017). Flavonoids have a group characteristic of a benzo-γ-pyrone structure (Kumar, Pandey 2013). The flavonoid family is subdivided into many different classes. Within the flavonoid family, a prolific class, known as flavones, has been shown to be a particularly important and potent source of medicines for human health. The flavones have provided a broad spectrum, from UV-B radiation (Schmitz-Hoerner, Weissenbock 2003) to leukemia (Vargo, et al. 2006), of medicines for the treatment of a wide range of medical conditions.

Flavone research is immensely active in the field of diabetic research. Flavones such as Cirsium japonicum, (Liao, et al. 2012) Artepellin C. (Sun-Sil, et al., 2011), and Puerarin (Ok-Hwan 2010) have all been found to be effective in the treatment of diabetes through restoring adipogenesis. Experimental results showed that these compounds displayed anti-diabetic effects through altering adipocyte function at multiple levels. Cirsium japonicum affected the adipocytes through stimulating differentiation within the cells, as well as, glucose uptake by activating peroxisome proliferator-activated receptor gamma (γ) (Liao, et al. 2012). Peroxisome proliferator-activated receptor γ, or PPARγ, is a transcription factor that has been observed to be one of the first responders during the upregulation of gene expression that occurs during adipogenesis (Siersbæk, et al.). Cirsium japonicum also alters the signaling pathway that is utilized by insulin (Liao, et al. 2012). Cirsium japonicum, Artipellin C, and Puerarin each have been shown to cause an increase in the expression of PPARγ, adiponectin, and GLUT4 (Liao, et al. 2012; Lee; Sun-Sil, et al., 2011)). Thiazolidinediones, or TZDs, are a group of compounds that have been found to be some of the most powerful regulators of insulin signaling, and they have been used therapeutically to treat T2DM due to their ability to reduce insulin resistance. Their only known action is potent activation of PPARγ (Hauner 2002). Although once considered a "blockbuster" drug, TZD usage for T2DM has been greatly reduced because of a liver toxicity issue. The commonalities amongst the TZDs and these flavones follow the notion that a successful compound for the treatment of T2DM is one that will increase GLUT4 activity and adiponectin secretion, while also suggesting that PPARγ will have to be activated.

These flavones are only some of the many flavonoid compounds used in medicine. The universality of flavones is being increasingly recognized in terms of versatility and helpfulness in treating human ailments. The flavones purified from botanical extracts contain a rare subclass of isomeric compounds called aurones (Boumendjel 2014). Limited research into aurones has shown potential therapeutic use for anti-viral and anti-cancer, among other things. Minimal exploration of the role of aurones in diabetes has been conducted.

For this study, aurones were investigated for their ability to initiate adipogenesis in preadipocytes. Additionally, the impact of various aurones on mature adipocytes was studied. As already stated, aurones belong to a family of flavonoids, specifically a subclass called flavones (Boumendjel 2014). Aurones -like most botanical extracts- are naturally occurring and are found in yellow-pigmented flowers, such as snapdragons, and other plants such as mosses, and ferns (Eiichiro, et al. 2006). They can also be created synthetically. Aurones are also known as (Z)-2-benzylidene-benzofuran-3(2h)-ones and due to them being some of the least common flavonoids, they did not begin to be thoroughly explored until more recent years (Boumendjel 2014).

Of the studies done, the results have shown that aurones may represent a promising class of therapeutically active drugs. Studies that examined their effectiveness against the influenza virus were limited to the aurones: sulfuretin, 2-((*E*)-4′-hydroxyphenylidene)-6-hydroxy-2,3-dihydrobenzofuran-3-one, and 2-((*E*)-4′-hydroxyphenylidene)-4,6-dihydroxy-2,3-dihydrobenzofuran-3-one (Liu, Wang 2008). These aurones were proven to partially inhibit the function of the enzyme neuraminidase, which allows for viral mobility from the host cell (Liu, Wang 2008). In a study that

involved Hepatitis C, forty-two different aurones were tested with seven found to be effective against the virus (Haudecoeur, et al. 2011). The treatment resulted in the inhibition of RNA polymerase, which is important for viral replication. Another study assessed the effectiveness of aurones on breast cancer cells and found them to be proficient against ATP-binding cassette transports found in breast cancer cells (Sim 2008). ATP-binding cassette transports cause issues in these cells by suppressing the movement of cytotoxic drugs into the cells and preventing cell apoptosis. These limited studies show the versatility and effectiveness of aurones. This provides the evidence that further studies into aurones on disease need to be conducted. This project investigates the ability of aurones to regulate adipogenesis and the physiology of mature fat cells in hopes of providing an avenue for the formation of a new class of anti-diabetic drugs.

For the experiments of this study, the aurones that were used were not direct extracts from nature. Instead synthesized aurones were used that had been garnered through a coordination with Dr. Scott T. Handy at Middle Tennessee State University. According to one of his methods, to manufacture the aurones, he combines a solvent, also a catalyst for the reaction, that contains choline chloride and urea with two well-known reactants for aurone synthesis-coumaranone and an aldehyde (Handy 2015). The ability to synthesize aurones allows for a much wider plethora of these compounds than would otherwise be afforded to us due to their rarity in nature. Discovery of new ways to create aurones has been one of the stimuli in the increased interest in their capabilities as drug therapies.

The regulation of adipogenesis has become an area of intense focus with T2DM researchers. There is a strong correlation between blood glucose control and a modest

weight gain in patients that have started taking medicine to treat their T2DM. The current hypothesis states that T2DM develops when an individual's fat pads can no longer expand, that ability depends mostly on genetic factors. It is also thought that fat pads are not able to enlarge due to a lack of differentiation in preadipocytes. Some of the most common medications used to treat T2DM appear to work through initiating differentiation of preadipocytes into mature fat cells. These compounds simultaneously stimulate the insulin pathway, which allows for glucose uptake within these fat cells and causes the patient to acquire more fat and therefore gain weight. In fact, some of the most effective type 2 diabetes mellitus drugs are known to induce adipocyte differentiation in vitro (Garcia-Escobar 2011). Currently, one of the methodologies for developing new drugs for the treatment for T2DM is to find compounds that induce differentiation of preadipocytes into functioning mature fat cells.

During the preliminary stages, these compounds are screened by being run through various tests to assess their effectiveness in triggering adipogenesis. In this experimentation, this approach was utilized. The effect of synthesized aurones on preadipocytes was examined by seeing how the application of these compounds affected adipogenesis through scrutinizing the levels of lipid production, lipolysis, and adiponectin release in treated cells. These characteristics were assessed because lipid accumulation, basal lipolysis, and the production and release of adiponectin are hallmark characteristics of normally functioning mature adipocytes.

In patients with T2DM, basal lipolysis levels in mature fat cells are significantly elevated when compared to normally functioning fat cells in non-diabetics. Lipolysis is a cellular process that breaks down triglycerides and allows for the release of free fatty

acids into the blood stream as a means of providing energy for cells. Insulin is a known inhibitor of this process, so when insulin resistance occurs during T2DM, lipolysis loses a major regulatory control. This leads to rapid release of free fatty acids into the blood and exacerbates insulin resistance. The lipid dysfunction associated with T2DM is a major risk factor for the development of atherosclerosis, leading to cardiovascular disease, which is the number one cause of morbidity and mortality in the United States. In fact, a study has found that patients with diabetes and metabolic syndrome are more prone to lipid accumulation in the heart, an occurrence that is rare in the general population (Marfella 2009). Thus, any compound that will adequately treat T2DM will most likely lower elevated levels of lipolysis in T2DM patients and will, in turn, lower the risk of other complications from the disease.

In this study, a goal was to examine the differentiation potential of 3T3-L1 preadipocytes following treatment with synthetized aurones, which are similar to naturally-occurring members of the flavonoid family. It is important to note that the 3T3-L1 fibroblast cell is highly utilized in adipocyte and diabetes research. This cell line was isolated by Dr. Howard Green and Dr. George Todaro (Green, Todaro 1963). These cells are derived from mice and have the capacity to undergo adipogenesis in culture in a way that is similar to differentiation in an intact organism. Though their potential was first recognized decades before now, their ability to replicate the functionality of human adipocytes have made them invaluable to the scientific community, and they continue to be used to this day. The extent of their differentiation was visually examined by noting the confluence and overall appearance of the adipocytes using the Oil Red O method. This method stained for the lipids (triglycerides) present within the adipocytes, and they

were then compared to the control cells that had been stained but were non-treated. Lipid accumulation, as seen by the Oil Red O method, allowed non-effective compounds to be eliminated from the proceeding experimentation and focus on compounds that induced adipogenesis. The compounds that activated the adipogenesis cascade were further tested to determine their effect on mature fat cell function. Mature fat cells were treated with these aurones to determine if they impacted lipolysis and adiponectin production and secretion.

The hypotheses of this study are that there are aurones, both natural and synthesized, that will induce differentiation in the 3T3-L1 preadipocytes, and the aurones that are capable of inducing differentiation are also capable of reducing lipolysis in the mature fat cell, while increasing the production and secretion of adiponection. To test these hypotheses, lipid accumulation levels, glycerol levels, and adiponectin secretion levels of the aurones were analyzed. Aurones that meet these criteria would likely be excellent candidates for further future drug development research.

Methods and Materials

Oil Red-O staining, BCA Assay, lipolysis, and Western Blot Analysis was utilized to determine the extent of adipocyte differentiation and function. The cell line used for these experiments was the murine 3T3-L1 preadipocyte strain. Preadipocytes were cultured in (100 mL) dishes containing in a maintenance medium composed of Dulbecco's Modified Eagle's Medium (DMEM) with pen/strep which were purchased from Sigma Aldrich and 10% bovine calf serum which was purchased through Thermo-HyClone. The cells were cultured in a 5% carbon dioxide environment at 37°C. Preadipocytes were kept under these conditions until 100% confluent. The date of confluency was noted, and forty-eight hours later, the cells were induced to differentiate into mature fat cells.

To induce differentiation, cells were treated with around 40 milliliters of an MDI cocktail. This cocktail includes IBMX, 400 microliters of 3-isobutyl-1-methylxanthine (MIX), 40 microliters of dexamethasone (DEX), and 400 microliters of insulin as well as 36 milliliters of DMEM with pen/strep and 4 milliliters of 10% fetal calf serum. MIX, DEX, and insulin (MDI) were all purchased from Sigma-Aldrich while fetal calf serum was purchased from Thermo-Hyclone. MDI was prepared fresh and the remainder discarded after each use. After a period of 48 hours to 72 hours, the cells were then fed with a quarter-insulin medium with 45 milliliters of DMEM with pen/strep, 5 milliliters of 10% bovine calf serum, and one-fourth of the amount of insulin contained in the initial differentiation media. Forty-eight hours to seventy-two hours later, cells were refed with the same quarter insulin media. Then, forty-eight hours after the second treatment with quarter-insulin media, the cells were fed at the same amounts with every forty-eight hours

with the normal maintenance medium. To test their ability to induce differentiation, aurones were added during MDI treatment phase at doses 0.1 mM, 0.2 mM, and 0.4 mM. Cells were also treated with an induction cocktail without aurones to serve as a control.

