DET TEKNISK-NATURVITENSKAPELIGE FAKULTETET

BACHELOROPPGAVE

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Tittel på oppgaven: Effekt av CCCP, rotenon og oligomycin på pankreaskreftcellelinje HPAF-II Engelsk tittel: Effect of CCCP, Rotenone and Oligomycin on the pancreatic cancer cell line HPAF-II.	
Studiepoeng: 20	
Emneord: pancreatic cancer Cell line HPAF-II CCCP, Rotenonen, Oligomycin	Sidetall: 73 + vedlegg/annet: Stavanger, *(dato)* 25.05.2023

Abbreviations:

NADPH - nicotinamide dinucleotide phosphate
NADH – Nicotinamide adenine dinucleotide
NAD+ - oxidized nicotinamide adenine dinucleotide
FADH2 – reduced flavin adenine dinucleotide
ATP – adenosine tri phosphate
ADT – androgen derivatives
Acetyl CoA – Acetyl coenzyme A
mtDNA – mitochondria DNA
ETC – Electron Transport Chain
TCA – the citric acid cycle
CCCP: Carbonyl cyanide 3-chlorophenylhydrazone
FCCP - Carbonyl cyanid-4 (trifluoromethoxy) phenylhydrazone

Content:

Abstract

This bachelor thesis focuses on pancreatic cancer cells, HPAF-II, and their response to treatment with mitochondrial inhibitors such us CCCP, Rotenone and oligomycin.
To assess the cell response after treatment with CCCP, Rotenone and Oligomycin vi used Alamar Blue metabolic assay. To determine the optimal cell seeding density, we first ran an experiment with increasing cell density concentrations and evaluated using Alamar Blue. The HPAF-II cells were treated with different inhibitors for 24 hours and 48 hours before assessing viability. CCCP is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential, which further cause mitochondrial swelling and cell death. The results showed that CCCP affect the cells as the cell viability decreased when the CCCP concentration increased, with 50 μM CCCP at 48 hours showed the highest cell death. Rotenone is an inhibitor that inhibits complex I of electron transport chain, and it causes blockade of electron transport chain, which further cause inhibiting ATP synthesis. The results showed that Rotenone had negative effect on the cell as the cell viability increased when the Rotenone concentration increased. Oligomycin is another inhibitor that inhibits the ATP synthase that cells cannot produce ATP. The results showed that Oligomycin affect the cell viability decreased when the Oligomycin concentration increased, with 500 nM Oligomycin at 48 hours showed the highest cell death.
The seahorse analyzer measuring the two energy pathways of the cells, mitochondrial respiration, and glycolysis. This was done by measuring the rates of oxygen consumption (OCR) and lactate secretion in the medium surrounding the cells. By measuring the oxygen consumption and free protons in the extracellular flux we can determine the bioenergetic status of the cells. The result shows that the HPAF-II cell line is energetic and using both mitochondrial respiration and glycolysis to produce ATP and this cell line forms a lot of lactates and consumes more oxygen.

1. Introduction

1.1 Cancer:

cancer is the second highest cause of death worldwide. Cancer is a complex disease and involves changes and damages genes and gene regulation. If the genes that are responsible for cell growth are destroyed or do not receive a stop signal, then cells grow out of control and can lead to cancer. Cancer can occur due DNA damage. Abnormal growth of cells forms tumors, and they can be cancerous or not cancerous (benign). The cancerous tumors are move and spread to the other parts of the body and growth. But the benign tumors do not move and spread (Figure 1.1) (1).

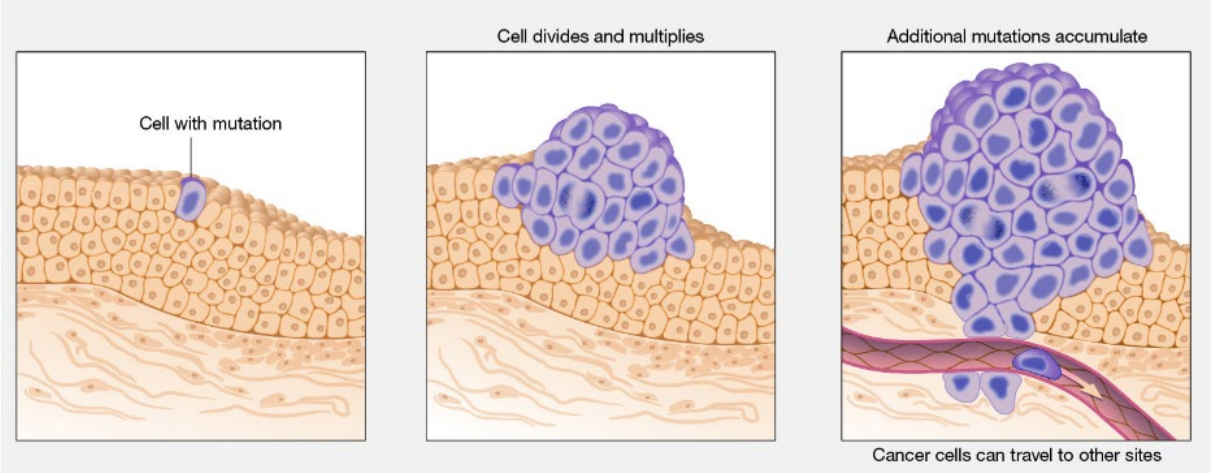


Figure (1.1): This figure shows the growth of cancer cells (2)

Cancer cells grow more than normal cells and therefore they need more nutrients than normal cells. for do this, cancer cells change the normal metabolic pathways.(3)
In recent decades, the understanding of cancer diseases has developed at an incredible pace and the development and discovery of new treatments is improving rapidly. There are a lot of type of treatments for example Chemotherapy, Hormone therapy, immunotherapy, Radiation therapy, Stem cell transplant, Surgery, and targeted therapy (*Figure (1.2)*). The type of treatment that you receive will depend on the type of cancer you have and how advanced it is (4).

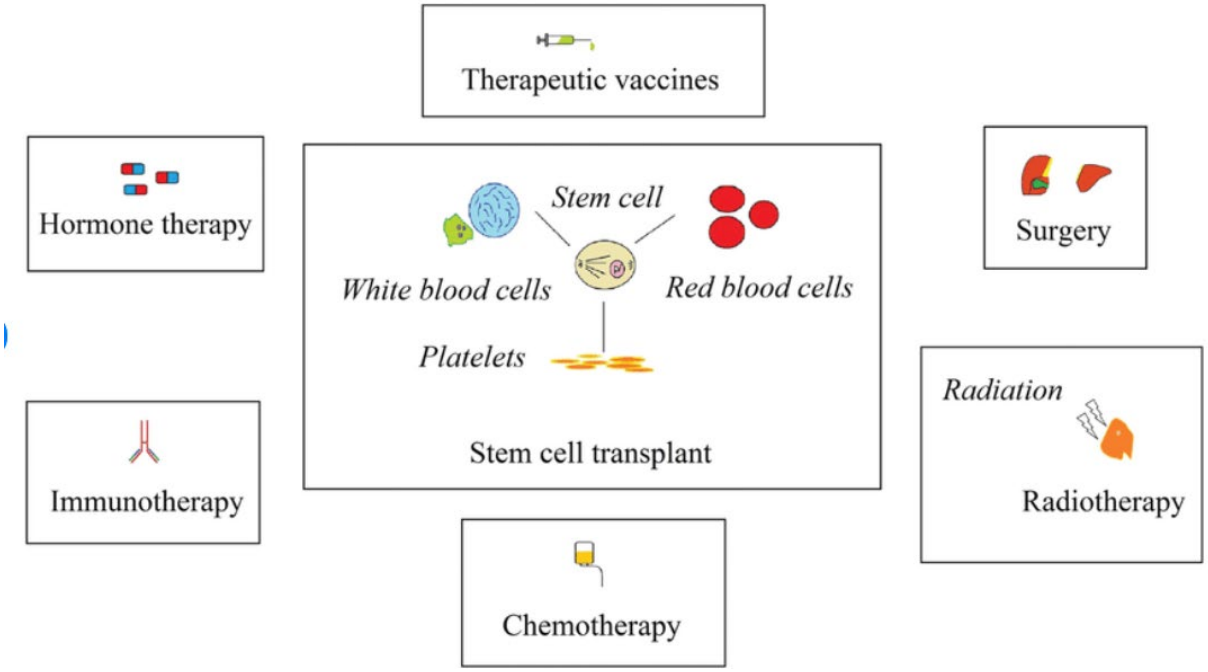


Figure (1.2): This figure shows the different types av cancer treatments (5)

This project focuses on investigating metabolic vulnerabilities in pancreatic cancer cells grown *in vitro* using inhibitors of electron transport chain.
Pancreatic cancer has the highest mortality rate in developed countries. It is more common for patients over 50- 60 to get this type of cancer than middle-aged patients. The median age are 73 years (6). The exact cause of pancreatic cancer is still unknown, but there are known risk factors that increase the risk of developing the disease. Cigarette smoking, a family history of pancreatic cancer or hereditary cancer syndromes, and chronic pancreatitis are some of these factors. In addition, certain pancreatic lesions such as Intraductal Papillary Mucinous Neoplasms (IPMNs) and Pancreatic Intraepithelial Neoplasia (PanIN) are considered precursors to pancreatic cancer (7)

1.2 Pancreas

The pancreas is an organ located behind the stomach in the upper left abdomen. It plays an essential role in converting the food we eat into fuel for the body's cells. It is spongy, about six to ten inches long, and is shaped like a flat pear extended horizontally across the abdomen. Pancreas is made of four main parts. The head of pancreas is positioned toward the center of the abdomen. The head of the pancreas is located at the juncture where the stomach meets the first part of the small intestine. This is where the stomach empties partially digested food into the intestine, and the pancreas releases digestive enzymes into these contents. The central section of the pancreas is called the neck or body and the thin end is called the tail and extends to the left side.
Almost 95 % of pancreatic tissue consists of exocrine tissue that produces pancreatic enzymes like chymotrypsin, amylase, and lipase for digestion. The remaining tissue is endocrine cells called islets of Langerhans. These cells look like grapes and produce hormones that regulate blood sugar and pancreatic secretions (7).

1.2.1 Pancreatic cancer cell:

Pancreatic cancer calls to tumors that starting in the cells of the pancreas, cancer develops when cells grow in uncontrolled manner and form tumors in the pancreas, and it can spread to other parts of the body. There is to types of tumors in the pancreas, exocrine tumors, and endocrine tumors. The exocrine tumors that also called pancreatic ductal adenocarcinoma, or PDAC, originates in the exocrine tissue. It is the most common type of pancreatic cancer (95%) and rises from abnormal cells lining the pancreatic duct. These cells may form glands, or a collection of cells surrounding an empty space. The endocrine pancreas constitutes less than 5% of the whole pancreas. They originate in the hormone-producing cells of the pancreas, the islet of Langerhans cells. Because endocrine tumors begin in cells that produce hormones, the tumors themselves may produce hormones that cause symptoms in addition to the problems caused by the presence of an abnormal mass (8).
Pancreatic cancer is difficult to treat and that it has been suggested that treating more targeted at its metabolism may be a solution.

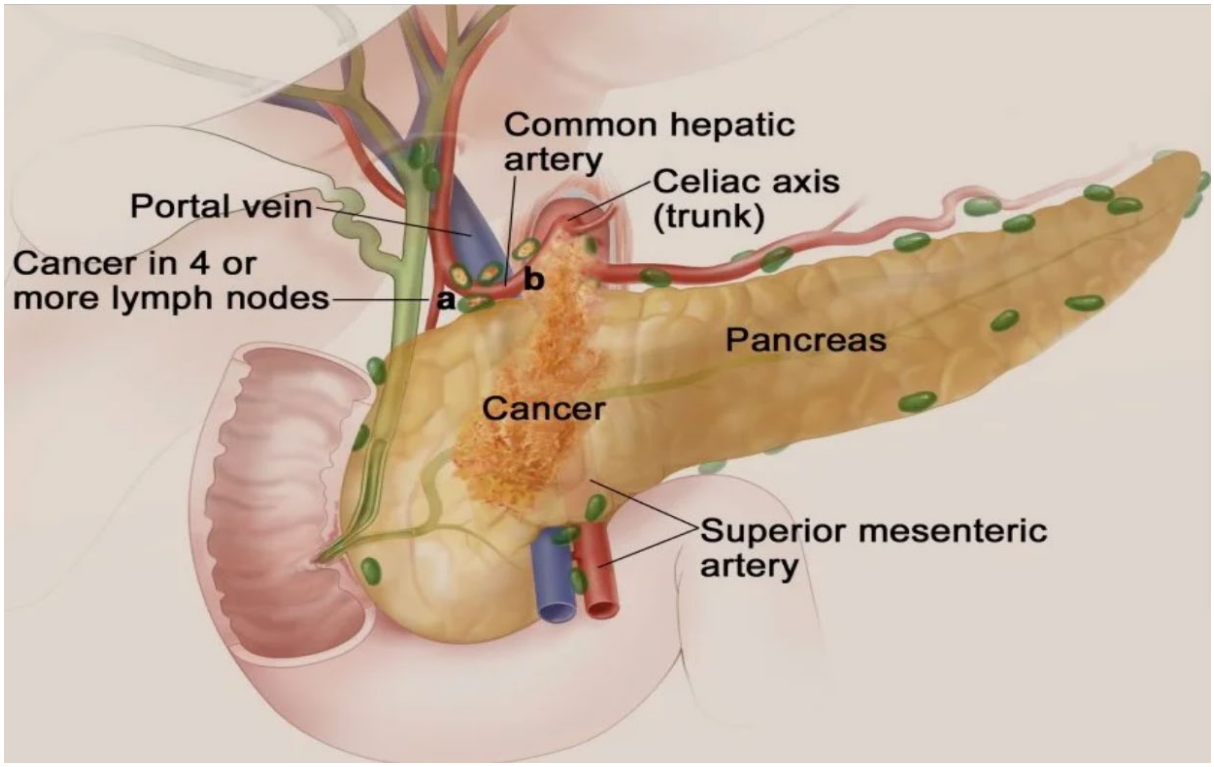


Figure (1.3): shows the pancreas and pancreatic cancer. It was taken from (9).

1.3 Normal cell metabolism:

Metabolism is the set of chemical reactions that occur in a cell, which enable it to keep living, growing, and dividing. Metabolism processes are classified as catabolism and anabolism. Catabolism is obtaining energy and reducing power from nutrients, and anabolism is production of new cell components, this process require energy.
By study the metabolism, we will find out that how the cells are able to make four important biomolecules like protein, fats, carbohydrate, and nucleic acids.
In humans, the most important metabolic pathways are glycolysis found in the cytoplasm, and the citric acid cycle (TCA-cycle) and oxidative phosphorylation that takes in the mitochondria, see *Figure (1.4).*

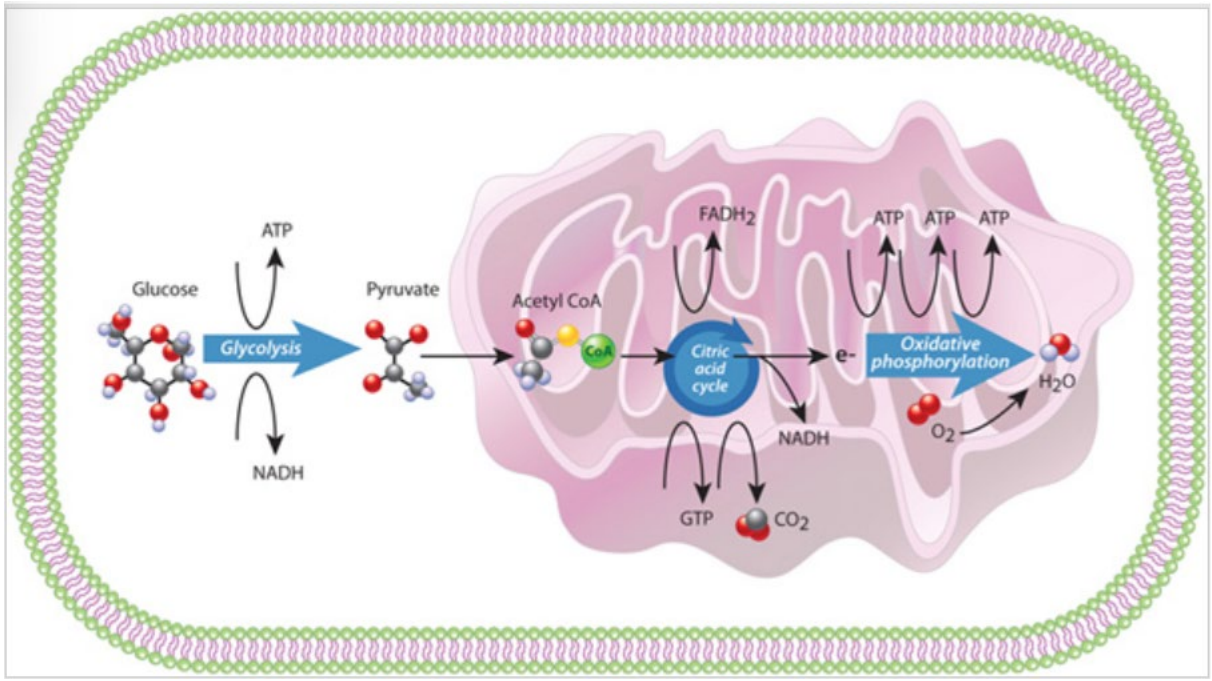


Figure (1.4): Overview of Metabolism in a eukaryotic cell: Glycolysis, the citric acid cycle, and oxidative phosphorylation (10)

1.3.1 Mitochondria:

A mitochondrion is an organelle found in the cell of most eukaryotes' organism; it has a key role in the generation of metabolic energy. Mitochondria are responsible for breakdown the carbohydrates and fatty acids and produce ATP by oxidative phosphorylation. Mitochondria have a double membrane, consisting of an inner mitochondrial membrane. The outer mitochondrial membrane is permeable to most small ions and molecules because of the channel protein mitochondrial porin. The inner membrane, which is folded into ridges called cristae, is impermeable to most molecules (*Figure 1.5).*The most of reactions of Citric acid cycle and fatty acid oxidation take place in the mitochondrial matrix, but the oxidative phosphorylation takes place in the inner mitochondrial membrane (11)

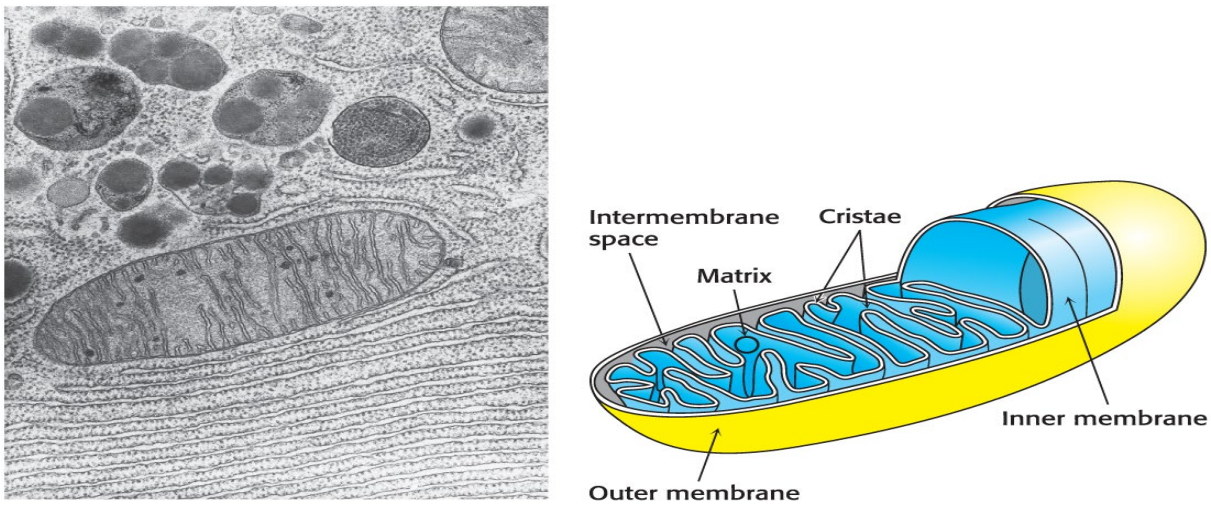
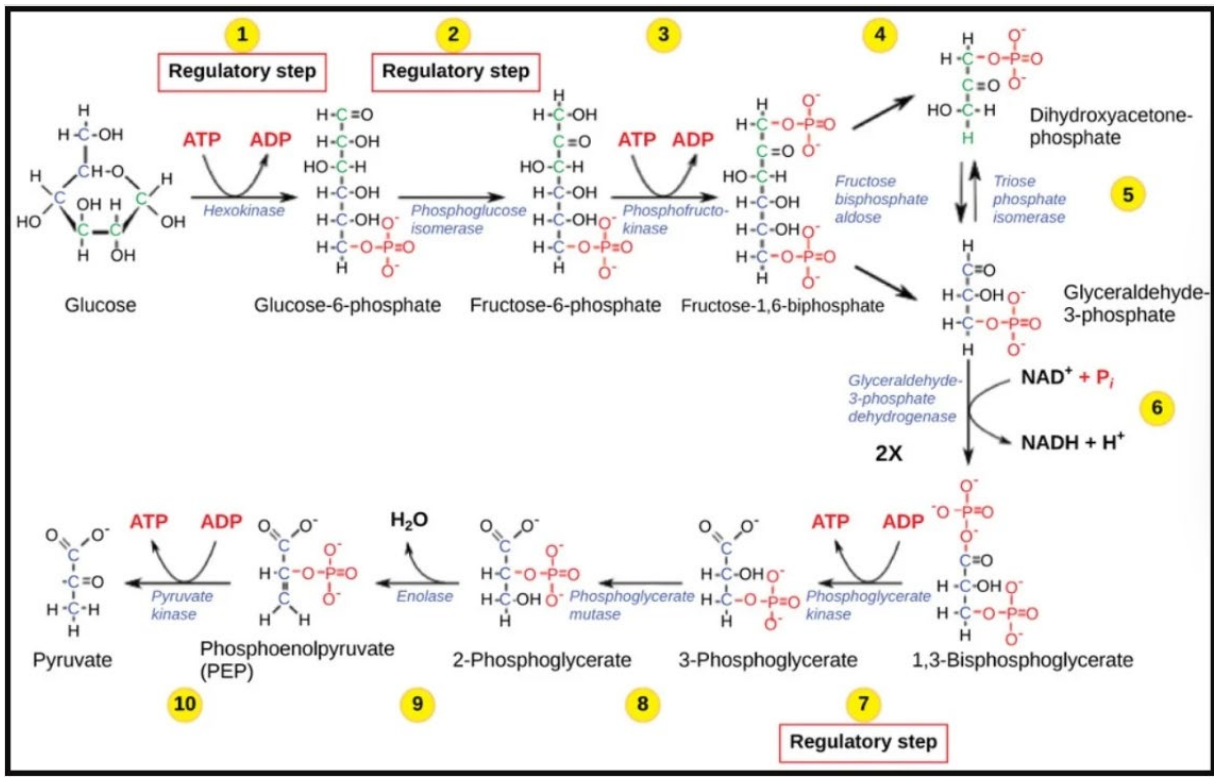


Figure (1.5): overview of mitochondria (11)

1.3.2 Glycolysis:

Glycolysis is the sequence of reactions that breakdown one molecule of glucose into two molecules of pyruvate, two molecules ATP and two molecules NADH (*Figure (1.6)*). This process is anaerobic because it does not require oxygen. These reactions take place in the cytoplasm of the cell. Glycolysis can be divided into two stages. Stage 1 traps glucose in the cell and modifies it so that it can be cleaved into a pair of phosphorylated-3 carbon compounds. In this stage glycolysis require energy in the form of two ATP. Stage 2 oxidizes the 3-carbon compounds to pyruvate while generating two molecules of ATP. In stage 2 it generates four ATP, giving a net production of two ATPs. Glycolysis also converts two molecules of oxidized nicotinamide adenine dinucleotide (NAD+) to Nicotinamide adenine dinucleotide (NADH) (11).
In the present of oxygen, NADH can pass its electrons into the electron transport chain and regenerating NAD^+ for use TCA. But in the absent of oxygen, the cells regenerating NAD^+ by process called fermentation, producing lactate. In this process NADH gives its electrons to an acceptor molecule in a reaction that does not make ATP but does regenerate NAD^+ so glycolysis can continue (12).



Figure(1.6): this figure shows the glycolysis steps (13)

1.3.3 Citric acid cycle:

Under aerobic conditions, the pyruvate produced in the glycolysis is transported into mitochondria by a specific transport protein located in the membrane of mitochondria. Here pyruvate is oxidatively decarboxylated by the pyruvate dehydrogenase complex to form an Acetyl CoA, carbon dioxide and high-energy electrons in the form of NADH. The pyruvate dehydrogenase complex links glycolysis to the citric acid cycle which is the final pathway for the oxidation of the fuel molecules such Carbohydrates, fatty acids, and Amino acids.
Acetyl CoA is the fuel for the Citric acid cycle. In the citric acid cycle, the acetyl CoA combines with a four-carbon molecule, and then it goes through a large series of reactions and ends up with regenerating the four-carbon molecule the circle started with, which can enter a new cycle. This cycle of reactions produces ATP, NADH and reduced flavin adenine

dinucleotide (FADH2) and releases carbon dioxide (*Figure (1.7)*). The net reaction of Citric acid cycle is (11):

$$\text{Acetyl CoA} + 3NAD^+ + FAD + ADP + P_i + 2H_2O \longrightarrow 2CO_2 + 3NADH + FADH_2 + ATP + 2H_2 + CoA \quad (11)$$

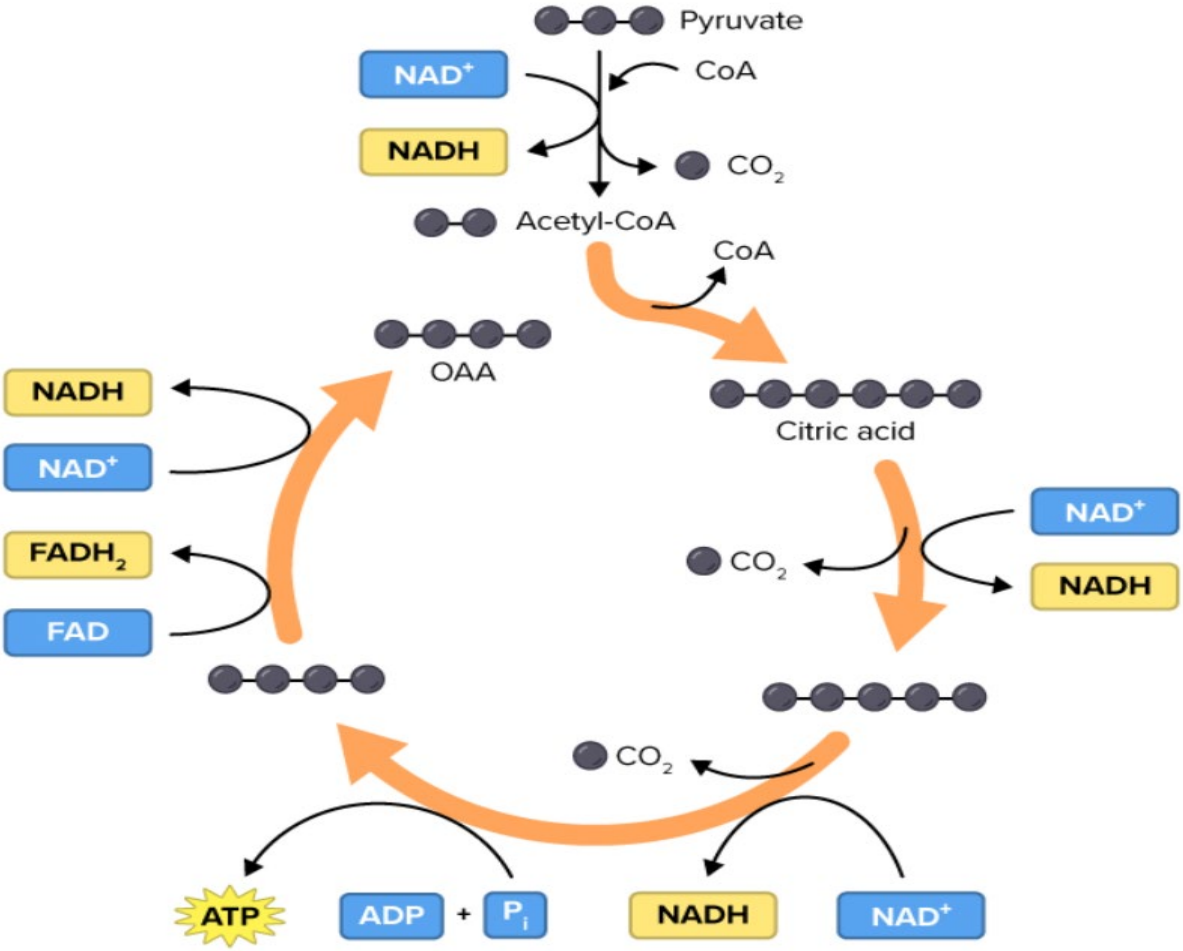


Figure (1.7): the reaction series of Citric acid cycle (14).

1.3.4 Oxidative phosphorylation:

The culmination of aerobic cell respiration is oxidative phosphorylation along the electron transport chain. The NADH and $FADH_2$ molecules formed in the Citric acid cycle are used to reduce oxygen and generate high energy ATP molecules. The electron transport chain consists of four major protein complexes and two electron carriers (*Figure (1.8)*).
Complex I also known as NADH dehydrogenase or NADH oxidoreductase, accepts high-energy electrons from NADH molecules. It also acts as a proton pump that use the movement of electrons to move hydrogen ions into the intermembrane space. Complex I inhibit by Rotenone. Complex II also called succinate reductase. The function of the complex II is to accept the high-energy electrons from $FADH_2$ which is synthesized in the Citric acid cycle. This complex is not proton pump, it does not move hydrogen ions across the inner membrane. Complex III is also called cytochrome c oxidoreductase, it accepts the electrons like complex I. It is a proton pump, and it inhibits by Antimycin. Complex IV also known as cytochrome c oxidase. This complex uses the electrons to pump H^+ ions out of the matrix. It transfers electrons on the oxygen and reducing it to form water. Coenzyme Q (ubiquinone) is a small

hydrophobic molecule located in the inner mitochondrial membrane, it acts as an electron carrier and shuttles electron from complex I/II to complex III. Cytochrome c is a small water-soluble protein that transfers electrons between complex III and complex IV (11)

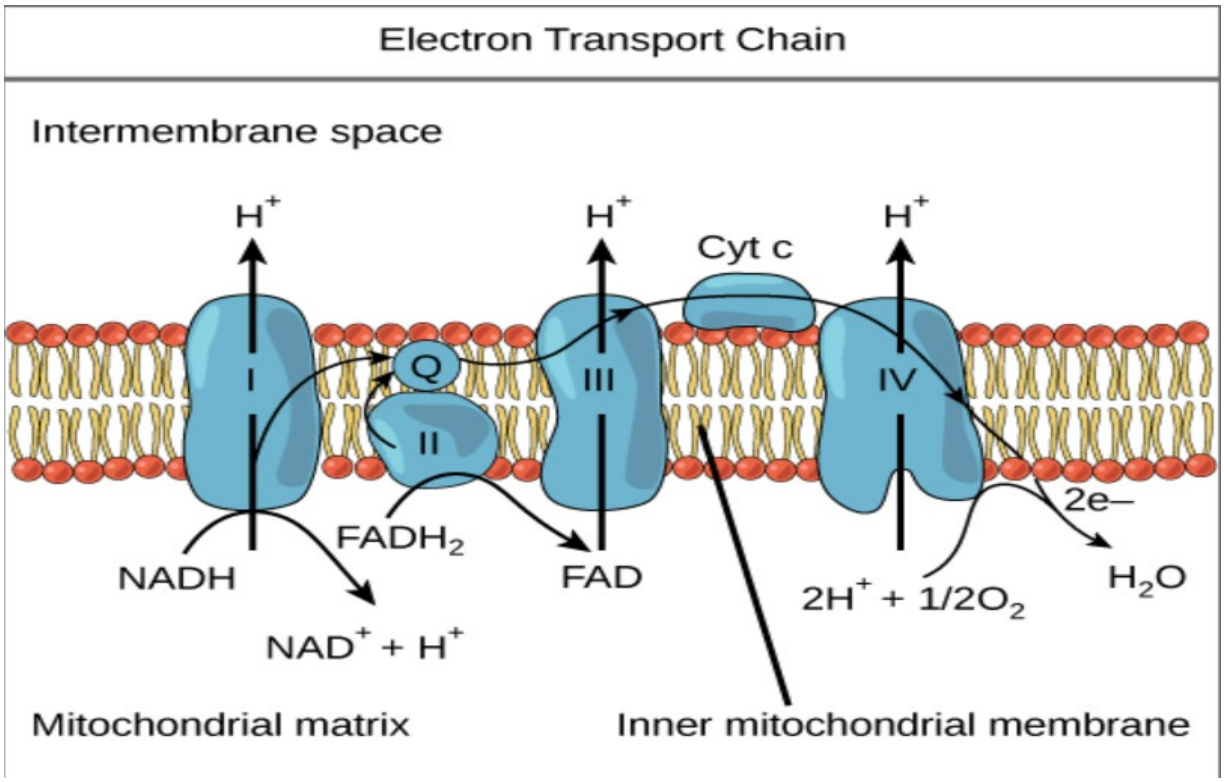


Figure (1.8): Overview of oxidative phosphorylation. The four protein complexes and two electron carriers of electron transport chain (15).