Differentiation from preadipocytes to adipocytes was determined by measuring lipid accumulation; this was assessed using Oil Red-O staining. Cells were treated with 31 different aurones initially and assessed visually. Successful aurones underwent this method again and were examined more closely. For the Oil Red-O analysis portion, the initial step was removal of the medium surrounding the mono-layer of cells. These cells were then washed with phosphate-buffer saline. Next, the cells were fixed with of a solution of 10% formaldehyde in PBS, or phosphate-buffered saline. Following fixation, formaldehyde was removed by being rinsed three times with distilled water. Following rinsing, any excess water was removed by blotting. The cells were then incubated in Oil Red O working solution for one hour. The Oil Red O working solution was prepared by mixing stock solution with purified water at a ratio of 6:4. This solution was then allowed to sit for 30 minutes and then filtered prior to use. The Oil Red O stock solution contained a mixture of 0.3 g of Oil Red O powder in 100 ml of 99% isopropyl alcohol. This was maintained at room temperature. After the one hour incubation, the Oil Red-O staining solution was aspirated off. Excess stain was then removed by rinsing with deionized water.

Colorimetric assay was then used to quantify Oil Red O staining. One milliliter of ethanol was added to each dish to extract the Oil Red O. The absorption of this solution was measured at a wavelength of 490 nanometers. The absorption of the treated cells was then compared to untreated, control cells. Treatments that resulted in an increase in

absorption correlated with an increase in lipid accumulation and indicated differentiation from the preadipocyte to the mature adipocyte. Aurones that were observed to have a positive impact on differentiation were then added to the newly differentiated cells to test for their impact on adipocyte function. Adiponectin expression and secretion and lipolysis activity were measured as a determinant of mature adipocyte function.

Adiponectin expression and secretion was determined by Western Blot Analysis. Cells were induced to differentiate. Seven days post-induction, cells were treated with Aurones 03, 10, 16, 17, and 20 for 96 hours, and samples were harvested. Doses of aurones were administered at 0.1 mM, 0.2 mM, and 0.4mM. Following treatment, one milliliter of medium from each dish was prepared for each treatment. To this sample, 150 microliters of IP (immunoprecipitation) buffer with protease inhibitors was added. The IP buffer contained Igepal CA-630, 10 mM Tris, 1 μM of phenylmethylsulfonyl fluoride, 10 μM of leupeptin, 50 trypsin inhibitory milliunits of aprotinin, 150 mM of NaCl, 2 mM of sodium vanadate, 1mM EGTA, 1 mM EDTA, and 1 μM of pepstatin. These samples remained on ice for 30 minutes succeeded by centrifuging at 15000 rpm done at 4°C. Following centrifugation, supernatant was saved and the pellet was discarded. Whole cell extracts were prepared by first removing the remaining medium and rinsing the dish in phosphate buffer saline. One hundred and fifty μL of the IP buffer with protease inhibitors were added to each dish. The cells were scraped in this buffer and added to a 1.5 mL centrifuge tube. The harvested cells were next homogenized by passing them through a syringe with a 22-gage needle five times. These samples remained on ice for 30 minutes succeeded by centrifuging at 15000 rpm done at 4°C. Supernatants containing the whole cell extracts were saved and the pellet was discarded. The protein

concentration for media samples and whole cell extract samples was determined using a BCA kit from Pierce as per their instructions.

Proteins from each treatment were separated by gel electrophoresis on a 10% polyacrylamide gel. Gel electrophoresis was conducted by method of Laemmli. For the media samples, 75 micrograms were added to each lane. For the whole cell extracts, 50 micrograms were added to each lane. Following protein separation, the protein bands on the gels were then transferred onto nitrocellulose membranes utilizing a 25 mM Tris, 192 mM glycine, and 20% methanol transfer buffer. Following transfer, the membranes were placed in 4% milk and 10% tween blocking solution. The transfers ran overnight in a cold room. Following blocking, the blots were washed twice for fifteen minutes in phosphate buffer containing tween-20. Blots were then incubated with primary anti-adiponectin antibody for 90 minutes. Blots were washed twice with washing solution and incubated with a secondary antibody conjugated to horseradish peroxidase for 90 minutes. Visualization of the protein bands containing adiponectin was enhanced by chemiluminescence. The antibodies that were used for this experiment were purchased from Abcam. All chemiluminescense kits were purchased from Thermo Scientific.

In addition to looking at expression and secretion of adiponectin as a marker of mature adipocyte function, the breakdown of triglycerides, lipolysis, was also analyzed. The lipolysis assay used is a colorometric assay that measures liberated glycerol produced from the breakdown of triglycerides during lipolysis. This assay was executed in accordance with the specifications of Sigma Aldrich. For the lipolysis assay, the culture medium was removed from the cells, and cells were washed in phosphate buffer saline (PBS) to remove extracellular lipids. Cells were then put in a serum-free

incubation medium, which consisted of 1% Bovine Serum Albumin (BSA) and 20 mM HEPES suspended in glucose-free DMEM containing pyruvate and glutamine. After the cells were put in this incubation media, the aurones were added. The aurone treatments were administered at a concentration of 25 nM, 50 nM, and 100 nM. All treatments were conducted in triplet. Two sets of controls were used during these experiments. Untreated cells served as a negative control. Ten mM isoproterenol was included as a positive control and was used to compare normal and treated cells with amplified lipolytic activity. Cells were incubated at both three-hour and six-hour time intervals and the activity was reported. The cells were incubated for six hours. Twenty-five mM of each mixture were transferred to the wells of a 96 well plate. Controls were untreated.

For the glycerol assay, a glycerol standard curve was produced by doing a serial dilution of the glycerol stock to produce standard concentrations from 260 μg per microliter to 1.625 μg/μL. For each standard concentration and for the treatment and control, 25 μL of incubation solution were added to a well of a 96 well plate in triplicate. One 40 mL bottle of free glycerol reagent was added to 40 ml of distilled water for later use in the assay. Two hundred μL of free glycerol reagent was added to each well. Free Glycerol Reagent is "used for quantitative enzymatic determination of glycerol" (Sigma-Aldrich c2017). The assay was conducted at room temperature for fifteen minutes. Following incubation, the absorption for each well was measured at 540 nm. The absorption values for the treated and control samples were compared to the standard curve to determine the concentration of glycerol.

Results

Oil Red O Results

Thirty-one aurones were investigated for their ability to induce differentiation of preadipocytes into adipocytes. Of the thirty-one aurones, five aurones induces preadipocyte differentiation due to their ability to stimulate lipid accumulation, which was determined by Oil Red O staining. The aurones that were of interest, Aurones 03, 10, 16, 17, and 20, see table 1, were further studied. A dose response utilizing these five aurones was conducted. The doses used for each of the aurones were 0.25 mM, 0.50 mM, and 1.0 mM. There was a dose dependent increase in Oil Red O staining with each of the five aurones tested. Normalizing the results to percent of its vehicle-control, Aurone 03 produced the best results of all aurones with its highest and middle doses (Figure 1). The highest dose, 1.0 mM, resulted in 334% increase in Oil Red O staining when compared to control, while the middle dose, 0.50 mM resulted in a 265% increase (Figure 1). The last dose for Aurone 03, 0.25 mM, resulted in a 167% increase (Figure 1). Aurone 10 held the best result of all aurones for its initial dose of 0.25 mM (Figure 2). The initial dose demonstrated a 180% increase from its vehicle, and its middle and highest dose showed a 214% increase and 252% increase, respectively (Figure 2). For Aurones 16, 17, and 20, there were similar results overall. Aurone 16 had 115% for the first dose (0.2 mM), 165% for the second dose (0.5 mM), and 259% for the third in the series of doses (1.0 mM) (Figure 3). For Aurone 17, it had a 135% increase for the first dose (0.2 mM), 186% for the second dose (0.5 mM), and 231% for the third dose (1.0 mM) (Figure 4). The last of this group, Aurone 20, showed results of: 108% for the initial dose, 163% for the second dose, and 200% for the final treatment dose (Figures 5). An untreated control was

compared to vehicles were dissolved in DMSO, and the differences were found to be negligible (Figure 6). Raw data and graphs of this experiment can be located in Table 2 and Figures 11, 12, 13, 14, 15, and 6 in the Appendix. Of the thirty-one aurones examined, these five aurones were potent inducers of adipocyte differentiation. The next series of experiments examined the ability of these five aurones to impact mature fat cell function.

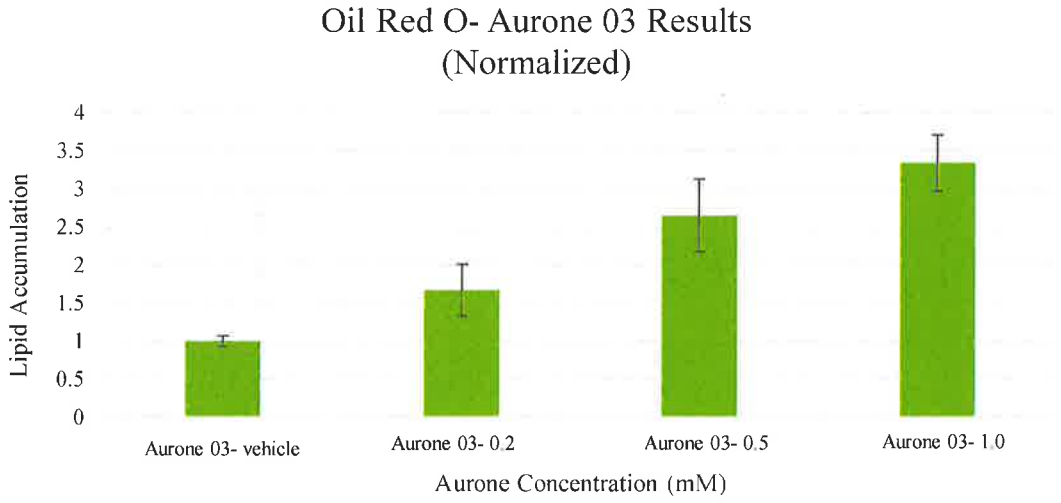

Figure 1: Aurone 03 at doses 0.2 mM, 0.5 mM, and 1.0 mM on plates of adipocytes. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. Absorption of this solution measured at a wavelength of 490 nanometers.

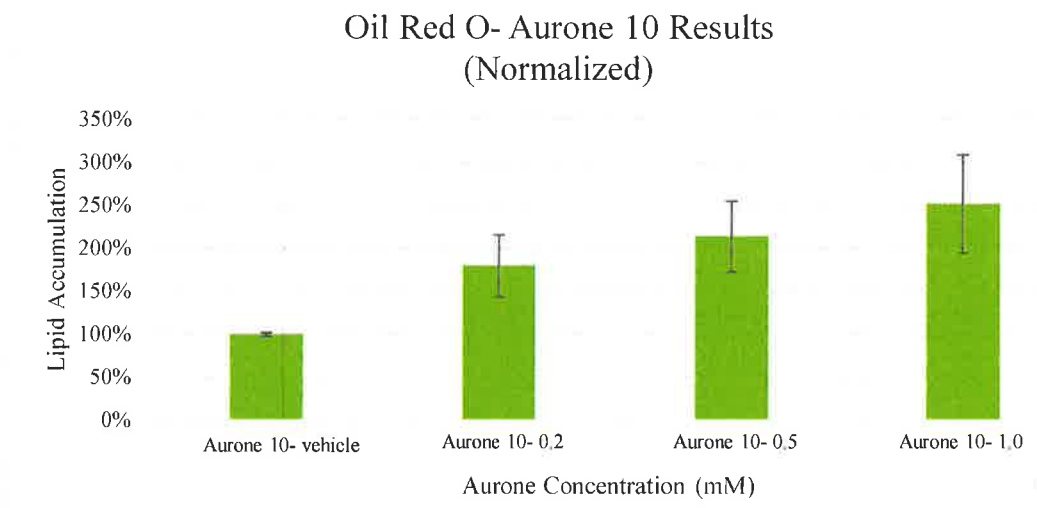

Figure 2: Aurone 10 at doses 0.2 mM, 0.5 mM, and 1.0 mM on plates of adipocytes. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. The results were normalized against the control. Absorption of this solution measured at a wavelength of 490 nanometers.

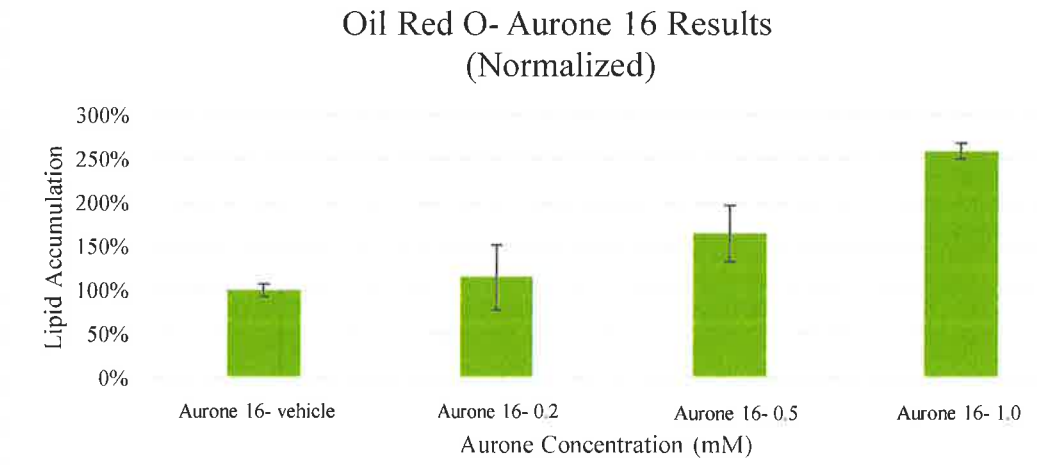

Figure 3: Aurone 16 at doses 0.2 mM, 0.5 mM, and 1.0 mM on plates of adipocytes. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. The results were normalized against the control. Absorption of this solution measured at a wavelength of 490 nanometers.

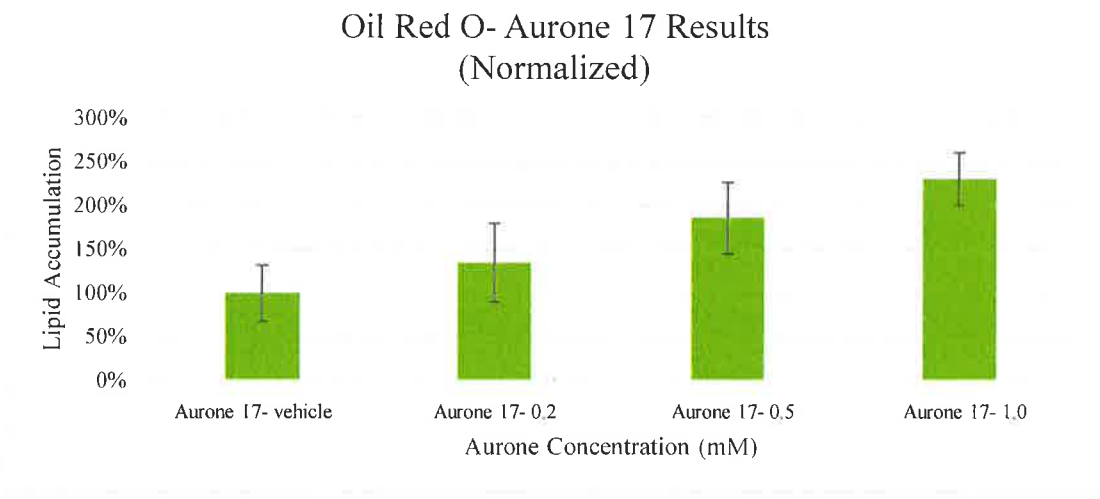

Figure 4: Aurone 17 at doses 0.2 mM, 0.5 mM, and 1.0 mM on plates of adipocytes. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. The results were normalized against the control. Absorption of this solution measured at a wavelength of 490 nanometers.

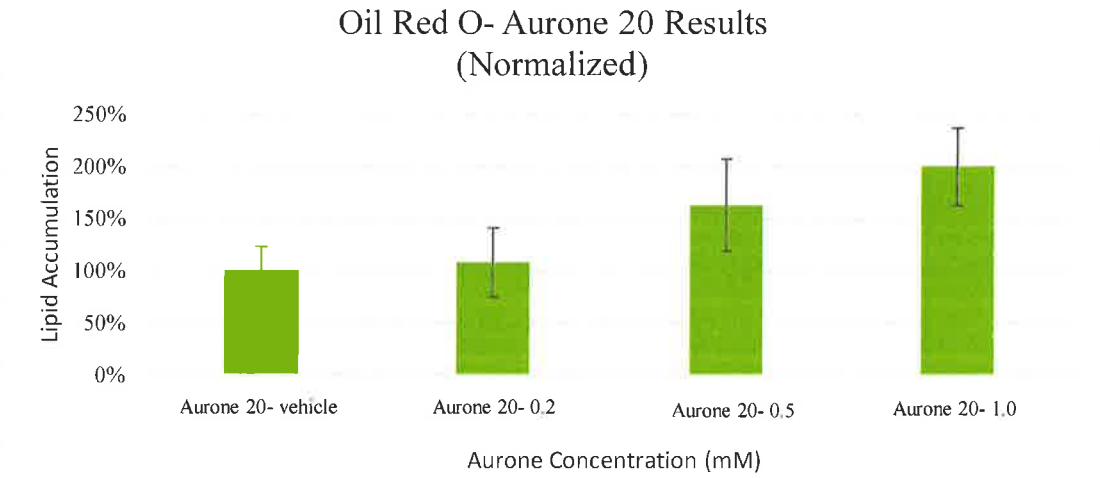

Figure 5: Aurone 20 at doses 0.2 mM, 0.5 mM, and 1.0 mM on plates of adipocytes. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. The results were normalized against the control. Absorption of this solution measured at a wavelength of 490 nanometers.

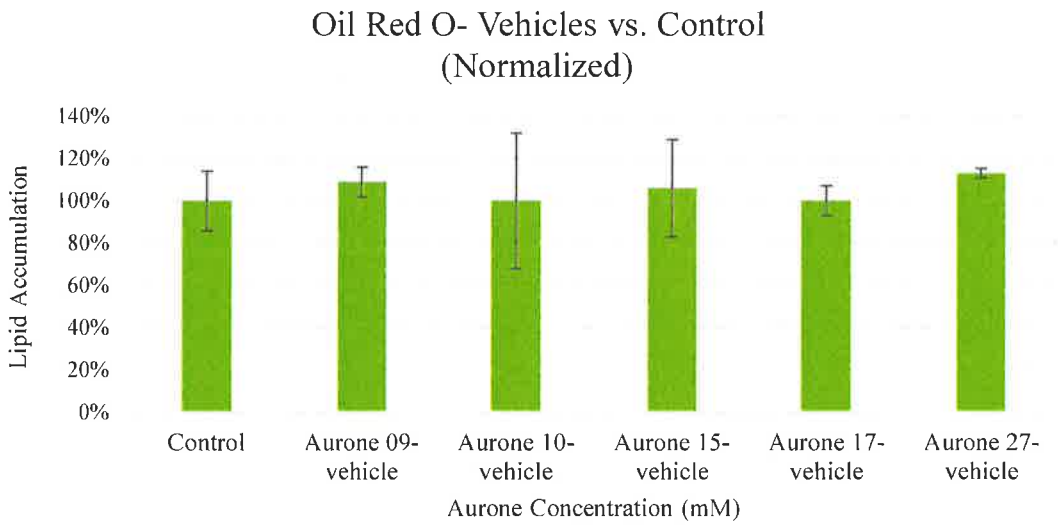

Figure 6: Control and vehicles for all aurones. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. The results were normalized against the control. Absorption of this solution measured at a wavelength of 490 nanometers.

Lipolysis Results

Mature fat cells were treated with concentrations of 25, 50, and 100 nM of Aurones 03, 10, 16, 17, and 20 and subjected to a lipolysis assay. Three hour results of the lipolysis assay for Aurone 3 and Aurone 10 showed similar patterns (Figure 7 & Table 3). For Aurone 03, at doses 25, 50, and 100 nM, the values obtained were 5.092, 4.870, and 5.549 μg/μL. For Aurone 10, at doses 25, 20, and 100 nM, the values acquired were 1.956, 1.782, and 2.477 μg/μL. Overall, both aurones had no dose response across the first two doses, with an increase in the third dose (Figure 7 & Table 3). Aurones 16 and 20 both showed similarities as well (Figure 7 & Table 3). Both contained a drop from the first dose to the second dose, with an increase at the third dose (Figure 7 & Table 3). For Aurone 16, at doses 25, 50, and 100 nM, the values shown were 2.124, 1.464, and 1.55 μg/μL. For Aurone 20, at doses 25, 50, and 100 nM, the values obtained were 2.192, 1.877, and 1.956 μg/μL. Aurone 17 showed an increase across the first two doses and a slight decrease at its final dose (Figure 7 & Table 3). At doses 25, 50, and 100 μM, Aurone 17 had values of 1.783, 2.426, and 2.343 μg/μL. All comparisons and values are shown below in Figure 7. Raw data from this experiment are available in Figure 17 and Table 3 in the Appendix.

For the six hour results, Aurone 20 showed little change from the first to second dose, with an increase in the final dose (Figure 8 & Table 4). The doses were the same as at the three-hour interval. The values for Aurone 20 were: 4.462, 4.972, and 5.993 μg/μL. Aurones 10 and 17 showed a slight increase across all doses (Figure 8 & Table 4). This means that it is likely that the threshold for toxicity resulting in cell death was not been reached. For Aurone 10, the values were shown were 5.059, 5.846, and 6.846 μg/μL. For

Aurone 17, the values were 3.965, 6.285, and 7.882 μg/μL. Aurones 03 and 16 showed an increase from their first to middle doses with a decrease at their final doses (Figure 8 & Table 4). For Aurone 03, the values obtained were 9.847, 12.04, and 10.464 μg/μL. For Aurone 16, the values were 4.403, 6.196, and 5.644 μg/μL. These results demonstrated the proclivity of some aurones to inhibit lipolysis to some degree. Of the five aurones that were noted for having the strongest ability to induce differentiation, four of these strongly inhibited lipolysis. The fifth aurone (Aurone 03), while still inhibiting lipolysis, did not do so as strongly. These comparisons are all seen below in Figure 8. Raw data from this experiment is available in Figure 18 and Table 4 in the appendix.

For these aurones, their ability to initiate adipogenesis seemed to be correlated to their ability to inhibit lipolysis. As this test was being conducted, simultaneously, another test assessing the adiponectin secretion of treated cells was being observed as well. Adiponectin secretion was examined through Western Blot Analysis.