The electron transport chain is a series of proteins that receive the high-energy electrons from NADN and $FADH_2$ molecules and move those electrons along the inner mitochondrial membrane and onto the final electron acceptor, namely oxygen. In the process, a proton gradient is established, this is that used to produced ATP molecules via the activity of a special enzyme called ATP synthase. High-energy electrons are transferred onto the electron transport chain, the movement of electrons stimulates the movement of H^+ ions out of the matrix and into the intermembrane space of the mitochondria. This establishes an electric potential difference between the two sides. The electrons are captured by oxygen to form water. The unequal distribution of H^+ down their electrochemical gradient and back into the matrix, this flow of ions helps generate the ATP.
The oxidation of fuel molecules in glycolysis and Citric acid cycle is coupled to the oxidative phosphorylation of ADP to ATP by the establishment of the hydrogen ion gradient along the ETC (electron transport chain) (11).

1.3.5 ATP synthase:

ATP synthase also known complex V, generates the ATP molecules and uses the proton motive force to release the ATP molecules into the matrix of the mitochondria. The ATP

synthase inhibits by Oligomycin. The ATP synthase is consisting of two major regions. The F_0 region and the F_1 region (*Figure (1.9)*)
F_1 region (catalytic unit) places in the matrix of the mitochondria, it is made of five types of polypeptides chains: α, β, γ, Ɛ and δ chains.
F_0 region (proton pump) is mostly hydrophobic and lies within the inner membrane of the mitochondria. It is making of 10-14 **c** subunits and single **a** subunit (11).

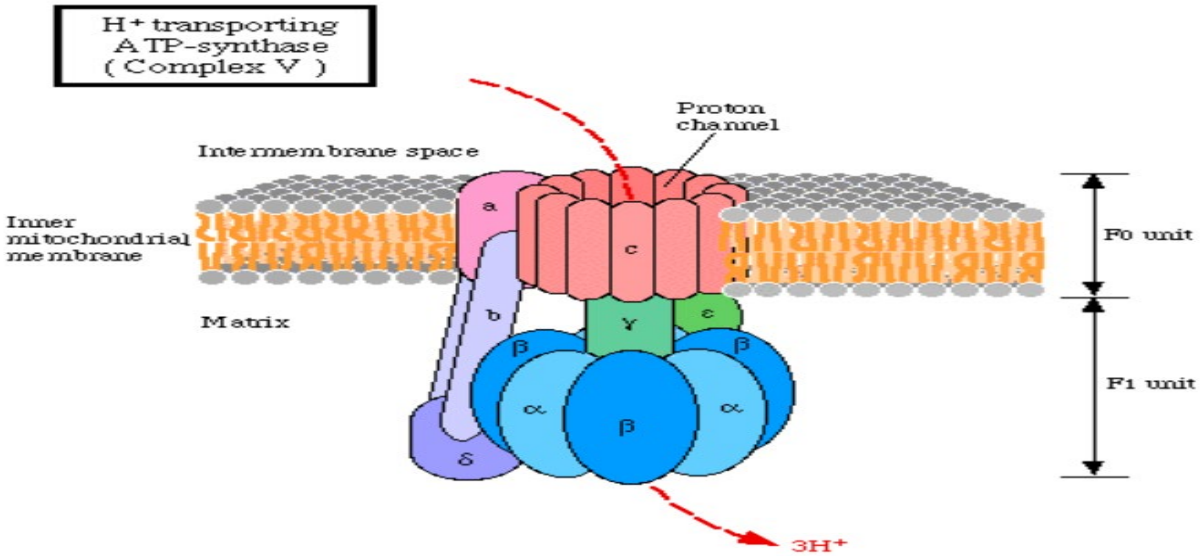


Figure (1.9). shows the different units of ATP synthase. Take. Taken of (16)

1.4 Cancer metabolism:

Mitochondria are intracellular organelles that have important role in energy production, cell metabolism and cell signaling.
They are also very essential in tumor development and metastasis; therefore, they have important role in cancer treatment. Cells are dependent on two main energy pathways: oxidative phosphorylation and glycolysis (17).
Cancer cells change their metabolism to proliferation rapidly. The common altered cell metabolism in cancer cells is the increased glucose uptake and fermentation of glucose to lactate when oxygen is present, and even they have a functionating mitochondria. This phenomenon is known as the Warburg effect and was discovered by Warburg in the 1920s (12). The rate of production of ATP in the aerobic glycolysis is much higher than anaerobic glycolysis, but the metabolism of glucose to production of lactate occurs 10-100 times faster than the complete oxidation of glucose in the mitochondria (18). So can say that cancer cells were performing lactic fermentation, even in aerobic conditions. Therefore, it was concluded that, to kill tumor cells, They should be deprived of energy, both glucose and oxygen (19).
Mitochondria are dysfunctional in various cancers due to somatic mutations of mitochondrial DNA (mtDNA) and defects of mitochondrial enzyme leading to tumorigenesis and tumor progression due to abnormalities of the metabolic pathway or resistance to apoptosis
Mitochondria are essential for physiological processes in eukaryotes, including production of adenosine triphosphate (ATP), regulation of calcium dependent signaling, generation of reactive oxygen species (ROS), and regulation of apoptosis (20).
Mitochondria are dysfunctional in various cancers due to somatic mutations of mitochondrial DNA (mtDNA), the mitochondrial enzyme becomes defective due to abnormalities in the metabolic pathway or resistance to apoptosis, and it leads to tumorigenesis and tumor

progression. Generation of ROS, oxidative stress, and other mutations in mtDNA stimulate mutations of mtDNA (20). The increasing level of the ROS in the cells is leaded to tumorigenic through promotion of genomic instability (21).
Hypoxia-inducible factor 1α (HIF1α), which is overexpression in many cancer cells is stabilized by defects in metabolic enzymes such as succinate dehydrogenase (SDH) and fumarate hydratase (FH) (20).
Tumor cells can generate nicotinamide dinucleotide phosphate (NADPH) through the pentose phosphate pathway (PPP) by metabolizing glucose, that it can ensures the cell's antioxidant defenses against a hostile microenvironment and chemotherapeutic agents and therefore protects the cells. NADPH can contribute to fatty acid synteses (22). Cancer cells use a lot of glucose for anabolic reactions, and they can also use intermediates of the glycolytic pathway ((22)). All these alterations help cancer cells to survive and divide in nutrient depleted conditions and proliferate rapidly.
NADH generated through the TCA cycle in the mitochondrial matrix is oxidized by respiratory complex I and complex II. They two complexes have important role in the production of ATP. The production of ATP hindered by inhibition of complex I and complex II.

1.4.1 Pancreatic cancer cell models

In this project there was studied three pancreatic cancer cell-lines. The first one is Panc-1 from a 56-year-old male, the growth properties were adherent. The second cell-line we used were HPAF-II from a 44-year-old male, the growth properties were adherent, and the third cell- line we were used BxPC-3 from a 61-year-old female, the growth properties were adherent. They are epithelia.

1.5 Mitochondrial uncouplers and inhibitors:

This project deals with studying three different cancer cell lines from the pancreas after treatment with the inhibitors that change cell metabolism (oligomycin, CCCP, Rotenone).

1.5.1 Oligomycin:

Oligomycin is an antibiotic that inhibits ATP synthase. This inhibition occurs by blocking the proton channel (F_0 subunit), which is very important for oxidative phosphorylation of ADP to ATP, and because of that the electron transport chain will stop. The concentration of proton increases because of inhibition of electron transport chain and the free energy released by biological oxidation of substates is not enough to pump any more protons against the steep gradient (23).
The oligomycin is a group of Streptomyces macrolides that was found for the first time in 1954 (*Figure (1.10)*). There will find different isomers of oligomycin (oligomycin A-G) that are specific for the disruption of mitochondria metabolism (24). Oligomycin is toxic and storage at -20c under nitrogen. It is Soluble in DMSO at 300mg/ml and EtOH at 200mg/ml (25)
Oligomycin A is an inhibitor of mitochondrial F_1F_0-ATP synthase that binds to the oligomycin sensitivity-conferring protein (OSCP) at the F_0 subunits 6 and 9 in the F_1F_0-ATP synthase complex, suppresses the TNF-induced apoptosis (24). Oligomycin inhibits HIF-1alpha expression in hypoxic tumor cells. The experiments on the 60 human cancer cell lines from the National Cancer Institute showed that oligomycin is among the top 0.1% of the most cell line selective cytotoxic agents of 37,000 molecules (26)

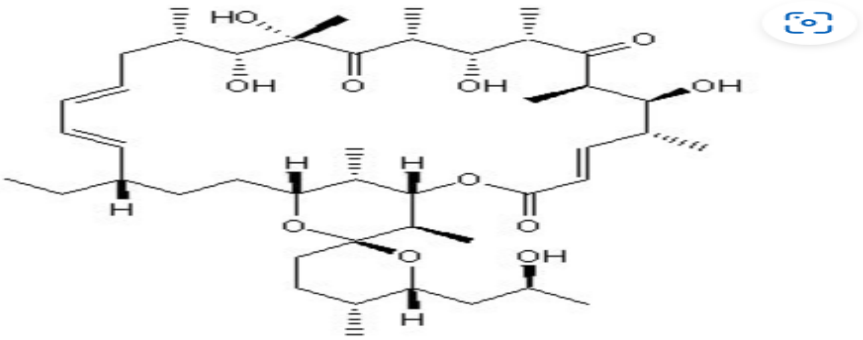


Figure (1.10): Chemical structure of oligomycin A, is taken av (27)

1.5.2 CCCP:

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is a toxic chemical with molecular formula ($C_6H_5ClN_4$) (Figure 7). Can store it at - 20 ℃ in powder form and - 80 ℃ in solvent form (28).

CCCP, a chlorinated is another classical protonophore, it is lipophilic weak acid that is used to study mitochondrial functions in cells. CCCP induced reversible mitochondrial permeability transition pore (mPTP) opening that cause mitochondrial swelling and cells death and therefore can say at CCCP is toxic to many cancer cells (29). CCCP can create pores on the mitochondrial membrane and ions can go in and out of those pores.

Uncoupler of mitochondrial oxidative phosphorylation, CCCP is using to study the function of mitochondria in cellular function. The proton gradient that is establishing during the normal activity of electron carriers in the electron transport chain is inhibiting due to CCCP. It increases the mitochondrial membrane potential (30). The mitochondrial inhibitor (CCCP) is used in study of intracellular Ca^{+2} homeostasis because mitochondria have important role in regulation of intracellular Ca^{+2} by uptake and release Ca^{+2}. Mitochondria by buffering Ca^{+2} can make Ca^{+2} signals in specific cellular locations. Under physiological level of intracellular Ca^{+2} buffering, energized mitochondria are essential for a sustained activation of Ca^{+2} release activated Ca^{+2} channels (CRAC). According to this, CCCP inhibits store-operated Ca^{+2} entry (SOCE)in different of cell types (30).

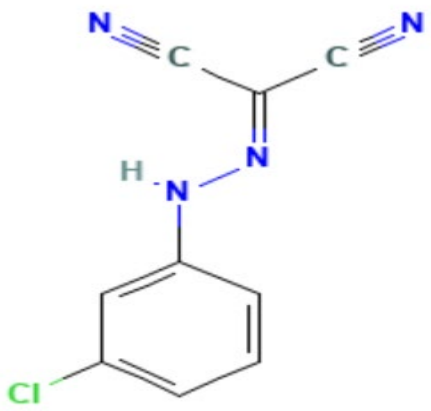


Figure (1.11) shows the chemical formula of CCCP. Taken of (31)

1.5.3 Rotenone:

Rotenone, an organic pesticide that isolated from the roots and barks of the Derris and Lonchorcarpus species. Rotenone inhibits complex I of the electron transport chain, resulting in the generation of reactive oxygen species (ROS) (32). Rotenone causes mitochondrial dysfunction and ultimately cell loss in the nigrostriatal pathway and display anticancer activity through the induction of apoptosis in various cancer cells (33). The studies shows that rotenone can stop the development of cancer such as lung cancer and hepatic cancer, by impairing the autophagic flux and inhibition cancer cells proliferation (34).
Rotenone inhibits the transfer of electros from the iron-sulfur centers in complex I to ubiquinone, leaded to a blockade of oxidative phosphorylation by inhibited ATP synthesis. This incomplete electron transfer to oxygen causes to the formation of reactive oxygen species (ROS). ROS that produced by rotenone damages mitochondrial DNA and may ultimately led to apoptosis (35).
Rotenone with low concentration inhibits the movement and invasion of oral cancer cells by regulating tumor nuclear factor- κB (NF-κB) activity and matrix metalloproteinase (32).

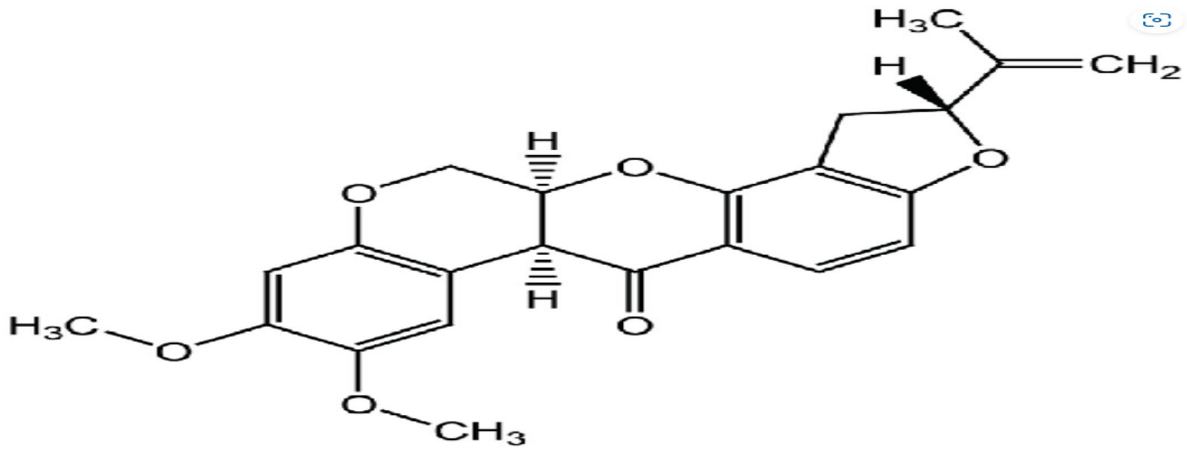


Figure (1.12) shows the chemical structure of Rotenone (36)

1.6 Laboratory instruments and equipment used in this project

1.6.1 AlamarBlue® Cell Viability Assay:

Alamar Blue is used to quantify cellular metabolic activity and determine the concentration of viable cells in a given sample. In this experiment it was used to measuring the growth of a cell papulation over time by measuring it at two points (24 hours and 48 hours) of the sample.
The reagent has an oxidation- reduction indicator that is response to chemical reduction due to the cell growth. The Alamar Blue both fluoresces and change color in response to this chemical reduction.
Alamar Blue has two forms: oxidize and reduced.
The Alamar Blue (resazurin) dye in its oxidized form is blue in color and non-fluorescent. when the resazurin enters the cells reduced to resorufin, which produced red fluoresce. The continued growth of viable cells maintains a reducing environment (fluorescent, red) and inhibition of growth maintains an oxidized environment (non-fluorescent, blue), which can be detected using a fluorescence or absorbance detector (37), see (*Figure (1.13)*).