Aurones: 3-Hour Interval Graphs

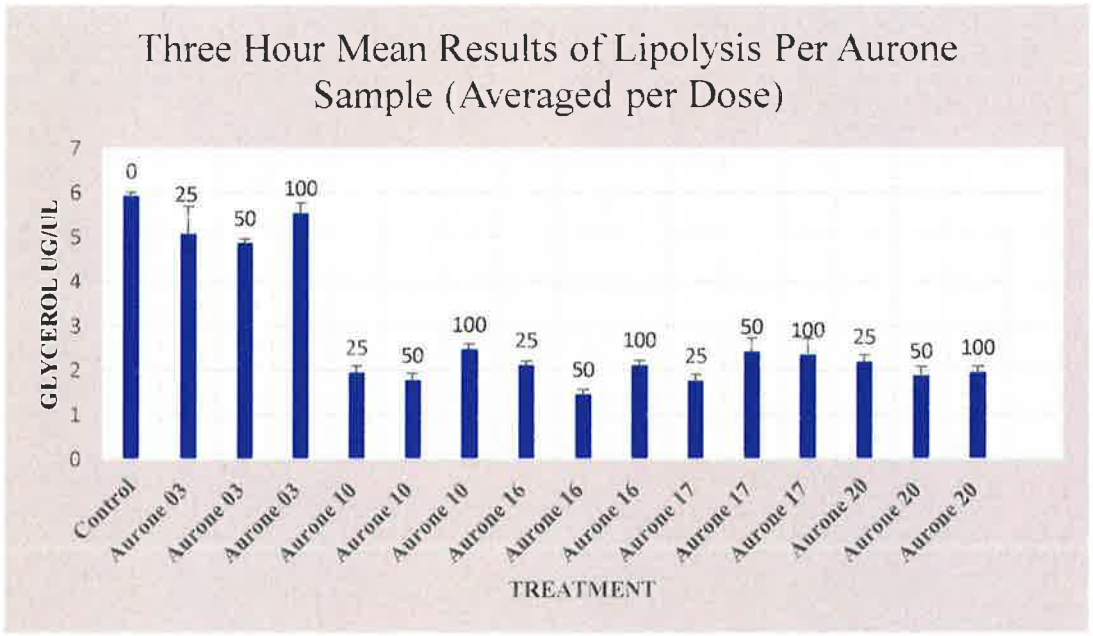

Figure 7: Averaged results of two experiments conducted in triplicate, with standard deviation calculated. Doses for each aurone were administered at 25, 50, and 100 nM. These results are the cells at the three-hour interval. For each standard concentration and for the treatment and control, 25 μL of incubation solution were added to a well of a 96 well plate in triplicate. 200 μL of free glycerol reagent was added to each well. Following incubation, the absorption for each well at 540 nm was measured. The absorption values for the treated and control samples were compared to the standard curve to determine the concentration of glycerol.

Aurones: 6 Hour Interval Graphs

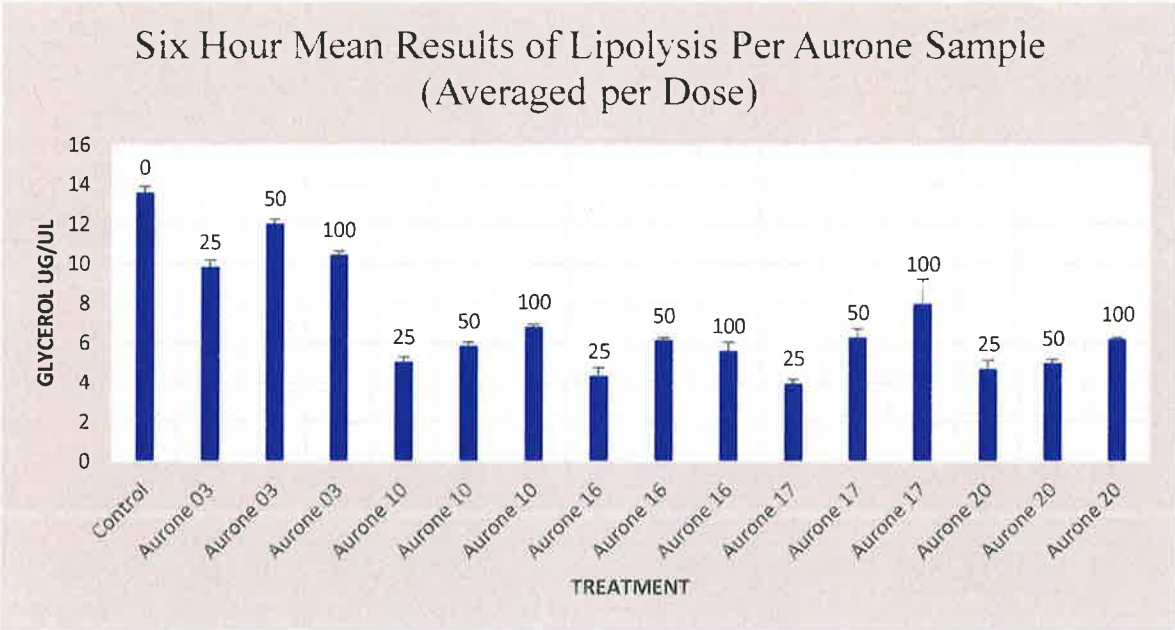

Figure 8: Averaged results of two experiments conducted in triplicate, with standard deviation calculated. Doses for each aurone were administered at 25, 50, and 100 nM. These results are the cells at the six-hour interval. For each standard concentration and for the treatment and control, 25 µL of incubation solution were added to a well of a 96 well plate in triplicate. 200 µL of free glycerol reagent was added to each well. Following incubation, the absorption for each well at 540 nm was measured. The absorption values for the treated and control samples were compared to the standard curve to determine the concentration of glycerol.

Western Blot Analysis Results

Whole cell extracts of control cells and vehicle-treated cells were compared to whole cell extracts of aurone-treated cells (Aurones 03, 10, 16, 17, & 20) to determine if aurone treatment induced the expression of adiponectin. These data showed adiponectin levels in both the whole cell extracts, or WCE, and the surrounding media. There were two controls used: one a simple untreated batch and the other is DMSO. Treatments were administered at doses of 0.1 mM, 0.2 mM, and 0.4 mM. Figure 9 below shows the comparisons in adiponectin expression. Aurone 03 displayed an induction in the expression of adiponectin at dose 2, while dose 1 and dose 3 were similar to control. Aurones 10 and 16 produced similar results as well. Aurones 17 and 20 displayed an increased expression of adiponectin in all three doses when compared to control. As observed in the other aurones, dose 2 did provide the largest induction of adiponectin expression. These results show that these five aurones were able to induce adiponectin expression.

Media samples of control cells and vehicle-treated cells were compared to media samples of aurone-treated cells (Aurones 03, 10, 16, 17, & 20) to determine if aurone treatment induced the adipocytes to secrete adiponectin into the media. Figure 10 shows the comparisons of adiponectin secretion. Aurone 03 produced a modest secretion across all three doses when compared to control. Aurones 10 and 16 treatment induced a stronger secretion of adiponectin across all three doses. For Aurone 10, the largest secretions occurred at doses 1 and 2, with dose 3 still showing an increase from control. For Aurone 16, its largest increase occurred at dose 2, with doses 1 and 2 showing similarly more modest increases when compared to control. Aurone 17 also produced a

modest increase in the secretion of adiponectin but only at dose 1. Secretion was reduced at doses 2 and 3 when compared to control. Treatment with Aurone 20 resulted in a reduction of adiponectin when compared to control. The aurones that were examined by western blot analysis were all capable of strongly inducing adipogenesis. They were also capable of mildly to strongly reducing lipolysis. All five of these aurones were capable of a modest to strong induction of the expression of adiponectin. Aurones 03, 10, and 16 increased the secretion of adiponectin into the culture media when compared to control. Paradoxically, treatment with Aurone 17, and particularly, Aurone 20, resulted in a reduction in secretion of adiponectin when compared to control.

Aurones- Western Blot Analysis Figures

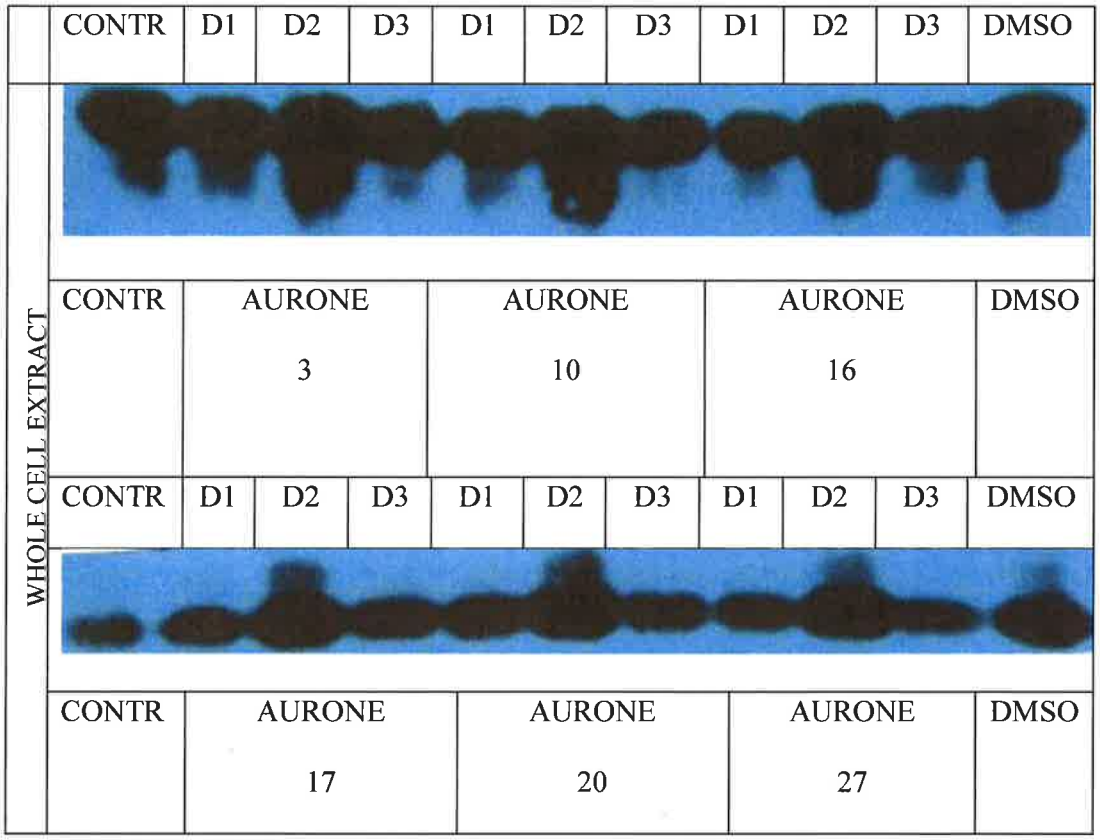

Fig 9: Western Blot Analysis was performed on whole cell extracts prepared from mature adipocytes that were harvested one week post-induction. Cells were treated for 96 hours at Dose 1, Dose 2, and Dose 3 for Aurones 03, 10, 16, 17, 20, 27. Dose 1 = 0.1 mM, Dose 2 = 0.2 mM, and Dose 3 = 0.4 mM. Cells were harvested in IP buffer. 75 micrograms of protein was added to each lane. Proteins were separated by gel electrophoresis on a 10% polyacrylamide gel. Following protein separation, the protein bands on the gels were then transferred onto nitrocellulose membranes. Following transfer, the membranes were blocked and incubated with an anti-adiponectin antibody. Visualization of adiponectin was by enhanced chemoluminescence.

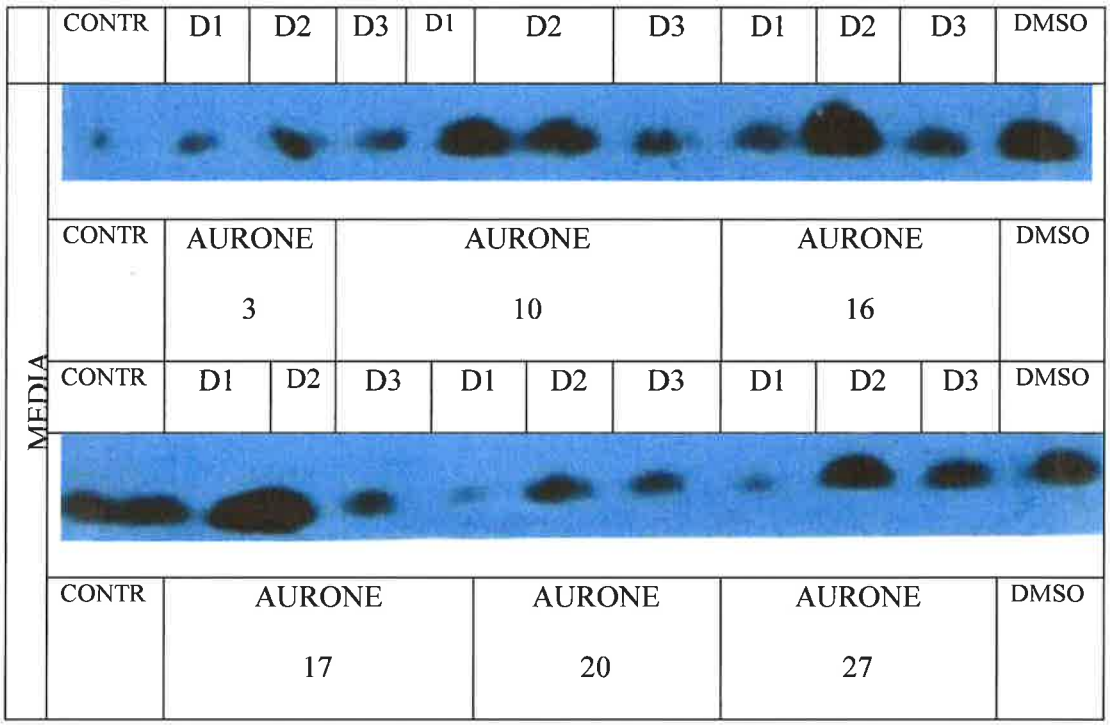

Figure 10: Western Blot Analysis was performed on media extracts prepared from mature adipocytes that were harvested one week post-induction. Cells were treated for 96 hours at Dose 1, Dose 2, and Dose 3 for Aurones 03, 10, 16, 17, 20, 27. Dose 1=0.1 mM, Dose 2=0.2 mM, and Dose 3=0.4 mM. Cells were harvested in IP buffer. 75 micrograms of protein was added to each lane. Proteins were separated by gel electrophoresis on a 10% polyacrylamide gel. Following protein separation, the protein bands on the gels were then transferred onto nitrocellulose membranes. Following transfer, the membranes were blocked and incubated with an anti-adiponectin antibody. Visualization of adiponectin was by enhanced chemoluminescence.

Discussion

Type 2 diabetes mellitus is a disease that is one of the largest impediments on the advancement of human health today. In the United States, as well as all over the globe, millions suffer from this ailment, and many die from it or its complications. Treatments for this disease and advancements in the field of diabetic medicine is of the utmost importance so that one day, we may have the propensity to more adequately treat, or maybe even cure, those suffering from diabetes. In the general public, the World Health Organization estimates that, in Africa, 80% of the population worldwide use traditional medicine, herbs, as a means of treatment for disease (World Health Organization 2003). It also estimates that in North America and Europe, 50% of people have tried some means of traditional medicine at least once during their lifetime (World Health Organization 2003). Coincidentally in science, there is a resurgence in the examination of natural sources ranging from botanical extracts to designer molecules whose formulas are based upon botanically extract compounds. Currently, flavonoids are one of the most significant families of biologically active botanical compounds. These compounds are one of the most utilized family of compounds in the field of research right now. Aurones are a group of compounds classified as a subclass of flavones, which are a part of the flavonoid family. This research investigated the impact aurones have on adipocyte function and the possible implications in drug therapy development for diabetes.

For the treatment of type 2 diabetes mellitus, or T2DM, research has shown a correlation between increased differentiation of adipocytes and better treatment outcomes of type 2 diabetes patients. In this study, various synthesized aurones were tested for their possible impact on the differentiation of preadipocytes in hopes that it would lead to

future treatments for T2DM. Also, the impact of aurones was observed on lipolysis and adiponectin release in mature fat cells as a measure of adipocyte function. In doing this, a large group of synthesized aurones were screened, and five aurones were identified with the potential for future testing to see if they may someday help fight one of the most impactful diseases on humans to date.

Oil Red O (ORO) staining is the standard experimental protocol for determining preadipocyte differentiation into mature adipocytes. From the initial screening of thirty-one aurones, five aurones were selected to further investigate based on Oil Red O staining. The Oil Red O results from these five aurones were comparable with the results of a successful adipogenesis experiment using a flavonoid, Rutin (Naowaboot 2015). For the related experiment, Rutin increased the lipid accumulation as measured by Oil Red O of the adipocytes by 120%-145% when issued at dosages of 3, 10, 30, and 100 μM. These percentages were normalized against control values, which were set at 100%. During the experimentation, cells were treated with 50 different aurones. Of these aurones, all five demonstrated results comparable to or exceeding the results (Figures 1, 2, 3, 4, 5, and 6) of Rutin treatment, which was considered an experiment with noteworthy results. Aurone 03, specifically, exceeded Rutin by more than doubling the effects that Rutin displayed on adipocyte differentiation. A concern of experiments of this nature is drug toxicity. During the experiments, these five aurones were investigated at three dosing concentrations, and there were no morphological changes in the cells and no changes in growth rate or mortality. Of the thirty-one compounds initially screened, some did display immediate and strong toxicity and were excluded from future experiments.

All aurones used in the experiments were dissolved in dimethyl sulfoxide (DMSO). DMSO has been shown to trigger differentiation in other cell lines, including the neuroblastoma cells. To minimize the impact of DMSO on the results, DMSO concentrations never exceeded 1% in the culture media, and a DMSO control was always incorporated in the experimental design. However, DMSO is an accepted solvent in scientific research; the experiment that tested the ability of Rutin to cause differentiation utilized DMSO as a solvent as well.

The ORO results of the thirty-one aurones initially screened served as a catalyst for future investigations into the five aurones that produced the best ORO results. These five aurones displayed a strong ability to induce differentiation of preadipocytes. They were next analyzed for their impact on the lipolysis activity of mature adipocytes. These five aurones were further tested on mature adipocytes to determine their ability to induce the expression and the secretion of adiponectin.

One of the major functions of a mature fat cell is the breakdown of triglycerides and release of fatty acids. This process is referred to as lipolysis. A hallmark of patients suffering from T2DM is the manifestation of elevated basal lipolysis rates. A reduction in basal lipolysis rates is consistent with successful medical therapies used to treat T2DM. For example, Metformin, a well-known anti-diabetic drug, when administered to diabetic patients was shown to significantly lower basal lipolysis rates (Zhang 2009). In these *in-vivo* experiments, lipolysis rates were assayed by measuring circulating glycerol in the blood plasma (Flechtner-Mors, et al. 1999). Similar *in-vivo* results have been shown with the insulin analog Glargine (Lactus), which is the number one prescribed drug in the world for T2DM (Hedrington et al. 2017; Pharmaceutical-technology 2016; Dagogo-

Jack, et al. 2000). Due to the strong correlation between drug therapy and reduced lipolysis rates, we next determined if treatment with Aurones 03, 10, 16, 17, and 20 would impact lipolysis rates in mature fat cells.

Basal lipolysis rates were measured three-hours and six hours post-treatment with Aurones 03, 10, 16, 17, and 20. All aurone treatments reduced lipolysis. Aurones 10, 16, 17, and 20 all produced a large reduction, approximately 60%, in lipolysis rates within three hours when compared to control cells, and the drop in lipolysis rates were consistent at the six hour assay. Aurone 03 did not significantly reduce lipolysis rates until six hours post-treatment, where the reduction was approximately 35%- 40%. Another flavonoid, cyanidin-3-O-β-glucoside, has also been reported to inhibit lipolysis in cultured adipocytes, a correlation seen in with the aurones of this study as well (Björk 2016). It demonstrated a reduction of around 18% of lipolysis rates *in-vivo* (Björk 2016). While lower than any of the percentages of Aurones 03, 10, 16, 17 and 20, the studies were conducted in different environments. The percentage of cyanidin-3-O-β-glucoside may be more reflective of the typical reduction that occurs in patients. It can be concluded from these results that all five of these aurones display the ability to reduce basal lipolysis rates similar to that seen in *in-vivo* treatment with Metformin and Glargine.

A difficulty with comparing this study to these previous studies is that these experiments were conducted in cell culture, *in-vitro*, and were not in the animal, *in-vivo*. An additional limitation to the experimental design is that the cells that were examined were not "diabetic" cells, so it is difficult to compare the results to a pathology like T2DM. It is probable that treatment of "diabetic" cells with these five aurones would

result in an even larger decrease in lipolysis rates. During the course of the lipolysis experiment, cell morphology and cell survival did not appear to be altered, but the concern of toxicity still exists and would need to be addressed with future experiments. At the six-hour interval, there was an increase across each dose consistently for Aurones 10, 17, and 20. This argues against cell death. If cell death was occurring, triglycerides would be released instead of glycerol. This would mean a continuous decrease at all doses of the assay for a toxic compound, or a very large decrease from one dose to the next would occur. Applying this idea, Aurones 03 and 16 had a slight decrease from the second treatment dose to the last dose. However, the decrease seen with aurones did not seem large enough to warrant an argument for toxicity. Conducting a more complete dose response toxicity assay would help to better clarify and reinforce our findings. Also, a continuation of lower doses may be beneficial as well. It may help remove the possibility that aurones are oversaturating the cells. Future investigations into the anti-lipolytic characteristics of these aurones should allow for longer intervals of treatment as a more thorough exploration of possible toxicity and the long-term inhibitory effects.

Adipocytes are a major endocrine tissue, producing and releasing numerous hormones. Adiponectin is a hormone released from healthy adipocytes and the most common hormone in blood plasma. Adiponectin levels correspond with good cardiovascular health. Patients with T2DM have reduced plasma levels of adiponectin. Diminished cardiovascular health is one of the major long-term concerns of T2DM. Five aurones were identified that have a strong propensity to induce adipogenesis. All five of the aurones all have a strong ability to reduce lipolysis rates. The next series of

experiments investigate the abilities of these five aurones to impact adiponectin production and secretion.

Results of the Western Blot Analysis of whole cell extracts demonstrated an increase in adiponectin expression for all of the aurones tested. For Aurones 03, 10, 16, 17, and 20, at least one treatment dose induced the expression of adiponectin above control values. However, it is interesting that all not dosings induced an increase in the expression of adiponectin. Aurones did not show an expected dose response as seen earlier in the lipolysis experiments.

The results presented here are similar to previous experiments testing the flavonoid, cyanidin-3-O-β-glucoside (C3G). Using ELISA assay and Western Blot Analysis, Liu, et al., 2008, reported that C3G increased adiponectin secretion when administered to mice (Liu, et. al 2008). Despite the overall accord with other literature, however there are some concerns about the results.