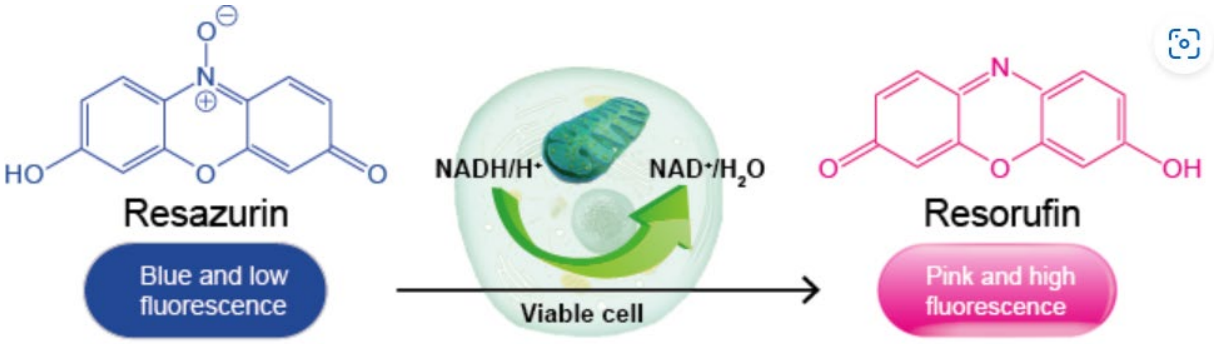


Figure (1.13). Conversion of Alamar Blue dye from oxidized to reduced form. Taken from (38).

The list of laboratory machines that was used in this experiment.
In this experiment were used Muse® Count & Viability kit (200X), SpectraMax® iD5 and Seahorse XF.

1.6.2 Muse® Count & Viability kit (200X):

The Muse® Count & Viability kit (200X) uses for absolute cell count and viability determination of cell sample. This machine finds viable cell count (cells/ml), total cell count (cells/ml) and present of the cell sample. To count these uses the regent that contains the same fluorescent DNA-binding dyes used in the Muse® Count & Viability kit (200X), but in a highly concentrated formulation. The dyes have different permeability to viable and non-viable cells, and both are different stained according to their permeability to the DNA-binding dyes in the regent. The result will be showing in a dot plot on the screen of machine, see *(figure 1.14)* (39).

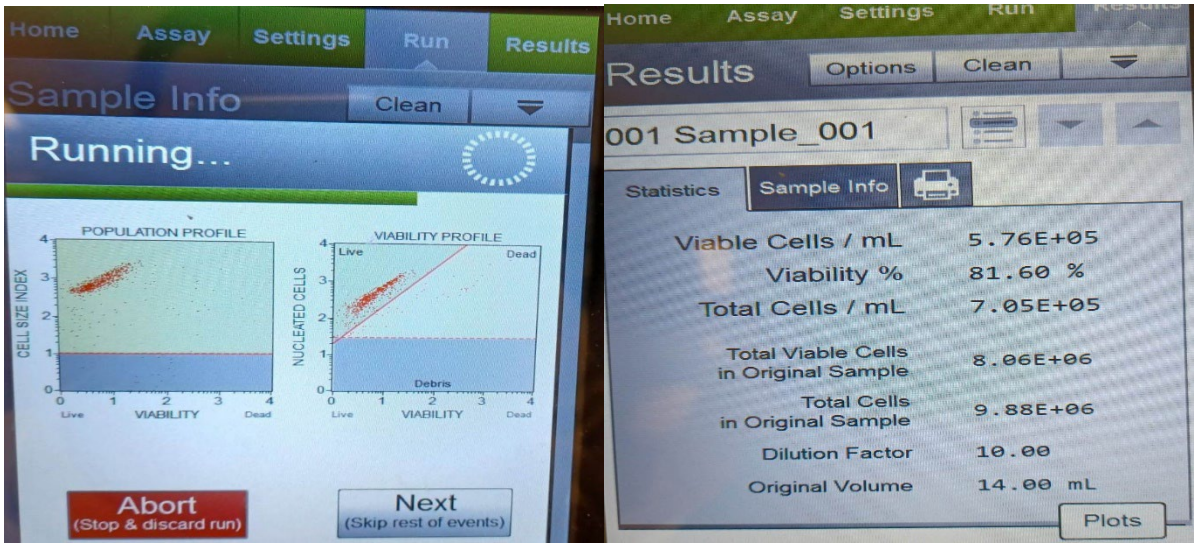


Figure (1.14). the picture of the screen of the Muse count and viability machine from the laboratory UIS.

1.6.3 SpectraMax® iD5:

The SpectraMax® uses to measure the absorbance, fluorescence, time-resolved fluorescence (including HTRF), fluorescence polarization, AlphaScreen®, AlphaLISA®, and luminescence measurements (40). see *Figure (1.15)*.
This machine uses to measure the viable cells by measuring the absorbance and fluorescence with different light wavelengths. The wavelengths that we used here is (540-590 nm).

Figure (1.15). shows the SpectraMax® iD5, taken from (41).

1.6.4 Seahorse XF:

The Seahorse XF analyzer is an instrument used in measuring the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) in live cells. The OCR and ECAR have important role in metabolism and give information about the function of metabolism in the cell. the OCR measures of the mitochondrial function and shows the factors triggering the change from normal oxidative phosphorylation to aerobic glycolysis in cancer cells. The ECAR defines glycolysis by measuring the extracellular acidification rate of surrounding tissues, the excretion of lactic acid after its conversion from pyruvate (42).
To measuring the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) in live cells we well do Agilent Seahorse XF Cell Mito Stress Test by adding modulators of respiration into the wells to find out the key parameters of mitochondrial function. The modulators are Oligomycin, Carbonyl cyanid-4 (trifluoromethoxy) phenylhydrazone (FCCP), Rotenone and Antimycin (43), see *Figure (1.16)*.
The XFe sensor cartridges of Seahorse XF have built-in injection ports that uses for adding the modulators of respiration into the wells (44).

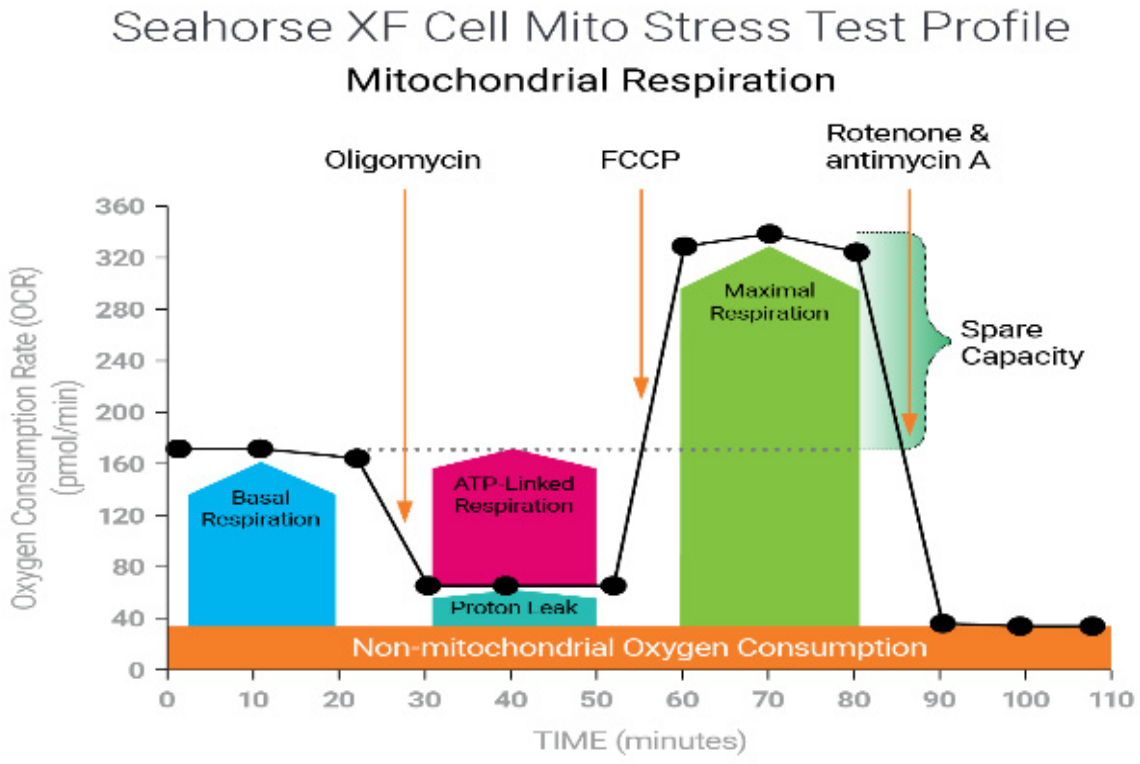


Figure (1.16): shows the expected oxygen consumption rate in pmol/min in the mitochondrial respiration when the modulators oligomycin, FCCP, Rotenone and antimycin A are added (43).

The modulators that adding on Seahorse XF cell Mito stress test inhibiting the electron transport chain in different ways. The oligomycin is the first injection after basal measurements that inhibits the complex V that producers av ATP accrue her. The second injection FCCP is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential. (43).
Electron transport chain is couplet with ATP synthesis. Electron transport chain creates the gradient of hydrogen. The NADH and $FADH_2$ are oxidized, and the electrons are transferred in subsequences step by several complexes, and it used to reduce oxygen to produce water, but couple with this process there is proton pumping in the mitochondrial intermembrane space and that is way amount of proton increases in the intermembrane space, while inside of the membrane matrix the number of protons is less that creates the proton gradient. Using the proton gradient the ATP synthase complex generates ATP, so electron transport chain and ATP synthesis are interlinked and dependent on each other.
The uncoupling agents that who can break this link between electron transport chain and ATP synthesis. One of the uncoupling agents is FCCP that bounds on the intermembrane of mitochondria and allows the hydrogen ions to get in inside the mitochondrial matrix. As a result, the ATP synthesis shut off.
The oligomycin inhibits the hydrogen ions to inter in the mitochondrial matrix but the FCCP allows the hydrogen ions to get in the mitochondrial matrix. As a result, electron flow through the electron transport chain is uninhibited, and oxygen consumption by complex IV reaches the maximum. The FCCP-stimulated OCR can then be used to calculate spare respiratory

capacity, defined as the difference between maximal respiration and basal respiration. Spare respiratory capacity is a measure of the ability of the cell to respond to increased energy demand or under stress.
The third injection is a mixture of rotenone and antimycin A. Rotenone inhibits the complex I and antimycin A inhibits the complex III. This combination inhibits the mitochondrial respiration and consulates the nonmitochondrial respiration outside the mitochondria (43) see *Figure (1.17)*.

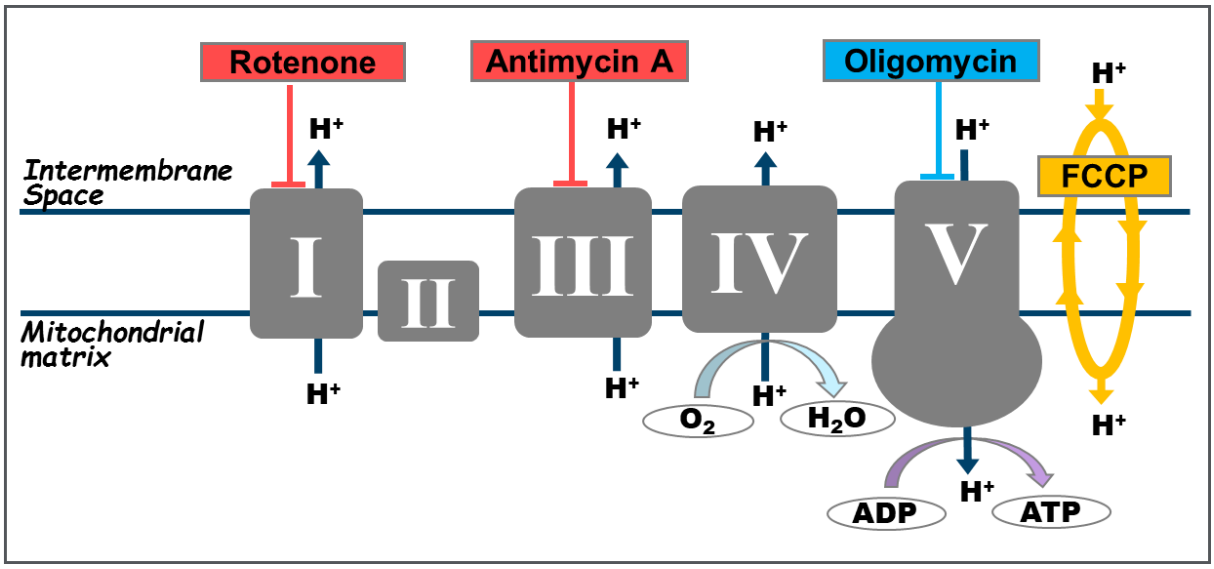


Figure (1.17): Agilent Seahorse XFe Cell Mito Stress Test modulators of the electron transport chain (43).

2. Methods

2.1 Cell Lab working environment:

The cell laboratory where the experiments are performed must be free from bacteria and pathogens and avoid any contamination. To inter the cell laboratory, shoes cover, and lab clothes must worn. There are two doors to entering and only one door could be open at the time. While working in the laboratory, we must wear gloves and disinfected with 70 % ethanol before handling products to prevent contamination. The cancer cells were handled in a biosafety cabinet with an integrated UV-function and laminar air flow that protects the cells from the contamination. The cabinet and everything that is using in cabinet should be cleaned with ethanol before putting it in.

2.2 Chemical equipment and Materials:

All materials and chemicals used in this project were provided from associate professor Hanne Røland Hagland and her lab team. Everything is listed in tables, see table (2.1) and (2.2).

The table (2.1) and (2.2) shows all chemicals, equipment and machines that have used in this project.

Table (2.1): Table of all the chemicals used in this project

Product	Company	Product Number
DMEM (Dulbecco's Modified Eagle's Media) without glucose, L-glutamine and sodium pyruvate	Corning	17-207-CV
Fetal bovine serum, heat inactivated, south American origin	Biowest SAS	S181H-500
Penicillin: Streptomycin solution 6,0/10,0 g/L 100X	Biowest SAS	L0022-100
L-glutamine, 200mM	Corning	25-005-Cl
PBS	ThermoFisher	189112-014
Trypsin EDTA 1X	Corning	25-053-Cl
AlamarBlue	VWR	MFCD00005036
CCCP	Sigma- Aldrich	C2759
Rotenone	Merck Life Science AS	PS99
Oligomycin	SIGMA-ALDRICH	O4876
HPAF II	ATCC	CRL-1997
Antimycin A	Sigma- Aldrich	A8674

Table (2.2): List of important equipment, materials, and machines.

Sterile bench	Electric Pipette	Aspiration-machine
Rack for tubes	Serological pipettes	Vortex mixer
50 mL tube	96-wells plate (96 Wells-F, Surface Treated, Sterile)	SpectraMax® Paradigm® Multi-mode Microplate reader:
15 mL tube	Water bath	Muse® Count & Viability kit (200X):
T75 flasks	Incubator with 5% CO_2	Seahorse XF
Eppendorf tube	Fridge	Centrifuge
Automat pipette	Freezer	

2.3 Cell culturing techniques:

Cells are grown "*in vitro*" that means they are growing outside the body.
Cancer cells are grown in cell culturing flask (T-50 or T-75) with growth media (see figure 2.1). The cell culture media contains important ingredients for the cell growth and proliferation. The cells are kept in an incubator at 37 C and 5% CO_2. The temperature must be like body temperature because cells are growth best in this temperature.

The cells need nutrient from the medium, so the media in the flask is changing regularly with fresh media. When the cells reached a certain confluency, they must be passaged to avoid competition for nutrients and unwanted cell death.

Figure (2.1): Shows the cell culture flaks med media, taken from (45).

2.4 Media:

Cell is the smallest unit that can live on its own and that makes up all living organisms and the tissues of the body. The cell grows and reproduces and needs nutrients to do this. That is why it need the complete cell culture media, that we have made with different chemicals. The table shows the composition of complete cell culture media.

Table (2.3): All components to fix a DMEM complete media on a total volume of 562,22 ml.