The concerns for the Western Blot Analysis data is as follows: the copious amount of secretion for our DMSO control, the lack of a correlation between adiponectin secretion and expression for some aurones (Aurone 20), and the similarities between the levels of the responsive doses with the DMSO control levels. This is more of a failure in technique, i.e. overexposure, air bubbles in the gels, etcetera, than a failure of the experiment. At the time, there was little tissue remaining, and the volumes of aurones were almost completely depleted. To collect sufficient tissue and compounds for the Western Blot Analysis process was both time consuming and expensive, which prohibited an immediate repeat of these experiments. When this experiment is repeated, these factors will be accounted for, and the results will be clearer.

Of the three approaches at this question, Western Blot Analysis results are the weakest. Here, evidence did show that the aurones were able induce the expression of adiponectin and cause secretion. However, when the data strength is compared amongst all experiments, the results of Western Blot Analysis are modest, at best. Although the western blot analysis results were not as impressive as the other data, they none-the-less support the conclusion that the five aurones that were best at inducing adipogenesis, that were able to reduce basal lipolysis were also generally speaking able to induce adiponectin secretion and expression.

Of the thirty-one aurones initially analyzed for their ability to initiate differentiation of preadipocytes, five aurones were identified as successful. These five aurones, Aurones 03, 10, 16, 17 and 20, were subjected to additional analysis through a glycerol assay and Western Blot Analysis. The five aurones showed proficiency in increasing the expression and secretion of adiponectin and excelled in inhibiting lipolysis. The results of these experiments indicate that these five aurones should be considered for additional investigations into their ability to be a drug therapy for T2DM.

While the progress made in the field of science is exciting, it is important to continue to search for more alternatives for the increasingly distressing problem of type 2 diabetes mellitus. There is currently no medical cure for type 2 diabetes mellitus once it is acquired. Because of the unique way in which each individual reacts to certain compounds entering their body, there needs to be continued work towards a various array of cures and alternatives. In the last few decades, synthesis research has advanced dramatically, and the advancement seen in this field has helped explore even further the different capabilities of each family and classes, such as flavonoids and flavones. The

increase in investigations on botanical compounds may lead to rapid growth in treatment for human ailments, especially with flavones and aurones. Due to our current ability to synthesize aurones and their potential effectiveness as a treatment for T2DM, future, more personalized methods of treatment are possible with these compounds. The variety in future medicinal treatments will also compensate for any individual intolerance that may occur due to allergies, incompatibility, etcetera. With these future chemotherapeutics, researchers should and will strive for equally effective alternative methods of managing T2DM. Until that goal is achieved, it will remain important to do research on adipocytes and adipogenesis in hopes of one day attaining that goal. The future and current advancement with the class of flavones are both promising and exciting in that aspect. It suggests that further research into this class, or perhaps a subclass of this group, is more likely to yield promising results. It gives us more reason to keep exploring these compounds.

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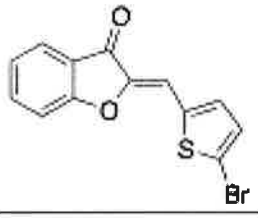
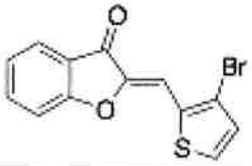
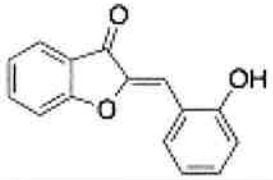
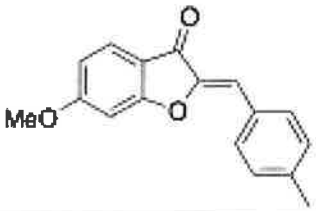
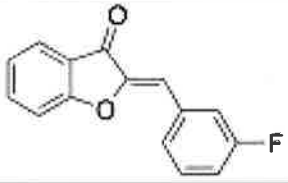
Appendix 1: Tables and Figures

Table 1: Aurones and Their Structures

Aurones That Worked	
Structure	Assigned (Original) Number and Experimental Numbers
	9058→ 09→ 16
	9060→ 10→ 17
	9088→ 15→ 20
	5002→ 17→ 03
	9024→ 27→ 10
Aurones That Did Not Work	
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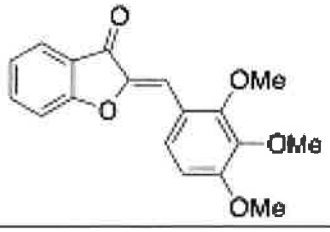
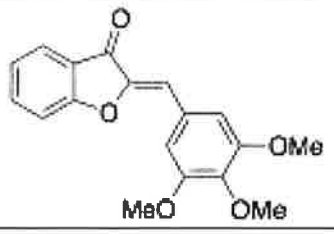
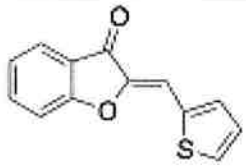
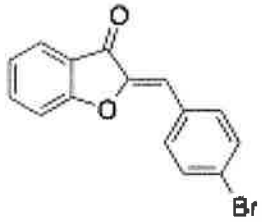
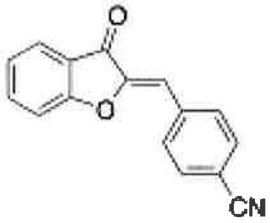
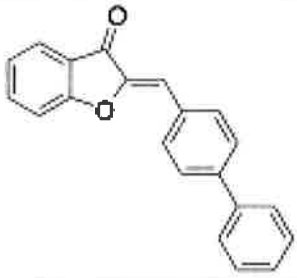
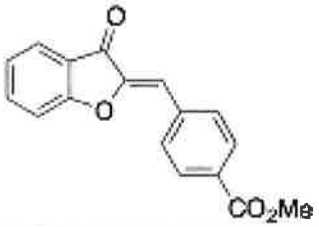
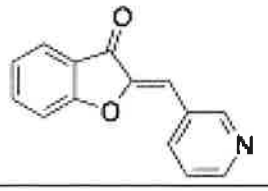
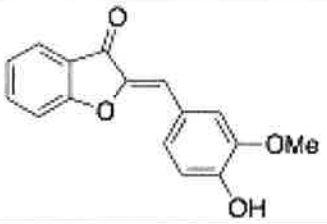
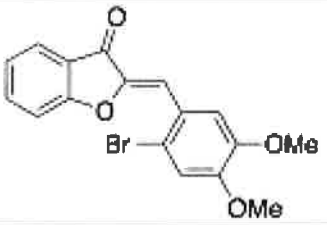
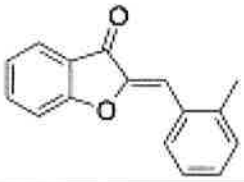
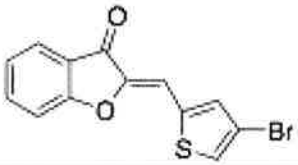
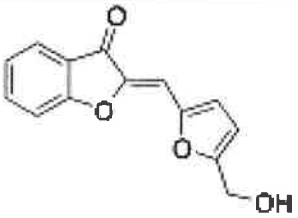
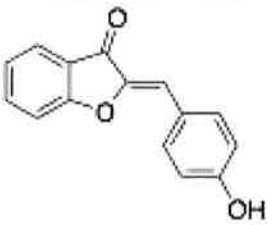
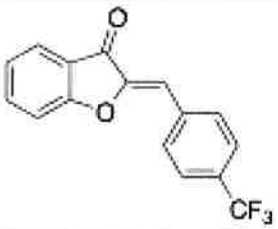
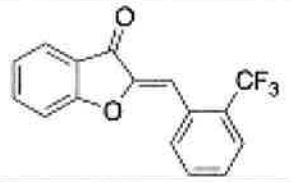
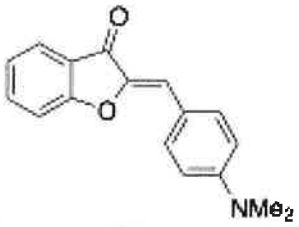
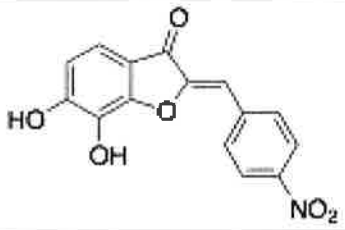
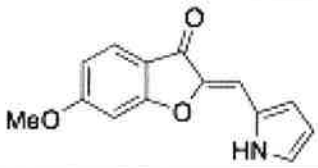
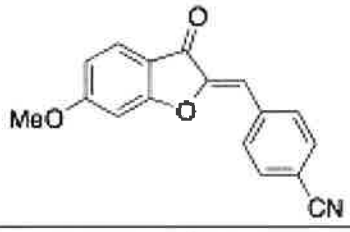
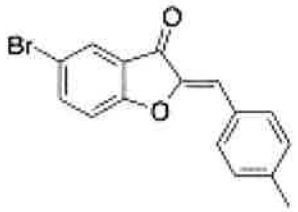
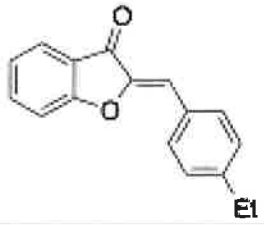
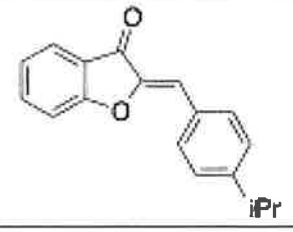
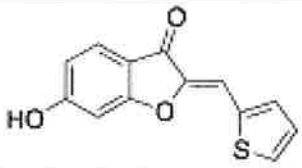
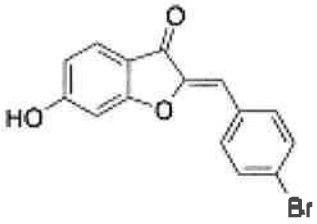
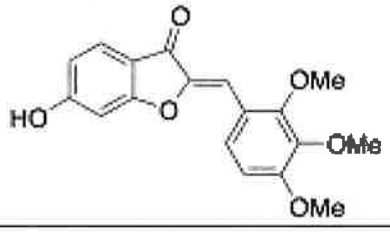
	A

	B
	D
	I
	N
	R
	9047
	9051

	9053
	9056
	9057
	9061
	9067
	9076
	9084

	9086
	9087
	1005
	5003
	5006
	6000
	8001

	8002
	Exp 1
	Exp 2
	Exp 12

Oil Red O Tables

Table 2: Oil Red O- Raw Data

Aurone & Dose	Average Value	Standard Deviation
16 0.2X-1	0.452	0.004
16 0.2X-2	0.264	0.007
16 0.2X-3	0.314	0.01
16 0.5X-1	0.588	0.005
16 0.5X-2	0.431	0.004
16 0.5X-3	0.46	0.009
16 1X-1	0.784	0.011
16 1X-2	0.752	0.025
16 1X-3	0.785	0.004
16 veh 1	0.279	0.009
16 veh 2	0.293	0.007
16 veh 3	0.324	0.003
17 0.2X-1	0.456	0.006
17 0.2X-2	0.248	0.008
17 0.2X-3	0.412	0.004
17 0.5X-1	0.559	0.016
17 0.5X-2	0.4	0.009
17 0.5X-3	0.575	0.012
17 1X-1	0.679	0.004
17 1X-2	0.553	0.003
17 1X-3	0.675	0.015
17 veh 1	0.312	0.023
17 veh 2	0.255	0.004
17 veh 3	0.258	0.002
20 0.2X-1	0.407	0.02
20 0.2X-2	0.261	0.002
20 0.2X-3	0.272	0.002
20 0.5X-1	0.581	0.01
20 0.5X-2	0.345	0.004
20 0.5X-3	0.489	0.001
20 1X-1	0.684	0.015
20 1X-2	0.538	0.004
20 1X-3	0.513	0.006

Aurone & Dose	Average Value	Standard Deviation
20 veh-1	0.351	0.034
20 veh-2	0.26	0.006
20 veh-3	0.255	0.006
03 0.2X-1	0.532	0.007
03 0.2X-2	0.365	0.011
03 0.2X-3	0.477	0.003
03 0.5X-1	0.826	0.004
03 0.5X-2	0.597	0.003
03 0.5X-3	0.758	0.008
03 1X-1	0.932	0.006
03 1X-2	0.813	0.021
03 1X-3	0.996	0.023
03 veh-1	0.292	0.031
03 veh-2	0.257	0.009
03 veh-3	0.275	0.002
10 0.2X-1	0.653	0.015
10 0.2X-2	0.443	0.006
10 0.2X-3	0.569	0.009
10 0.5X-1	0.766	0.003
10 0.5X-2	0.536	0.004
10 0.5X-3	0.685	0.006
10 1X-1	0.689	0.004
10 1X-2	0.695	0.029
10 1X-3	0.954	0.015
10 veh-1	0.315	0.02
10 veh-2	0.304	0.008
10 veh-3	0.307	0.004
CON-1	0.291	0.022
CON-2	0.229	0.004
CON-3	0.303	0.009
Rosi-1	0.677	0.025
Rosi-2	0.638	0.004
Rosi-3	0.602	0.01

Lipolysis Tables

Table 3: 3-Hour Interval of Lipolysis Assay

Sample	Dose	Result
Control	0	6.49
		6.314
		6.544
Control	0	5.927
		5.879
		6.042
Control	0	5.316
		5.455
		5.413
Iso	10	26.978
		25.569
		27.927
Iso	10	24.19
		23.47
		21.91
Iso	10	22.987
		24.008
		23.621
Aurone 03	25	4.306
		4.161
		6.308
Aurone 03	25	5.171
		5.292
		5.177
Aurone 03	50	4.736
		4.929
		5.014
Aurone 03	50	4.802
		4.905
		4.833
Aurone 03	100	5.159
		4.899
		5.002
Aurone 03	100	5.988
		5.8
		6.447

Sample	Dose	Result
Aurone 10	25	1.552
		1.739
		1.812
Aurone 10	25	2.29
		2.32
		2.024
Aurone 10	50	1.86
		1.697
		1.401
Aurone 10	50	1.957
		1.963
		1.812
Aurone 10	100	2.078
		2.066
		2.29
Aurone 10	100	2.701
		2.961
		2.767
Aurone 16	25	2.132
		2.126
		2.169
Aurone 16	25	2.266
		1.951
		2.102
Aurone 16	50	1.618
		1.389
		1.445
Aurone 16	50	1.552
		1.383
		1.395
Aurone 16	100	3.39
		3.167
		3.463
Aurone 16	100	0.85
		0.923
		0.959

Sample	Dose	Result
Aurone 17	25	1.909
		1.751
		1.885
Aurone 17	25	Masked*
		1.54
		1.83
Aurone 17	50	2.411
		2.538
		2.399
Aurone 17	50	1.854
		2.888
		2.465
Aurone 17	100	2.108
		2.616
		2.882
Aurone 17	100	2.151
		1.806
		2.495
Aurone 20	25	1.461
		1.467
		1.286
Aurone 20	25	2.894
		3.209
		2.834
Aurone 20	50	2.568
		2.241
		1.921
Aurone 20	50	1.479
		1.443
		1.612
Aurone 20	100	1.649
		1.57
		1.195
Aurone 20	100	2.417
		2.423
		2.483

Lipolysis Tables

Table 4: 6-Hour Interval of Lipolysis Assay

Sample	Dose	Result
Control	0	14.031
		14.429
		14.44
Control	0	13.323
		13.677
		13.965
Control	0	12.692
		12.747
		13.301
Iso	10	42.69
		44.261
		45.047
Iso	10	39.426
		38.939
		36.615
Iso	10	37.833
		39.326
		39.028
Aurone 03	25	10.357
		9.406
		9.372
Aurone 03	25	9.804
		10.07
		10.07
Aurone 03	50	11.22
		11.597
		11.386
Aurone 03	50	12.427
		12.98
		12.637
Aurone 03	100	10.899
		11.143
		11.043
Aurone 03	100	9.616
		9.937
		10.147

Sample	Dose	Result
Aurone 10	25	4.487
		5.173
		4.83
Aurone 10	25	5.394
		5.129
		5.339
Aurone 10	50	5.892
		6.136
		6.147
Aurone 10	50	5.804
		5.317
		5.782
Aurone 10	100	6.944
		6.899
		6.966
Aurone 10	100	6.567
		6.855
		Masked*
Aurone 16	25	4.144
		3.679
		Masked*
Aurone 16	25	4.498
		4.343
		5.35
Aurone 16	50	Masked*
		5.726
		5.915
Aurone 16	50	6.28
		6.49
		6.567
Aurone 16	100	5.074
		5.184
		6.346
Aurone 16	100	5.461
		5.826
		5.738

Sample	Dose	Result
Aurone 17	25	4.011
		4.299
		4.288
Aurone 17	25	3.513
		3.945
		3.735
Aurone 17	50	5.45
		5.97
		5.804
Aurone 17	50	6.567
		7.541
		6.379
Aurone 17	100	6.767
		8.958
		7.585
Aurone 17	100	6.999
		9.754
		7.729
Aurone 20	25	3.779
		4.365
Aurone 20	25	Masked*
		4.852
		4.852
Aurone 20	50	4.93
		4.985
		Masked*
Aurone 20	50	4.719
		5.428
		4.797
Aurone 20	100	5.527
		5.56
		5.749
Aurone 20	100	Masked*
		6.844
		6.789
		5.494

Oil Red O Figures- Raw Numbers

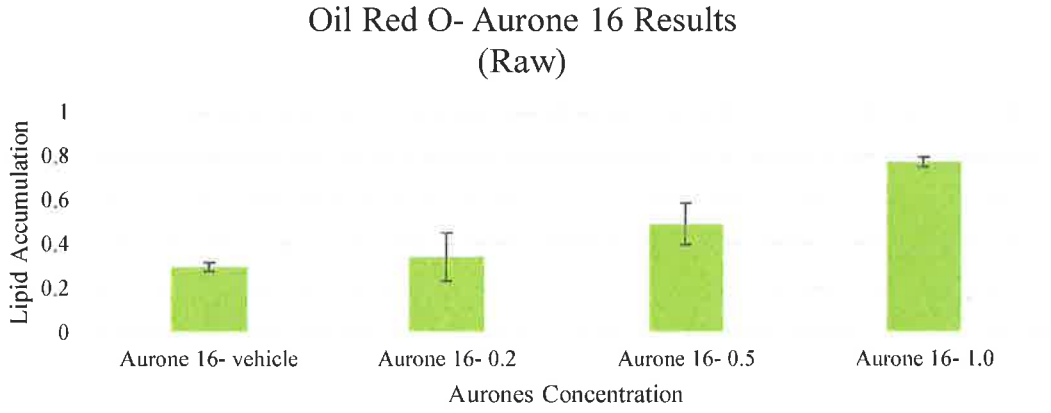


Figure 11: Aurone 16 at doses 0.2 mM, 0.5 mM, and 1.0 mM on plates of adipocytes. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. Absorption of this solution measured at a wavelength of 490 nanometers.

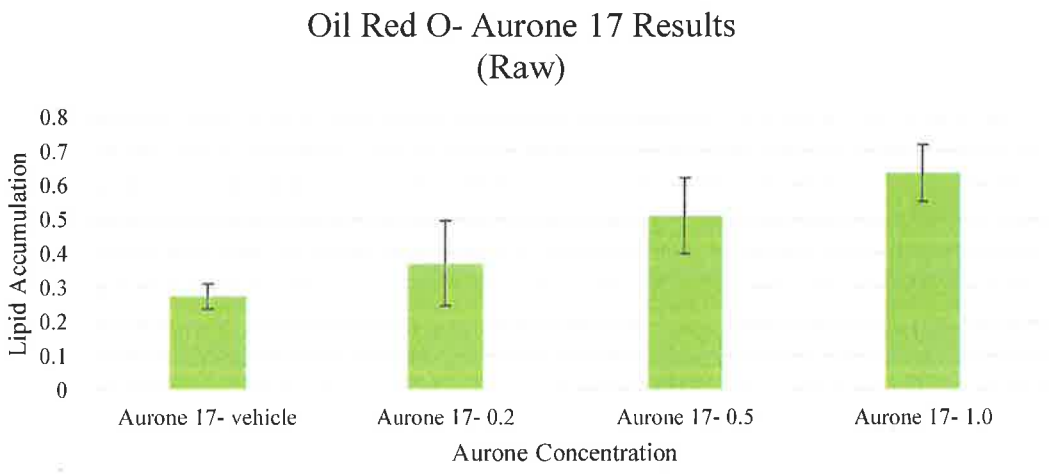


Figure 12: Aurone 17 at doses 0.2 mM, 0.5 mM, and 1.0 mM on plates of adipocytes. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. Absorption of this solution measured at a wavelength of 490 nanometers.

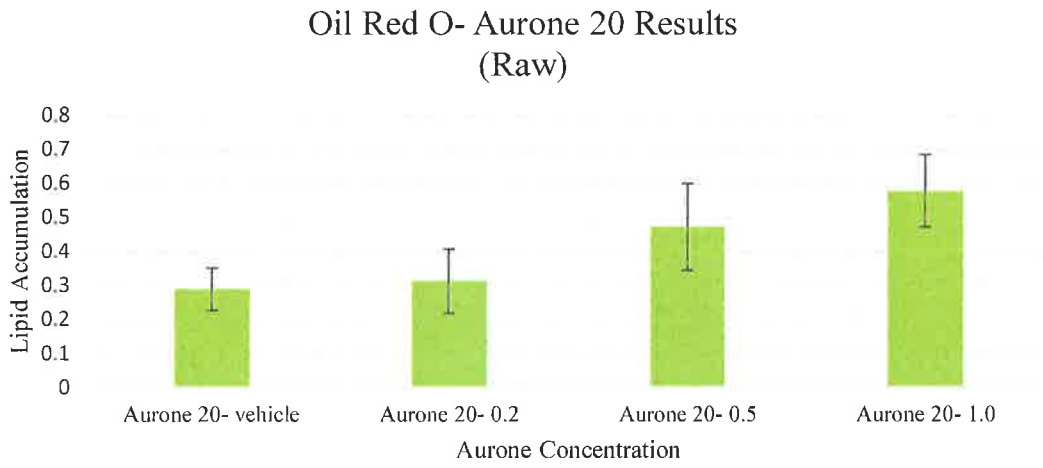


Figure 13: Aurone 20 at doses 0.2 mM, 0.5 mM, and 1.0 mM on plates of adipocytes. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. Absorption of this solution measured at a wavelength of 490 nanometers.