Components	Amount in mL
Dulbecco's Modified Eagle's Media, without glucose, L-glutamine and sodium pyruvate)	500
Fetal bovine serum, heat inactivated, South American origin	50
Penicillin; Streptomycin solution 6,0/10,0 g/L 100X	5
L-glutamine, 200 mM	5
Glucose, 1M	2,75

Fetal bovine serum (FBS) is blood serum drawn from a bovine fetus a common component of animal cell culture media. FBS has high level of growth factors and uses in vitro in the lab. FBS also contains a variety of small molecules like amino acids, sugars, lipids, and hormones (46). Glucose and glutamine are nutrients and energy sources for cells. Penicillin is an antibiotic and is used to protect cells from bacteria.

2.4.1 Media preparation:
All components to fix a DMEM complete media for cells are listed on table (2.3).

The glutamine, FBS and penicillin are ready to use on ice form in the freezer. Before using they were putted in the water bath until they reached the temperature of 37°C. The 500 mL Dulbecco's Modified Eagle's Media, without glucose, L-glutamine, and sodium pyruvate) is ready to use. But the glucose is not ready, and we made it.

Preparation of glucose:
to prepare the glucose solution, first we find that how many grams glucose needed to create 50 ml of 1 M glucose. *Equation 1* and *2* were used to fine the gram of glucose.

$$\frac{\boldsymbol{m}}{\boldsymbol{Mm}} = \boldsymbol{n} \qquad \textit{Equation 1}$$

$$\boldsymbol{n} = \boldsymbol{c} \times \boldsymbol{v} \qquad \textit{Equation 2}$$

Molecular weight of glucose = Mm = 180.16 g/mol

m = Mm x n = Mm x (c x v)
m = 180.16 g/mol x (0.05 L x 1 M) = 9.008 g

To make 50 ml glucose solution, 9.008 gr glucose was mixed with water and the solution was filtered through a 0.2 µm filter for the reduction of microbial and particle contamination. Only 2.75 ml of this solution is used to make cell culture media.

Each student used a 50 ml bottle of media that they labeled with their name and cell line. The cell media was changed every second day to ensure that the cells had enough nutrient. This was done in a sterile cabinet by aspirating the current medium and replacing it with 10 ml new DMEM complete medium. Before using the flask was putted in the water bath to reach the 37°C.

2.5 Cell passaging:
The chemicals that were used to cell passaging in this project are: Phosphate-Buffered Saline (PBS) and Trypsin.

2.5.1 Phosphate-Buffered Saline (PBS):
Phosphate-Buffered Saline (PBS) is a buffer solution used in biological research. It is water-based salt solution containing Sodium phosphate, Sodium chloride and potassium phosphate. This solution is buffered therefore it is non-toxic to most cells and used in cell enumeration as a diluent and rinsing cell (47). Her in this experiment it is used to wash and resuspend cells during the dissociation process.

2.5.2 Trypsin:
Trypsin is an enzyme that secreted by the pancreas in the small intestine which splits proteins to polypeptide chain (48). In the culturing flasks cells adhere to the walls by proteins in the extracellular matrix. These proteins need to be digested by the enzyme trypsin before cell

passage to break up adherent cells. In the splitting process of cells, the cells must not be long in trypsin because trypsin makes holes in the cell membrane and the cells die. Therefore, the trypsin should deactivate after at cells was detaching from cell culture flasks, and it is done by fetal bovine serum which is a widely used serum in cell culture medium.

2.5.3 cell passaging

The pancreatic cancer cells were cultured in the cell culture flasks with cell culture media. The cells grown and when the cell density in the flask reached a certain confluency, they started unwanted signaling and stop growing, because there is not enough room to grow more, and it is possible that many cells are damaged and die. The cells in the flask were passaged and splatted and the cell culture flasks were change regularly.
When the cell density reached 70-90 % confluency, they were passaged. It was done in sterile cabinet and all reagents and media used were putted in the water bath to reach at 37°C and then sprayed with ethanol and putted in the cabinet.
First the old media of the flask was aspirated and washed with 5 ml PBS. The PBS was aspirated carefully because it is possible to aspirate the cells. After that it was added 1-2 ml trypsin and putted the flask in the incubator for at cells reacted with this and was detached from the flask wall. The times is depending on the cell lines, but it was taken 5-10 min. A microscope was used to look the effect of trypsin, after detaching the cells it was added 5 ml DMEM complete media to stop the reaction of the trypsin. The solution of media and trypsin was mixed with pipette and then a volume of this was added to a new flask, or a certain volume was kept in the flask and the rest was removed, depending on the split ratio and if the cells needed a new flask. At last, DMEM complete media was added to have a total volume of 10 ml in the flask.

2.6 Test of correct seeding of cells density:

This part is very important to continue the experiment, and it was done many times (3 times on 24 and 48 hours). First the old media was aspirated and washed the cells with 5 ml PBS. The PBS was aspirated and added 1-2 ml trypsin. The cell culture flask was incubated for 2 min and checked in the microscope after the cells was detached of flasks wall, it was added 5 ml DMEM complete media to stop the reaction of the trypsin. The solution of media, cells and trypsin was mixed with pipette and then a volume of this was transferred to a 15 ml tube to keep the cells from adhering. Muse count cell viability Kit measured the cell density. To fine the cell viability, small amount of the solution of the media, cells and trypsin was transferred in the Eppendorf tube. The solution of the media, cells and trypsin was diluted with Muse count regent according to the table (2.4) and placed in the Muse count machine to fin the cell viability. If the percentage of cell viability is lower than 90% then do not continue the test, so transferred all cell suspensions on the cell culture flask and added media that the total volume becomes 10 ml and putted in the incubator and waiting for the cells to grow enough. The percentage of cell viability of the HPAF-II cell line was always low and I continued with the low value.
If the percentage of cell viability is 90% or more, then cells were seeded on the plate with different cell densities, table (2.4).

Table (2.4): Table of correct volumes of muse reagent contra cell suspension for measuring cell viability.

Conc. Of original cell suspension	Dilution factor	Cell suspension volume	Count and viability volume
$1\times105\ to\ 1\times106\ Cells/mL$	10	25 µL	225 µL

$1\times106\ to\ 1\times107\ Cells/mL$	20	10 µL	190 µL
$>1\times107\ Cells/mL$	40	10 µL	390 µL

This table was collected from the protocol av Muse count cell viability kit from cell laboratory. It shows that different cell suspensions use different volume of muse count reagent the dilution factors.

To calculate the new volume of the media, the cell concentration was adjusted to 100000 cells/ml. it is making easier the calculation of other concentrations that were used to making the Platt. See the calculation is on part I.

Part I:

C_1 = Viable cells/ml (from Muse count)
C_2 = 100000 cells/ml (adjusted cell density)
V_2 = 10 ml (total volume of cell suspension + media)
V_1 = volume of cell suspension
V_3 = volume of media

$$V_1 = \frac{C_2 \times V_2}{C_1}$$

$V_3 = V_2 - V_1$

$V_1 + V_3$ = solution of cell suspension and medium that was used to making the six different cell density.

To make 1 ml of six different cell densities, six Eppendorf tubes were used. first it was found that how much cell suspension and medium is needed. Calculation is shown on part II and volumes are listed in the table (2.5).

Part II:

C_1 = different cell density
C_2 = 100000 cells/ml (adjusted cell density)
V_1 = 1 ml (total volume of cell suspension + media)
V_2 = volume of cell suspension (($V_1 + V_3$) solution of part I)
V_3 = medium

$$V_2 = \frac{C_1 \times V_1}{C_2} \qquad \textit{Equation 3}$$

$$V_3 = V_1 - V_2 \qquad \textit{Equation 4}$$

The *Equation 3* and *4* were used to calculate the V_2 and V_3. These volumes and the six different cell density are listed on the table (2.5).

Table (2.5): Different cell density and volume of cell suspension and medium that is seeding on the plate to find correct cell density.

C_1	V_2 (µL)	V_3 (µL)
2500	125	875
5000	250	750
7500	375	625
10000	500	500
15000	750	250
20000	1000	0

Three parallels of each density (200 µl) were seeded out on a 96 well plate (96 Wells-F, Surface Treated, Sterile) with increasing cell number downwards. 200 µl PBS was seeded out in the remaining empty wells, except for the four outermost corners. The PBS is adding to prevent cells from drying out. The plate was placed in incubator to incubate for 24 hours in a cell incubator at 37°C until the next day to perform Alamar Blue assay. After incubation, 20 µL 484 µM resazurin at 37°C, which is active reagent in Alamar Blue assay that acts like un intermediate electron acceptor in the electron transport chain without interfering with the normal function of the chain. When Resazurin accepts electrons, it is reduced to the pink, highly fluorescent resorufin, was added to each of the three parallels of cell density. 220 µl of a positive control containing autoclaved medium + resazurin (44 µM Resazurin autoclaved) resorufin was seeded into the four empty outermost corners of the plate. The plate was incubated in the cell incubator at 37°C for 4 hours, see (figure 2.2). After incubation, the plate was read by Spectro Max iD5 to measure the fluorescence at 540-590 nm and hence the cell concentration.

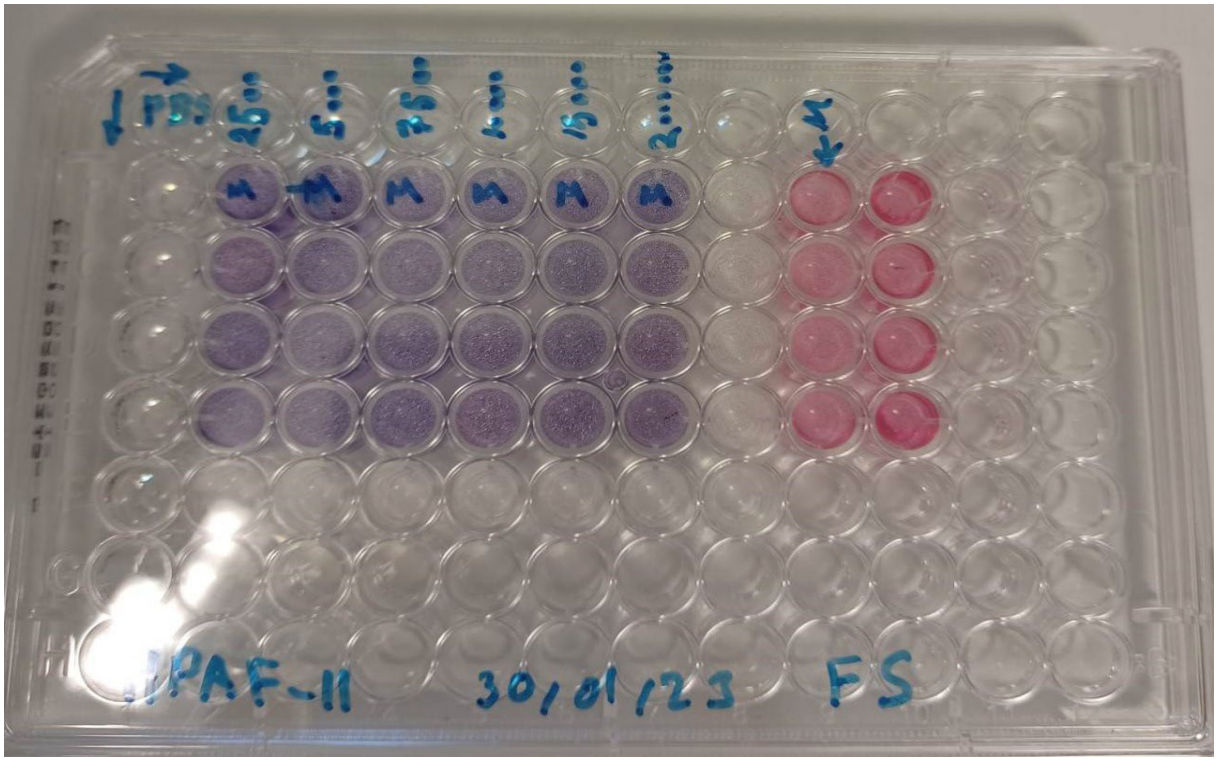


Figure (2.2): shows the plate for different concentrations of cell suspension with Alamar Blue assay after 4 hours.

Preparation of Resazurin:

For making Resazurin, the Resazurin powder with 484 µM concentration and 229 gr/mol Molecular weight was mixed with 44 ml or 0.044 L PBS. Fra *Equation 5* and *6*, the mass of Resazurin was calculated and mixed with 44 ml PBS.

Calculation:
C = 484 µM = 0.000484 Molar
M = 229.19 gr/mol
V = 44 ml = 0.044 L PBS

n = m/M *Equation 5* **C = n/v** *Equation 6*
From Equation 5,6:
m = C x V x M = 0.000484 mol/L x 0.044 L x 229.19 gr/mol = 0.005 gr

preparation of Resorufin:
Resorufin was made by mixing 1 ml of Resazurin and 10 ml PBS. The solution was autoclaved. The 100% reduced form of Alamar Blue reagent (Resorufin) uses as positive control in fluorescence measurements, and it prepared by autoclaving a sample containing 10 ml PBS and 1 ml Alamar Blue (resazurin) for 15 minutes. Resorufin was made by mixing 1 ml of Resazurin and 10 ml PBS. The solution was autoclaved.
Alamar Blue assay stores at 4°C and protects from light because it is light sensitive. If it exposed long time in the light will result in increased background fluorescence in the assay and decreased sensitivity (37).

The following assay controls are recommended for each assay plate set up (Table 2.6) (37):

Negative Control	Untreated Control	Positive Control (100% Reduced)
No Cell Control may be set up without the cells. This will serve as the Negative Control to determine background fluorescence that might be present.	Untreated Control may be set up with untreated cells. Same solvent used to deliver the test compound may be added to the control wells.	Positive Control with 100% reduced AlamarBlue is necessary to assay viability using fluorescence values. To prepare 100% reduced form of AlamarBlue Reagent, autoclave a sample containing cell culture media and AlamarBlue for 15 minutes.

2.7 Test of various mitochondrial inhibitors (CCCP, Rotenone and Oligomycin) in cells over time

Here, dilutions of the various mitochondrial inhibitors were made and evaluated to see how the cells react to these after 24 hours or 48 hours. This experiment was done three times. From the cell viability experiments, it was chosen which cell density (7500 cells/well) to use

for these experiments. The concentrations for the various inhibitors were found from previously published studies on cancer cells.
In this experiment the cells were seeded like cell density or cell viability but just with one cell concentration (7500 cells/ml). The cells were seeded on two plates that are identical in treatment, but one was added Alamar blue assay, which assesses cell growth, after 24 hours and measured the fluorescence and the other after 48 hours.
It was used six different concentrations for each inhibitor. It was used six Eppendorf tubes to making these concentrations for each concentration. To making these concentrations it was needed to fine the volume of these inhibitors that is calculated on the calculation part for each inhibitor.

- Cells were passaged and measured the cell viability with Muse count machine.
- It was made (7500 cell/will) cell concentration for each well, see part I and II, and it was seeded 200 µL in each well. Three or four parallels were made for each dilution.
- The plates were kept in the incubator.
- Next day, these different concentrations of inhibitors (table 2.5-2.7) were made, 100 µL av medium of the wells taken out and 100 µL of the concentrations of inhibitors that were made, added to the wells, but not in the one of the colons, because it was used for Untreated Control, without inhibitors.
- The plates were kept in incubator.
- The third day, it was added resazurin in the wells with different concentration of inhibitors, the colon without inhibitors and the colon (negative control) without cells just medium. One colon with four parallels resorufin. This day just one of the plates was used.
- The plat was kept in the incubator and measured fluorescence after 4 hours. The results were plotted. The graphs are in the Result part.
- The Alamar blue assay was added next day on the second plat and measured the fluorescence after 4 hours by Spectro Max.
- Figure (2.3) is an example of the plat with CCCP (C), Medium (M), Resazurin (RZ), Resorufin and PBS.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	
B	PBS	M+RZ	0C+RZ	0.1C+RZ	1C+RZ	5C+RZ	10C+RZ	25C+RZ	50C+RZ	Resorufin	PBS	
C	PBS	M+RZ	0C+RZ	0.1C+RZ	1C+RZ	5C+RZ	10C+RZ	25C+RZ	50C+RZ	Resorufin	PBS	
D	PBS	M+RZ	0C+RZ	0.1C+RZ	1C+RZ	5C+RZ	10C+RZ	25C+RZ	50C+RZ	Resorufin	PBS	
E	PBS	M+RZ	0C+RZ	0.1C+RZ	1C+RZ	5C+RZ	10C+RZ	25C+RZ	50C+RZ	Resorufin	PBS	
F	PBS	M	M	M	M	M	M	M	M		PBS	
G	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	
H												

Figure (2.3): Example of a plate with different concentration of CCCP. M (medium), RZ (resazurin), C (CCCP).

2.7.1 Calculation of different cell dilutions for different mitochondrial inhibitors:

- **CCCP:**

The stock concentration of the CCCP is 1 mM that was used in the experiment. The total volume (volume of CCCP + medium) is 500 µL. the volume of the CCCP was found from *Equation 7*. The concentrations of CCCP that were determined are: 0.1µM, 1 µM, 5 µM, 10 µM, 25 µM and 50 µM.

C_1 = 1 mM = 1000 µM (stock concentration of CCCP)
C_2 = (0.1µM, 1 µM, 5 µM, 10 µM, 25 µM and 50 µM)
V_2 = 500 µL
V_1 =? (Volume of CCCP)
Volume of medium = $V_2 - V_1$

$$C_1 \times V_1 = C_2 \times V_2 \qquad \textit{Equation 7}$$

The value of V_1 and the medium are listed on the table (2.7).

Table (2.7): the value of V_1 and medium.

Number	Concentration (µM)	V_1 (µL)	Volume of medium (µL)
1	50	25	475
2	25	12.5	485.5
3	10	5	495
4	5	50 µL of 50 µM	450
5	1	50 µL of 10 µM	450
6	0.1	50 µL of 1 µM	450

- **Rotenone:**

The stock concentration of the Rotenone is 2 µM that was used in the experiment. The total volume (volume of Rotenone + medium) is 1000 µL. the volume of the Rotenone was found from Equation 7. The concentrations of Rotenone that were determined are: 1 nM, 10 nM, 100 nM, 200 nM, 1000 nM, and 2000 nM.
The value of V_1(volume of Rotenone) and the medium are listed on the table (2.8).

Table (2.8): the value of V_1 (Rotenone) and medium.

Number	Concentration (nM)	V_1 (µL)	Volume of medium (µL)
1	2000	2	998
2	1000	1	999
3	200	100 µL of 2000 nM	900
4	100	100 µL of 1000 nM	900
5	10	100 µL of 100 nM	900
6	1	100 µL of 10 nM	900

- **Oligomycin:**

The stock concentration of the Oligomycin is 2 µM that was used in the experiment. The total volume (volume of Oligomycin + medium) is 1000 µL. the volume of the Oligomycin was found from Equation 7. The concentrations of Oligomycin that were determined are: 1 nM, 10 nM, 50 nM, 100 nM, 250 nM, and 500 nM.
The value of V_1(volume of Rotenone) and the medium are listed on the table (2.9).

Table (2.9): the value of V_1 (Rotenone) and medium.

Number	Concentration (nM)	V_1 (µL)	Volume of medium (µL)
1	500	50	950
2	250	25	975
3	100	10	990
4	50	100 µL of 500 nM	900
5	10	100 µL of 100 nM	900
6	1	100 µL of 10 nM	900

2.8 Seahorse analysis:

The Agilent Seahorse XF Cell Mito Stress Test:

The Agilent Seahorse XF Cell Mito Stress test measures two key energy metabolism pathways: Respiration and Glycolysis
Mitochondrial Respiration or oxidative metabolism is one of the keyways a cell generate energy. When oxygen is available cells respire to generate ATP, consuming oxygens, and many substrates such as fatty acids, glucose, and glutamine. Respiration is measured via decreases of oxygen concentration in the assay media. This called oxygen consumption rate or OCR.
cell also generates ATP through glycolysis, the conversion of glucose to lactate independent of oxygen. Lactate is the primary source of protons in the medium of cultured cells. Glycolysis measures by measuring the accumulation of protons in the assay media also known extracellular acidification rate or ECAR.
By measuring these two metabolic pathways will determine cellular phenotype and function in the real time.
In this experiment, it was used three kits, see table (2.10).

Table (2.10): Agilent Seahorse XF Cell Mito Stress Test Kit foil pouch contents

Compound	Cap color	Quantity per tube (nmol)
Oligomycin	Blue	63
FCCP	Yellow	72
Rotenone + antimycin A	Red	27 (of both)

2.8.1 Seeding the cells:

Cells were seeding in Seahorse XF Cell Culture Microplates by 20000 cells/ml cell density. The plates were kept in the incubator. The next day making the correct concentration of inhibitors (CCCP, Rotenone and oligomycin) and added on the wells. The stock concentration of inhibitors is 50 mM, but we just needed 10 nM of these. To obtain 10 nM concentration of inhibitors, it was diluted in three steps.

Step I

To obtain 1 mM:

$$V_1 = \frac{1mM \times 1000\ \ \mu l}{50mM} = 20\ \mu L,\ \ 980\ \mu L\ medium$$

Step II

To obtain 10 µM:

$$V_1 = \frac{10\ \mu M \times 1000\ \ \mu l}{1000\ \mu M} = 1\ \mu L,\ \ 999\ \mu L\ medium$$

Step III

To obtain 10 nM (final concentration):

$$V_1 = \frac{10\ nM \times 4000\ \ \mu l}{1000\ \mu M} = 4\ \mu L,\ \ 996\ \mu L\ medium$$

100 µL av medium of the wells taken out and 100 µL of the concentration of inhibitors that was made, added to the wells, but not in the one of the colons, because it was used for Untreated Control, without inhibitors. The plate was placed in the incubator at 37 °C with 5% CO_2. Figure (2.4) shows the Seahorse XF Cell Culture Microplates setup. The sensor cartridge in Seahorse XF Calibrant was hydrated at 37 °C in a non-CO2 incubator overnight. It is better to put a petri dish of water in the incubator to prevent cells from drying out. Agilent Seahorse XFe/XF Analyzer was turned on, and left it warm up overnight (minimum five hours). The Seahorse XFe should be ready for next deg, the day of assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bakgrunn (ingen celler	Panc 1 m/10nM Rot	Panc 1 m/10nM CCCP	Panc 1 m/10nM oligomycin	HPAF-II kontroll	HPAF-II m/10 nM Rot	HPAF-II m/10 nM CCCP	HPAF-II m/10 nM Oligo	BxPC-3 kontroll	BxPC-3 m/10 nM Rot	BxPC-3 m/10nM CCCP	Bakgrunn (ingen celler
B	Panc 1 kontroll	Panc 1 m/10nM Rot	Panc 1 m/10nM CCCP	Panc 1 m/10nM oligomycin	HPAF-II kontroll	HPAF-II m/10 nM Rot	HPAF-II m/10 nM CCCP	HPAF-II m/10 nM Oligo	BxPC-3 kontroll	BxPC-3 m/10 nM Rot	BxPC-3 m/10nM CCCP	BxPC-3 m/10nM Oligo
C	Panc 1 kontroll	Panc 1 m/10nM Rot	Panc 1 m/10nM CCCP	Panc 1 m/10nM oligomycin	HPAF-II kontroll	HPAF-II m/10 nM Rot	HPAF-II m/10 nM CCCP	HPAF-II m/10 nM Oligo	BxPC-3 kontroll	BxPC-3 m/10 nM Rot	BxPC-3 m/10nM CCCP	BxPC-3 m/10nM Oligo
D	Panc 1 kontroll	Panc 1 m/10nM Rot	Panc 1 m/10nM CCCP	Panc 1 m/10nM oligomycin	HPAF-II kontroll	HPAF-II m/10 nM Rot	HPAF-II m/10 nM CCCP	HPAF-II m/10 nM Oligo	BxPC-3 kontroll	BxPC-3 m/10 nM Rot	BxPC-3 m/10nM CCCP	BxPC-3 m/10nM Oligo
E	Panc 1 kontroll	Panc 1 m/10nM Rot	Panc 1 m/10nM CCCP	Panc 1 m/10nM oligomycin	HPAF-II kontroll	HPAF-II m/10 nM Rot	HPAF-II m/10 nM CCCP	HPAF-II m/10 nM Oligo	BxPC-3 kontroll	BxPC-3 m/10 nM Rot	BxPC-3 m/10nM CCCP	BxPC-3 m/10nM Oligo
F	Panc 1 kontroll	Panc 1 m/10nM Rot	Panc 1 m/10nM CCCP	Panc 1 m/10nM oligomycin	HPAF-II kontroll	HPAF-II m/10 nM Rot	HPAF-II m/10 nM CCCP	HPAF-II m/10 nM Oligo	BxPC-3 kontroll	BxPC-3 m/10 nM Rot	BxPC-3 m/10nM CCCP	BxPC-3 m/10nM Oligo
G	Panc 1 kontroll	Panc 1 m/10nM Rot	Panc 1 m/10nM CCCP	Panc 1 m/10nM oligomycin	HPAF-II kontroll	HPAF-II m/10 nM Rot	HPAF-II m/10 nM CCCP	HPAF-II m/10 nM Oligo	BxPC-3 kontroll	BxPC-3 m/10 nM Rot	BxPC-3 m/10nM CCCP	BxPC-3 m/10nM Oligo
H	Bakgrunn (ingen celler	Panc 1 m/10nM Rot	Panc 1 m/10nM CCCP	Panc 1 m/10nM oligomycin	HPAF-II kontroll	HPAF-II m/10 nM Rot	HPAF-II m/10 nM CCCP	HPAF-II m/10 nM Oligo	BxPC-3 kontroll	BxPC-3 m/10 nM Rot	BxPC-3 m/10nM CCCP	Bakgrunn (ingen celler

Figure (2.4): shows the Seahorse XF Cell Culture Microplates setup. Cells lines: Panc 1, HPAF-II and BxPC 3. m (with), Rot (Rotenone) and Oligo (Oligomycin).

2.8.2 Day of assay

- **Prepare seahorse assay medium:**
  1. 250 µL of 1 M glucose
  2. 500 µL of 200 mM of L-glutamine
  3. 60 µL of 1 M NaOH
  4. 49 ml + 190 µL of Seahorse base media

It was added all these ingredients on the base media to making the Seahorse assay medium. But NaOH should not be added at once, 20 ml was added time after time and check the PH, PH must be about 7.4. Assay media was putted in the water bath to reach at 37°C and it is ready to use.

- **Prepare Agilent Seahorse XF Cell Culture Microplate for assay:**

The Seahorse XF Cell Culture Microplate was removed from 37°C incubator with 5% CO2 and examined the cells under a microscope to confirm confluence. The assay medium was removed and placed on the cell lab cabinet. The cell culture growth medium was aspirated, and the cells washed with 100 µl seahorse assay medium one time and added 180 µL seahorse assay medium in the wells. The cell culture microplate was placed into a 37 °C non-CO2 incubator for 45 minutes to 1 hour.

- **Prepare compound stock solutions and working solutions:**

There were three tubes in the Seahorse XF Cell Mito Stress Test Kit box that was contained oligomycin (blue cap), FCCP (yellow cap), and rotenone/antimycin A (red cap). The tubes were placed in a small tube rack with glove hand. The cap from each tube by inserting the cap's tooth and the contents of each tube were resuspended with prepared assay medium in volumes described in table (2.11). The assay medium was pipetted in each tube and mixed very well to solubilize the compounds. These were the compound stock solutions.

Table (2.11): Stock solutions

compound	Volume of assay medium	Stock concentration
Oligomycin	630 µL	100 µM
FCCP	720 µL	100 µM
Rot/AA	540 µL	50 µM

The stock solutions were used to make compound working solutions for loading into the injection ports on sensor cartridges. The volumes that were used to make the working solutions and the volume of working solution that will added to the port indicated in table (2.12).

Table (2.12): Compound preparation for loading to XFe/XF96 sensor cartridges.

	Final well (µM)	Stock solution volume (µL)	Media volume (µL)	10X (Port) (µM)	Volume added to port (µL)
Port A Oligomycin	1.5	450	2.550	15	20
	0.5	150	2.850	5	20
Port B FCCP	1	300	2.700	10	22
	0.5	150	2.850	5	22
Port C Rot/AA	0.5	300	2.700	5	25

- **Load solutions into the ports on sensor cartridge:**

The working solution was loaded into the ports on sensor hydrated cartridge plate. The volume is indicted in table (2.10). Before inserting the cartridge in the Seahorse XFe, check the temperature of the machine and it should be 37 °C. The lid of the cartridge was removed and placed into the XFe 96 and clicked the prompt of software and the tray was closed. The machine was runed some validation steps to check it is correctly sensing oxygen and pH. This was taken

ca. 20 min. After that, the tray was opened, taken out the utility plate and placed the cell plate on the tray. The loaded cartridge was held inside the machine. After placing the cell plate on the tray clicked the prompt in the software and tray was closed and started to read the assay. The Seahorse XF Mito Stress Test Report Generator automatically calculates the Seahorse XF Cell Mito Stress Test parameters from Wave data that has been exported to Excel.
Once the analysis was complete, a software prompt appears to eject the tray and remove the cartridge. The top of the cartridge was discarded, and the cell plate was frozen to use for further experiments such as normalization assays.

2.8.3 Protein concentration test:

In this experiment it will be find that how many cells do we have in this concentration.
It was used 225 µL PBS to make a new plate to kip the balance in the centrifuge machine, and 200 µL working reagent that was ready to use in the lab.

- It was made a new cell plate, but without cells. In this plate it was added 225 µL PBS into each well.
- It was added 25 µL PBS on the cell plat with cell and after that added 200 µL working reagent on each well.
- The plates were placed in the centrifuge for 30 sec and at 25 °C.
- After that, the plate with cells was incubated at 37 °C for 30 min without CO_2. After incubating the color of the solution in the wells were changed to purple.
- The plate was placed in the Spectro Max R machine and measured the absorbance with wavelengths from 540–590.
- The data of absorbances were plotted resulting in the standard curve.

3. Result:

3.1 Pancreatic cancer cell growth:

The pancreatic cancer cell lines HPAF-II, PANC-I and BxPC-3 were used in this project. The cell line that I worked with is HPAF-II (Figure 3.1).
They grew in the cell culture medium throughout the experiments and passaged regularly when approaching approximately 70% confluency. HPAF-II grows slowly then the PANC-I and BxPC-3, and therefore it was passaged at a lower ratio to make sure the density of cells was kept at the number needed to complete our experiments, for example if the other cell lines were passaging 1:8, I was passaging 1:2.

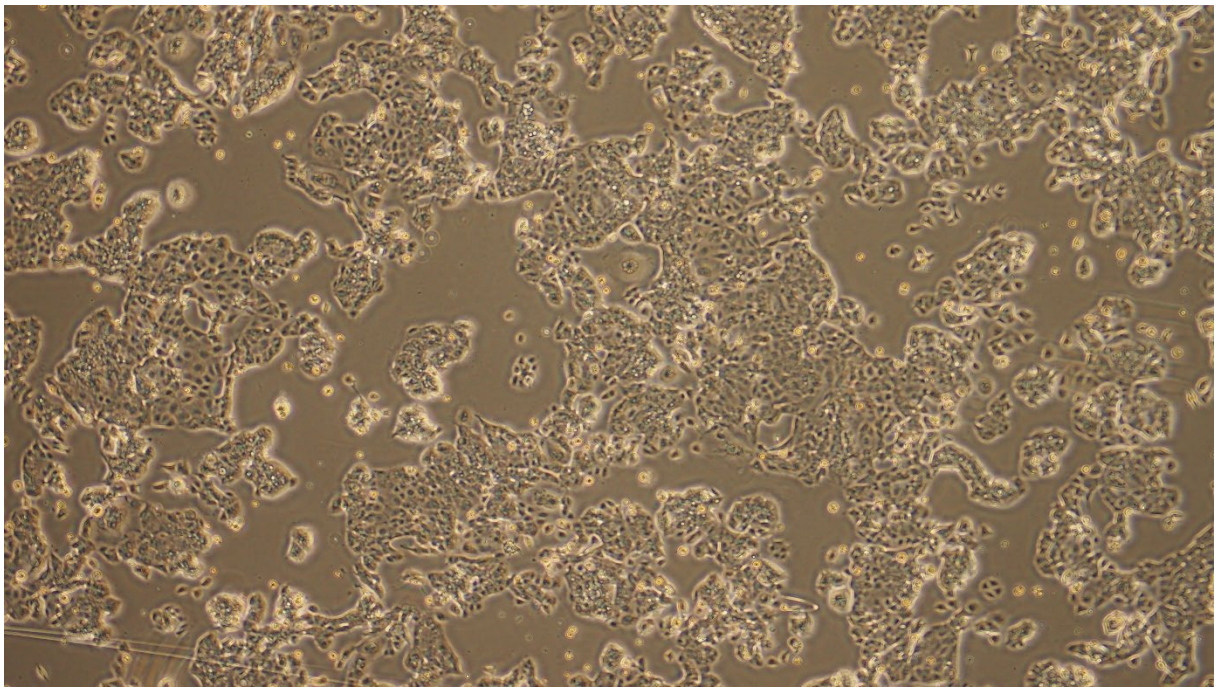


Figure (3.1): Shows the cell line HPAF-II.