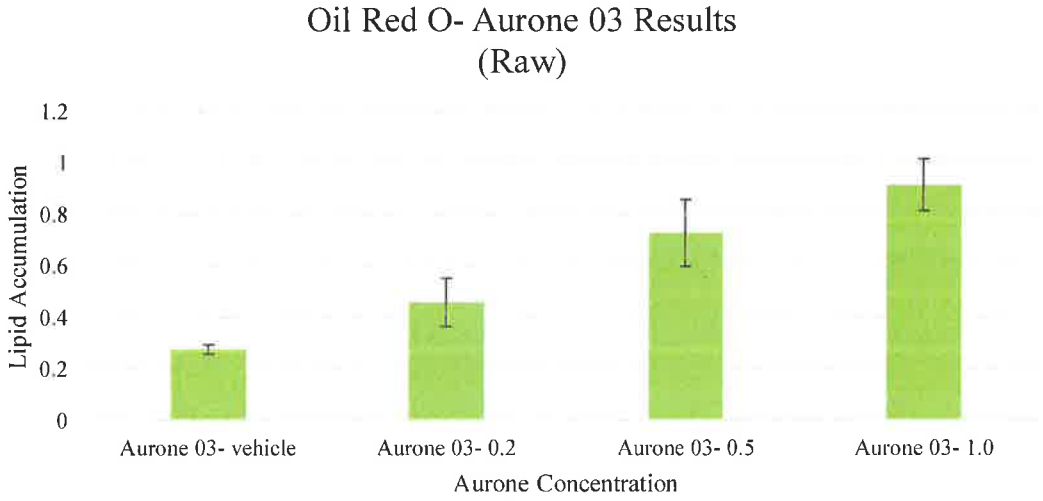


Figure 14: Aurone 03 at doses 0.2 mM, 0.5 mM, and 1.0 mM on plates of adipocytes. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. Absorption of this solution measured at a wavelength of 490 nanometers.

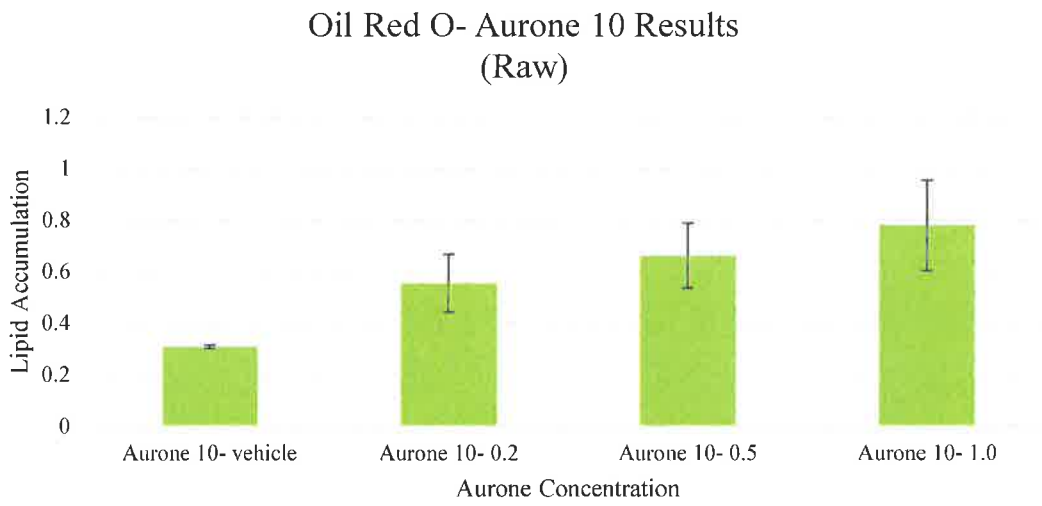


Figure 15: Aurone 10 at doses 0.2 mM, 0.5 mM, and 1.0 mM on plates of adipocytes. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. Absorption of this solution measured at a wavelength of 490 nanometers.

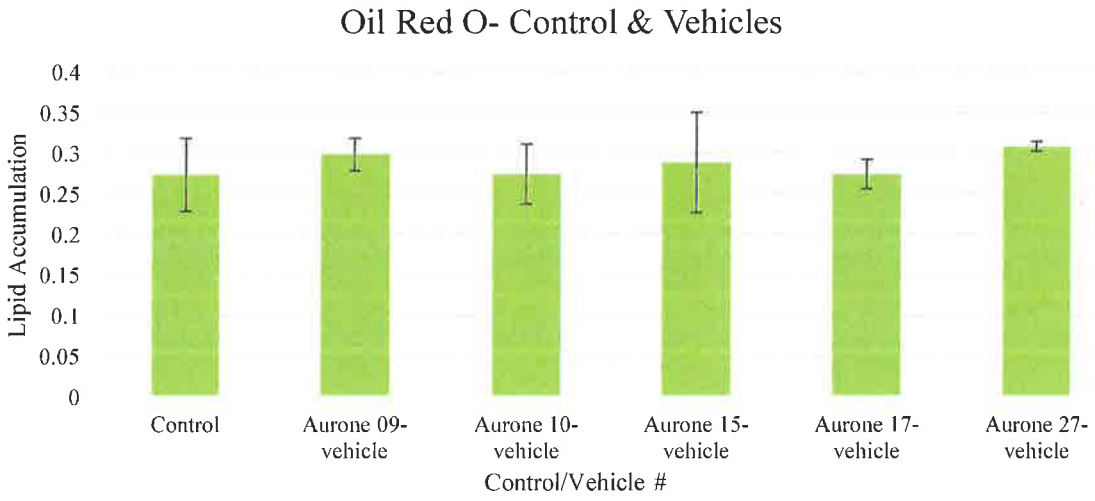


Figure 16: Control and vehicles for all aurones. These doses were done in triplicate. Values were averaged per triplicate and standard deviation was calculated. Absorption of this solution measured at a wavelength of 490 nanometers.

Lipolysis Figures- Raw Numbers

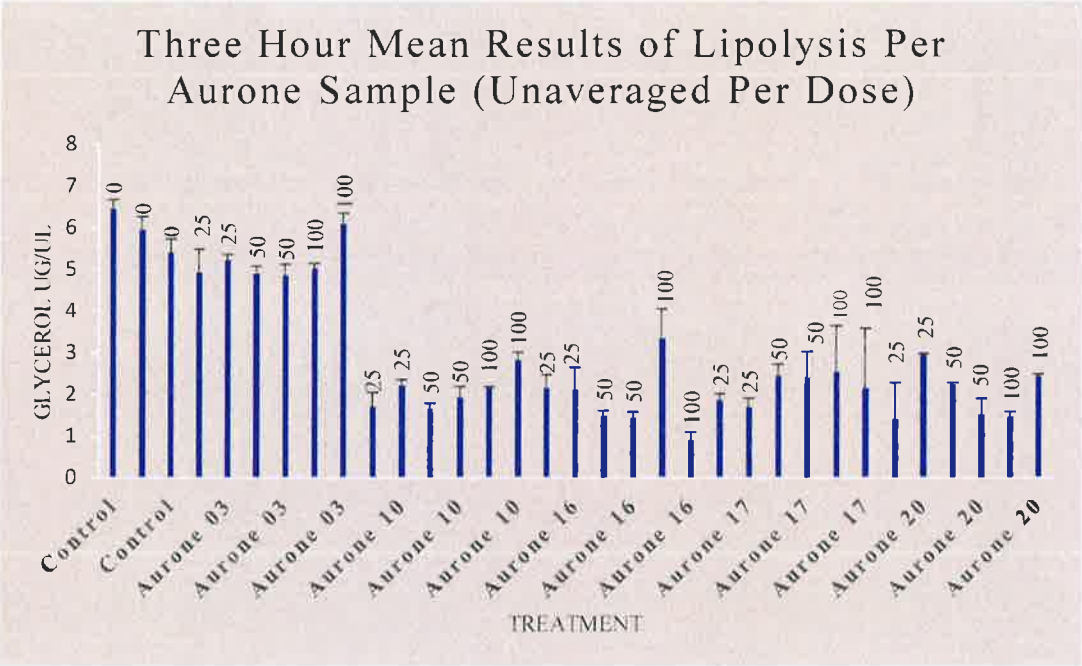


Figure 17: Results of two experiments conducted in triplicate. These results are averaged triplicates of each aurone, and standard deviation was calculated. Doses for each aurone were administered at 25, 50, and 100 nM. These results are the cells at the three-hour interval. For each standard concentration and for the treatment and control, 25 μL of incubation solution were added to a well of a 96 well plate in triplicate. 200 μL of free glycerol reagent was added to each well. Following incubation, the absorption for each well at 540 nm was measured. The absorption values for the treated and control samples were compared to the standard curve to determine the concentration of glycerol.

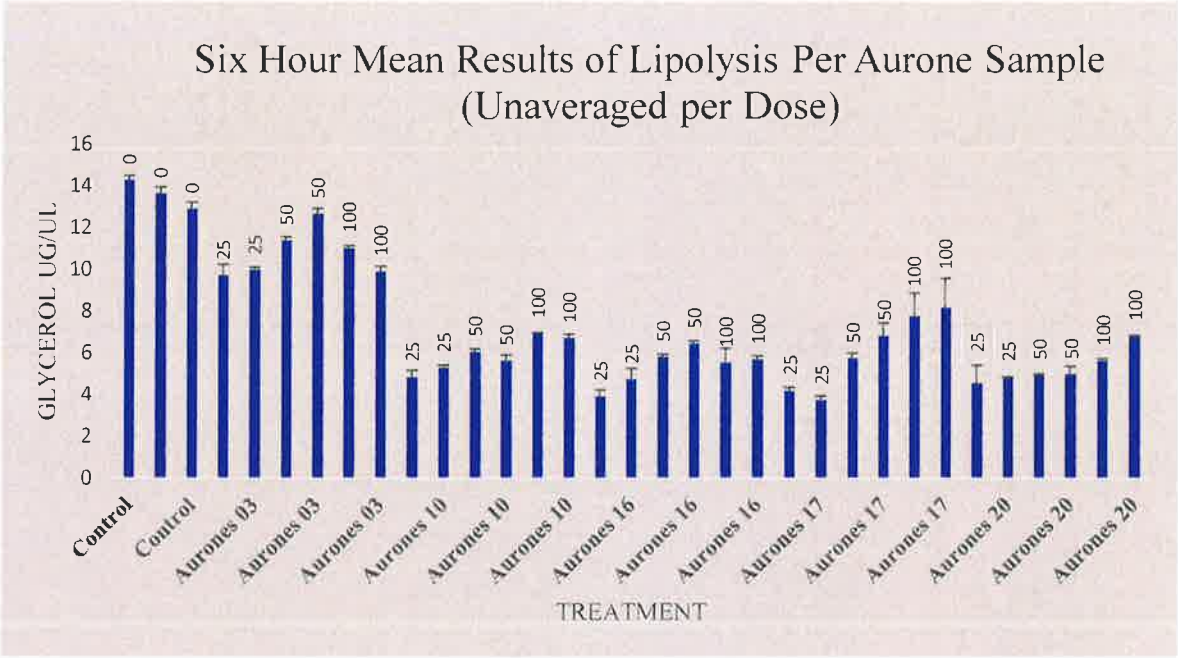


Figure 18: Results of two experiments conducted in triplicate. These results are averaged triplicates of each aurone, and standard deviation was calculated. Doses for each aurone were administered at 25, 50, and 100 nM. These results are the cells at the six-hour interval. For each standard concentration and for the treatment and control, 25 μL of incubation solution were added to a well of a 96 well plate in triplicate. 200 μL of free glycerol reagent was added to each well. Following incubation, the absorption for each well at 540 nm was measured. The absorption values for the treated and control samples were compared to the standard curve to determine the concentration of glycerol.