3.2 Test of correct seeding of cells density:

To find the optimal seeding density to be used for drug treatment experiments we seeded different cell concentrations in 96 well cell culture plates and incubated for 24 hours. After incubation added Alamar Blue assay and after 4 hours the fluorescence was measured at 540-590 nm in the Spectro Max. Results from Spectro Max were plotted in the excel (Appendix A). the x axis is cell amount, y axis is measuring the fluorescence.

I collected the results from the three weeks in one graph (*Figure 3.2*). we can see at fluorescence values increasing with increasing the cell concentration.

In the figure (3.2, week 1) we can see at the fluorescence increase with increasing the cell concentrations. There is not much different in the fluorescence between 2500 and 5000 cells. They have quite the same value.
In the *figure (3.2, week 2)* we can see that the fluorescence increasing also with increasing the cell concentration. But in this week, there is not much different between the 10000 and 15000 cells. The fluorescence did not change much from 10000 to 15000 cells.
In the *figure (3.2, week 3)*, we can see slightly different results. The graph is not like a straight line that rises with increasing concentration
Finally, we decided that we will use 7500 cells/well to perform the rest of the experiments with CCCP, Rotenone and Oligomycin. Because in this density there is enough place for cells to grow.

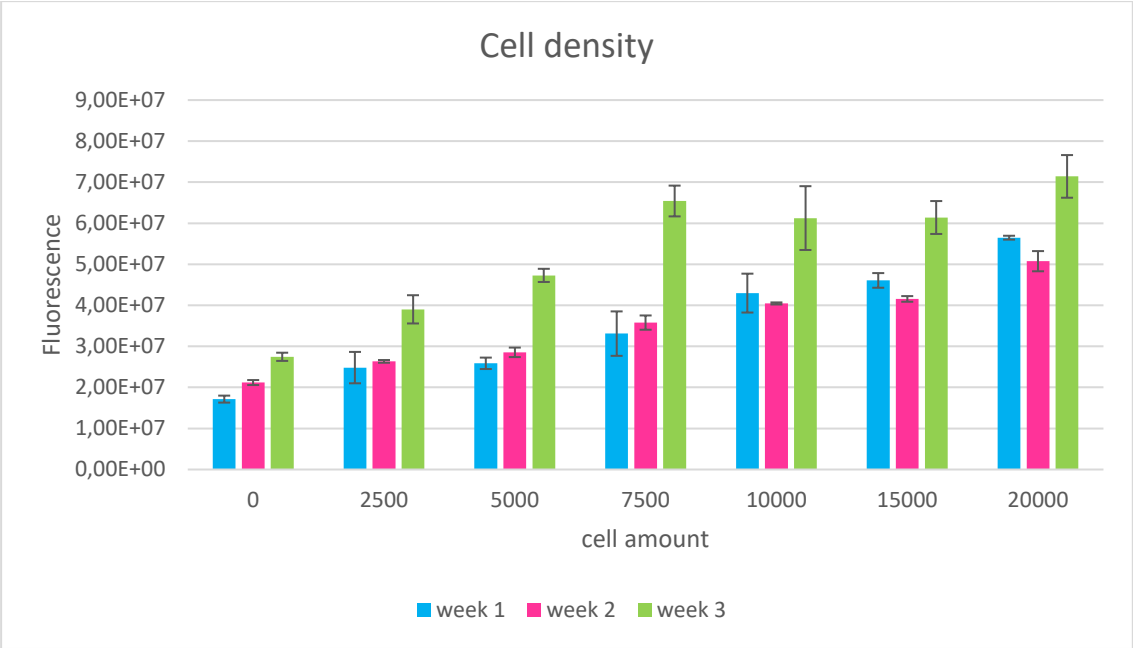


Figure (3.2): Alamar Blue cell viability assay results of three weeks.

3.3 Test of various mitochondrial inhibitors (CCCP, Rotenone and Oligomycin) in cells over time:

This test was done three times and on the different times (24 and 48 hours). The fluorescence was measured by Spectro Max, and the results were plotted. In the Appendix (B, C and D) you can find all the graphs and data from Spectro Max.
Here the data is normalized to control and presented as a function over all the three experiments for each inhibitor. See *Figure (3.3), (3.4), (3.5)*.

3.3.1 Effect of CCCP on 24 and 48 hours:

On the *Figure (3.3)*, we can see at with increasing the concentration of CCCP (0, 0.1, 1, 5, 10, 25,50 µM) the percent of cells are decreasing. But effect of the CCCP on the pancreatic cancer cell (HPAF-II) is more on the 48 hours. Here we can see that effect of CCCP with 25 and 50 µM is very high, because the cell viability of the cells is very low.

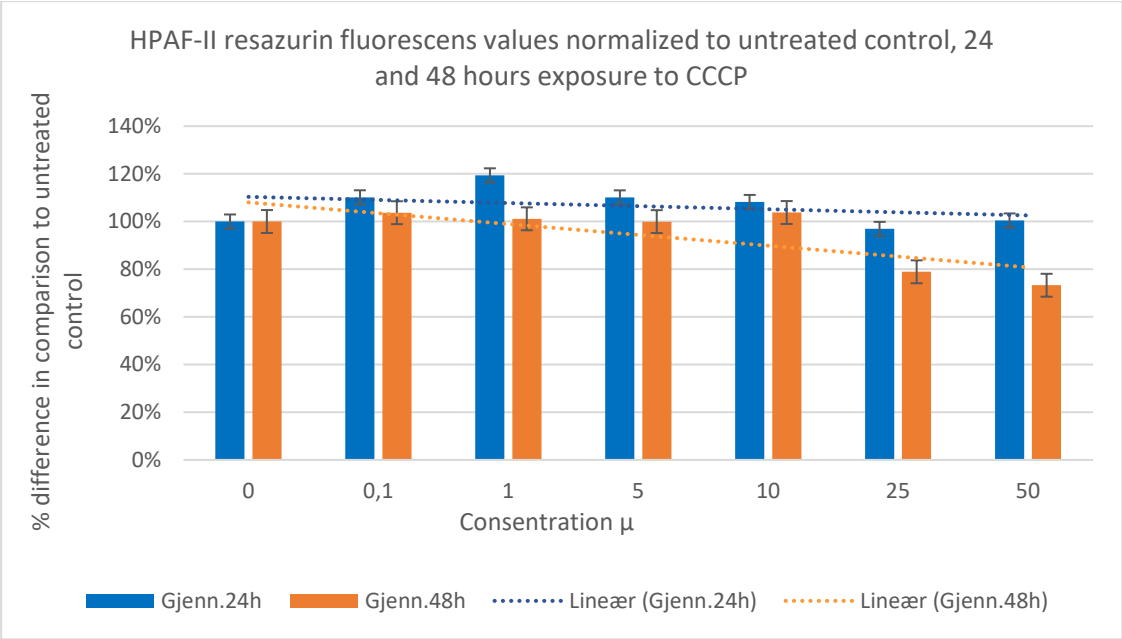


Figure (3.3): Shows the effect of CCCP on 24 and 48 hours.

3.3.2 Effect of Rotenone on 24 and 48 hours:

On the figure (3.6), we can see at with increasing the concentration of Rotenone (1, 10, 100, 200, 1000 and 2000 nM) the cell viability is also increasing. According on this experiment, Rotenone has negative effect on the cell line HFAF-II. But if see *Figure (3.4)*, cells were growth in the high concentration of Rotenone. The cell viability and the standard derivation on the wells with 1 and 10 nM Rotenone is higher at 48 hours than the others.

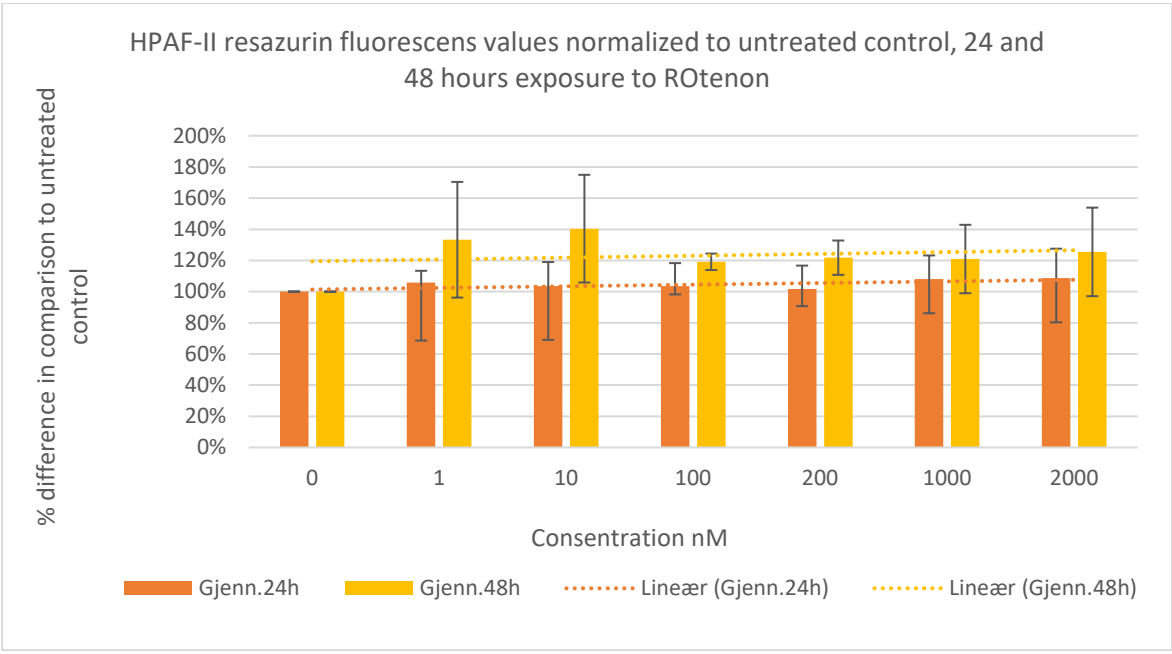


Figure (3.4): shows the effect of Rotenone on 24 and 48 hours.

3.3.3 Effect of Oligomycin on 24 and 48 hours:

Oligomycin inhibits the ATP synthase, and the cells cannot produce ATP by ATP synthase. If the cell cannot produce ATP will die. But *Figure (3.5)* shows that the cell viability of the cells is increasing with increasing the concentration of Oligomycin on 24 hours. The cell viability of cells is a little bit decreasing with increasing the concentration of Oligomycin on 48 hours. According to the *Figure (3.5)* we can say that Oligomycin on the 48 hours has positive effect, but it has negative effect on the 24 hours. The cell viability is increasing on the wells with (1, 10 nM) Oligomycin at 48 hours and they have high standards derivation. But it is decreasing on the wells with (50, 100 250 500 nM) Oligomycin at 48 hours.

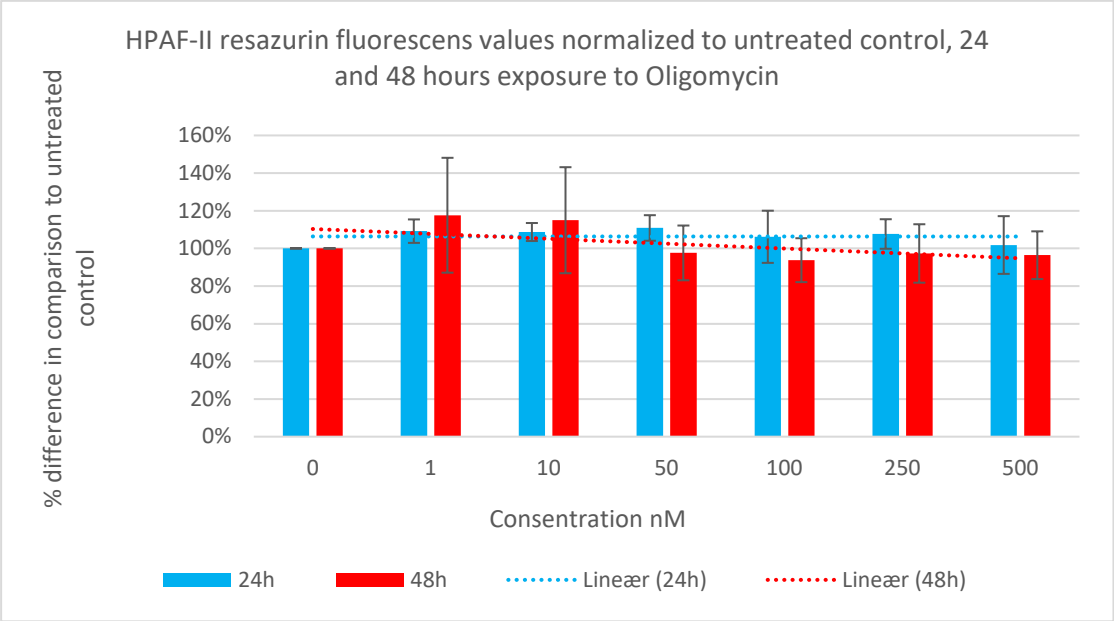


Figure (3.5): shows the effect of Oligomycin on 24 and 48 hours.

3.4 Seahorse analysis:

The Agilent Seahorse XF Cell Mito Stress Test:

In this experiment, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of live cells in a multi-well plate are measured by Seahorse XF. The cells were attached to the bottom of the plate and the lid of the plate contained detectors both oxygen sensors and pH sensors that were going to measured oxygen tensions and pH within the wells. The measurement units (sensors) go up and down and they do the measurements when they are down, and they are measuring three times for each measuring.
In this experiment, it was used three inhibitors and one uncoupler. (Oligomycin, FCCP and Rotenone/Anthocyanin).

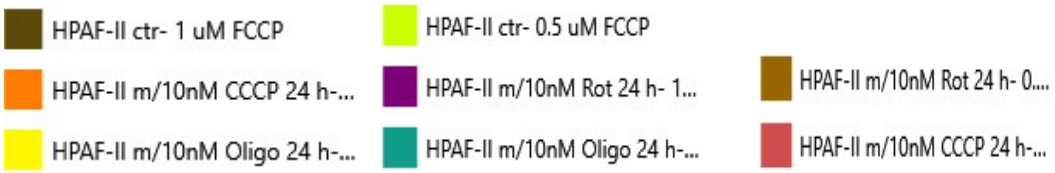


Figure (3.6): Shows the group list of the HPAF -II cell line. Control (untreated cells, without inhibitor with 1 uM FCCP, Control (untreated cells with 0.5 uM FCCP, cells treated with 10 nM CCCP and injected 0.5 uM FCCCP, cells treated with 10 nM and injected 1 uM FCCP, cells treated with 10 nM oligomycin and injected 0.5 uM FCCP, cells treated with 10 nM oligomycin and injected 1 uM FCCP, cells treated with 10 nM Rotenone and injected 0.5 uM FCCP, cell treated with 10 nM Oligomycin and injected 1 uM FCCP.

On the OCR graph (*Figure (3.7)*), initially here we have a respiratory rate which reflect the normal consumption of oxygen by this plate itself and it known as basal respiration. Basal respiration is the normal oxygen consumption rate, here it was not added any kind of inhibitors and it is between 75- 150 pmol/min (picomol/min). After adding the oligomycin the oxygen consumption rate is decreasing, and it is between 20 – 80 pmol/min. The second injection is FCCP. By adding the FCCP the oxygen consumption rate is increasing, and it is the maximal respiration of the cells. The OCR value is between 100 – 300 pmol/min. But after adding the Rotenone and Antimycin, the OCR value is decreasing significantly, and it is between 10 -25 pmol/min.

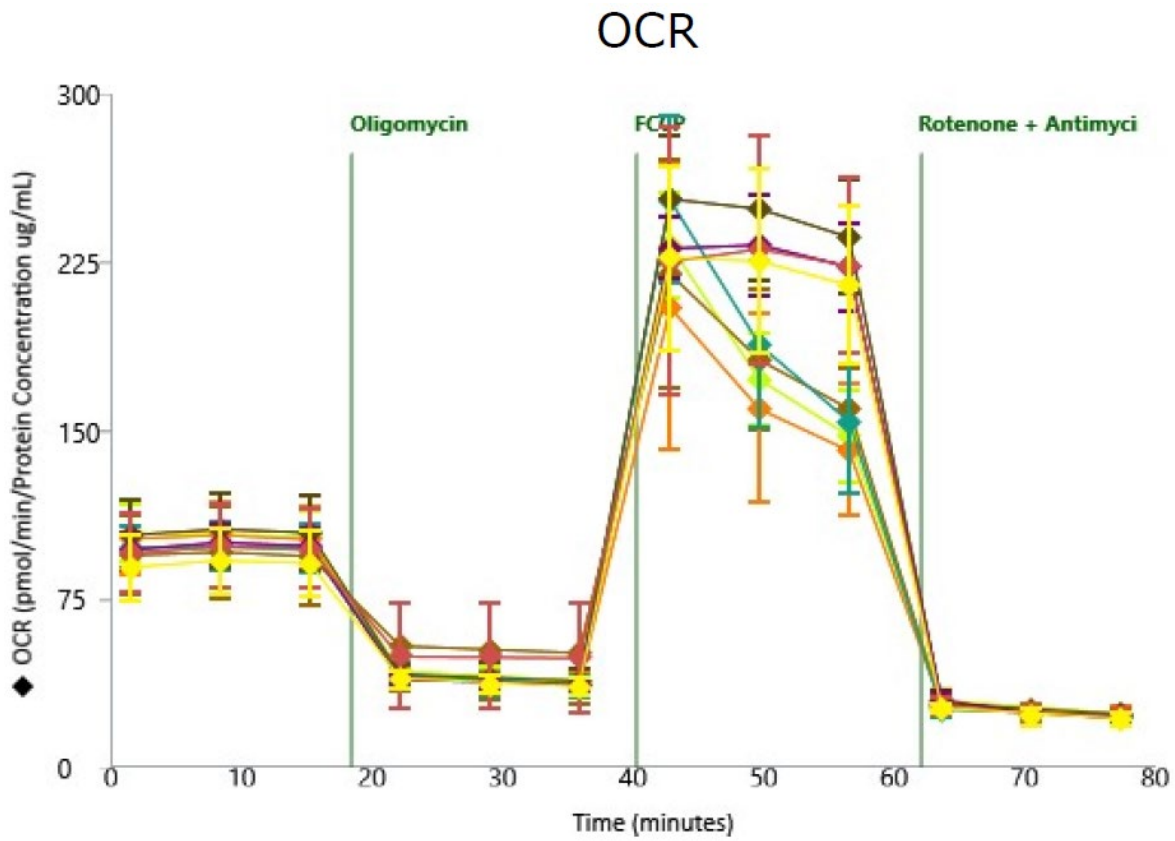


Figure (3.7): OCR shows the oxygen consumption rate of the cells over the time.

ECAR graph shows that how fast the cells produce lactic acid by glycolysis on the absent of oxygen. On the ECAR graph *Figure(3.8)*, the initially value of ECAR of the cells without any injection is between 35 -60 pmol/min. The extracellular acidification rate is increasing by adding the Oligomycin and it is between 40 – 85 pmol/min. By adding the FCCP, the extracellular acidification rate is first increasing, but on the second and third measuring the ECAR value is decreasing, and ECAR value is between 40 -100 pmol/min. By adding Rotenone/ Antimycin the ECAR value is decreasing, and it is between 40 -85 pmol/min.

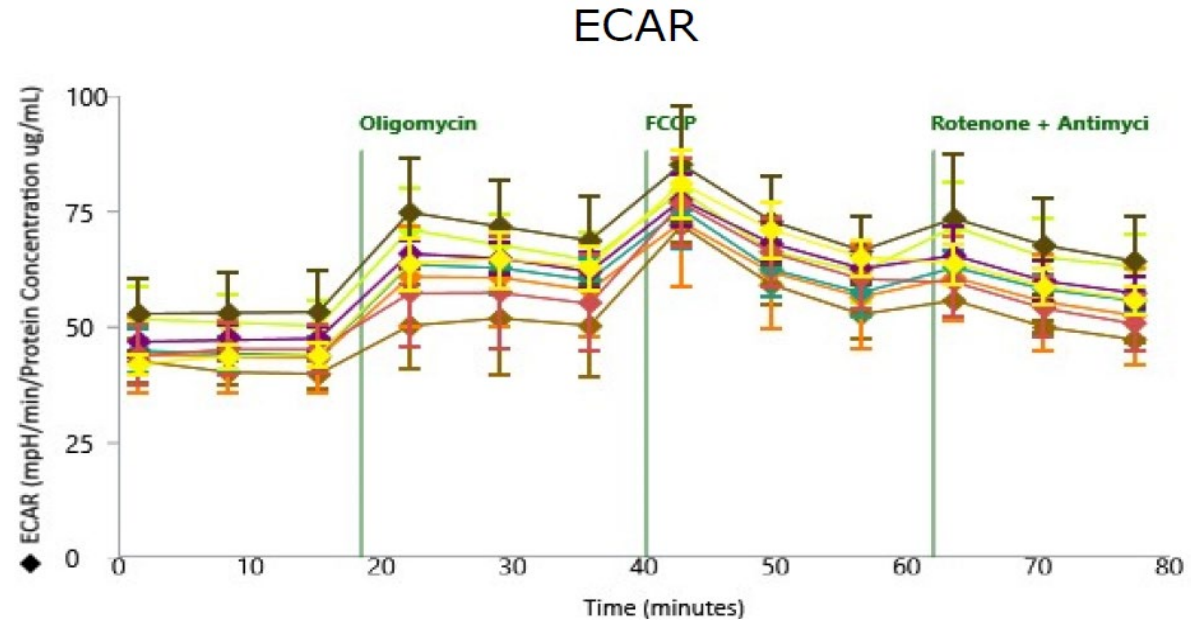


Figure (3.8): ECAR shows the extracellular acidification rate of the cells over the time.

Energy Map on *Figure (3.9),* shows that the HPAF-II cell line is very energetic. Energetic means that cells are very metabolic active, and both consume high amounts of oxygen and produce lactate, and ATP is producing both oxidative phosphorylation and glycolysis.

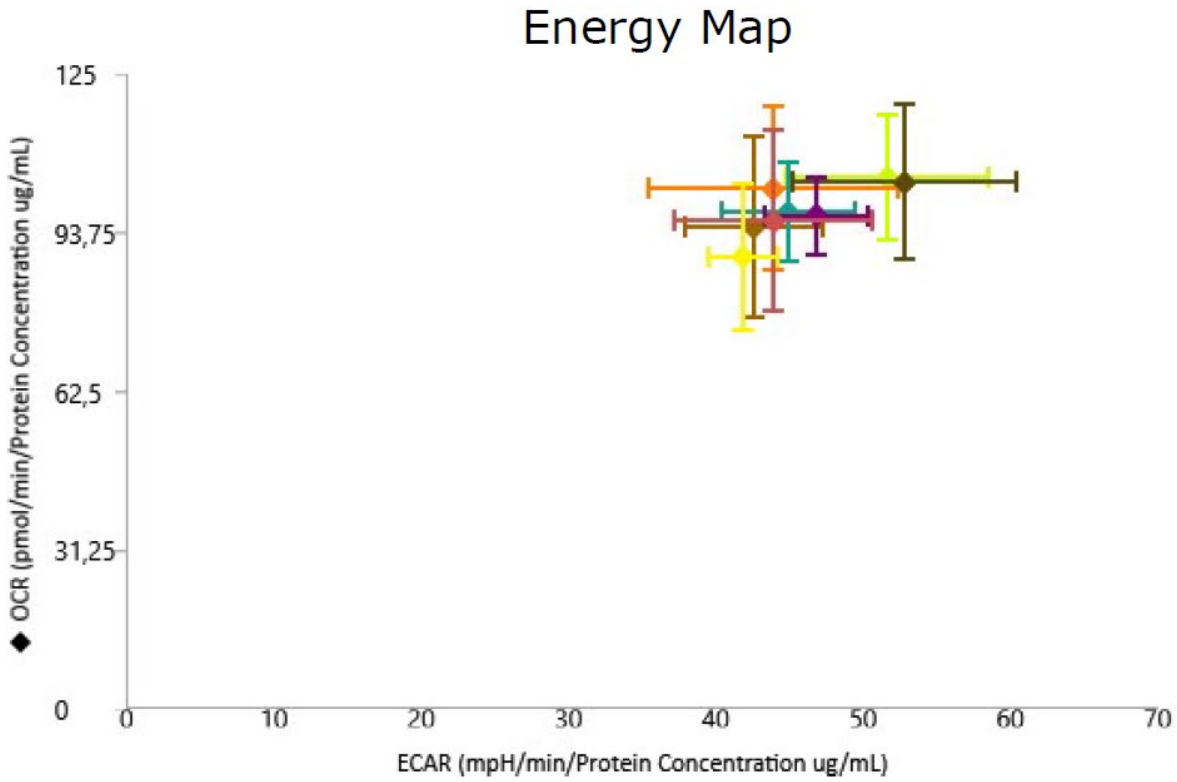


Figure (3.9): Shows the oxygen consumption rate of the cells over the time

4. Discussion:

4.1 Discussion of possible technical errors

In measuring the cell viability with Muse Count, the cell viability percent was always low then the other two cell lines Panc I and BxPC-3. I do not know, the HPAF-II was resistance against to trypsin. The cells did not react with trypsin so fast. It took 5 -10 min. I think in this period, I was missing the cells and because of that the cell viability percent was always low.

There might have been done mistakes in passaging the cells. The HPAF-II cell line grows slowly, and I passaged the cells some time at low confluency.

Test of correct seeding of cells density figure (3.2) shows the result of three weeks. Result of first and second week shows a nice line that increasing with increasing cell density. But the third week shows a higher cell viability in the 75 00 cell/well than in the 10000 and 15 000 cells/well. This is due to not mixing the cell suspension enough before seeding out on the plate or did not add correctly Alamar Blue. It can also be due to more proliferation in some wells than others. The standard deviations of well with 10000 cells /well is slightly higher than the others.

Other error can be that when we were takeout 100 µL of medium of plates, it is possible at it was taken out some cells of some wells. The other technician error can be pipetting. During making solution of cell suspension and medium to seeding on the plates, making different cell density, and making different concentration of inhibitors. To making these solutions, it is very important that the solutions should mixing very good. In cell suspension, the cells precipitating and before making different cell density it should maxing very good. When we are making solution of different concentration of inhibitors and medium, it is also very important to maxing very good before adding to the plats. Because of that we can have different results and standard deviation. It is very important when we added a solution on plate with pipette. Sometimes air gets into the pipette, and it does not work so well.

After adding the Alamar Blue, we always saw different colors on different wells while we added the same value in each well. One of the reasons for this variation could be that all the Alamar Blue could not enter the well and some of them got stuck in the wall of the well.

4.2 results discussion

4.2.1 Test of various mitochondrial inhibitors (CCCP, Rotenone and Oligomycin) in cells over time:

- **Effect of CCCP on 24 and 48 hours:**

CCCP is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential. That cause mitochondrial swelling and cells death and therefore can say at CCCP is toxic to many cancer cells. Because of disrupting the

mitochondrial membrane potential, the cell stopping to produce ATP and cells cannot proliferation and growth, and therefore they die. Figure (3.5) shows the effect of CCCP on 24 and 48 hours. Based to the function of CCCP on the cells, the cell viability should decrease with increasing the concentration of CCCP. But result on figure (3.5) shows that the cell viability of the cells is higher on the wells with 0.1, 1, 5, 10 and 50 µM CCCP concentration on 24 hours. And the cell viability of the cells is higher on the wells with 0.1, 1, 10 µM CCCP concentration on 48 hours. There might have been done mistakes in passaging the cells, mixing, aspirating, making different concentration of inhibitors or adding Alamar Blue.

- **Effect of Rotenone on 24 and 48 hours:**

Rotenone is an inhibitor that inhibits complex I of electron transport chain, that leaded to a blockade of oxidative phosphorylation. If the complex I blocked, the electrons cannot transfer through electron transport chain and oxygen cannot reduce to water molecule.
The figure (3.6) shows effect of Rotenone on 24 48 hours. But it shows that Rotenone has negative effect on the cells, and it is not logical because Rotenone inhibits the complex I of electron transport chain. The percentage of the live cells is increasing with increasing the concentration of Rotenone. Standard deviation of the wells with 1, 10, 1000 and 2000 nM concentration of Rotenone is very high than the others on 24 and 48 hours. There might have been done mistakes in passaging the cells, mixing, aspirating, making different concentration of inhibitors or adding Alamar Blue.

- **Effect of Oligomycin on 24 and 48 hours:**

Oligomycin is another inhibitor that we were used in this project, and it inhibits the ATP synthase that cells cannot produce ATP.
Figure (3.7) shows the effect of Oligomycin on 24 and 48 hours. Result shows that the percentage of live cells are increasing with increasing the concentration of Oligomycin on 24 hours and the standard derivation is slightly higher of the wells with 100 and 500 nM Oligomycin. But Oligomycin has positive effect on the cells on 48 hours because the percentage of live cells are decreasing with increasing the concentration of Oligomycin. But in the wells with 1 and 100 nM Oligomycin, the percentage of live cells are increasing with increasing the concentration of Oligomycin, and they have also high standard deviation. The errors might be mistakes in passaging the cells, mixing, aspirating, making different concentration of inhibitors or adding Alamar Blue.

4.2.2 Seahorse analysis:

In this experiment, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of live cells in a multi-well plate are measured by Seahorse XF.
Fra seahorse machine we plotted three graphs: OCR, ECAR and Energy MAP.
Figure (3.9) shows the result of oxygen consumption rate or OCR of the cells. Initially here we have a respiratory rate which reflect the normal consumption of oxygen by this plate itself and it known as basal respiration. Basal respiration is the normal oxygen consumption rate, here it was not added any kind of inhibitors. The first injection is Oligomycin. Oligomycin is an inhibitor of the ATP synthase. It inhibits ATP production. In mitochondria increased the inner mitochondrial membrane potential because protons are not returning to through the ATP synthase, and this decreases electron transport and oxygen consumption.

By adding the FCCP the membrane potential is decreasing because now we are allowing these protons to return into the mitochondrial matrix freely and therefor electron transport and oxygen consumption are increasing.
Finally adding Rotenone and Antimycin. Rotenone inhibits complex I on the electron transport chain, and Antimycin inhibits complex III of electron transport chain. They are going to slow down oxygen consumption completely in mitochondria, and we are only going to have residual oxygen consumption which is oxygen consumption from other cellular processes which is not mitochondrial. After adding Rotenone and Antimycin we do not have mitochondrial oxygen consumption anymore, so oxygen consumption and electron transport is totally inhibited. If it is totally inhibited the residual oxygen consumption that still happens here is non-mitochondrial oxygen consumption.
Figure (3.10) shows the result of extracellular acidification rate of the cells. Cells also generate ATP through glycolysis, the conversion of glucose to lactate independent of oxygen. The lactate is the primary source of free protons in the medium of the cultured cells. The initially value of ECAR of the cells without any injection is between 35 -60 pmol/min. The extracellular acidification rate is increasing by adding the Oligomycin because the oligomycin inhibits ATP synthase, but the cells producing free protons. By adding the FCCP, the extracellular acidification rate is first increasing, but on the second and third measuring the ECAR value is decreasing, because it makes pores on the membrane and the protons coming inn and the concentration of protons will come down in the medium. By adding Rotenone/ Antimycin the ECAR value is decreasing, because the complex I and III inhibiting by Rotenone and Antimycin.
The standard deviation is not so high that is means that the possibility of errors is very low.
Figure (3.11) shows the anergy map of the cells. It shows that the cells are energetic, and it means that the cells are metabolic active and produces ATP both by glycolysis and oxidative metabolism. But some of standard deviation is high.

The seahorse analysis was don just one time, and it is difficult to say anything about this. An experiment should don minimum three time that be sure about the result and find the errors.

5.Conclusion:

The optimal seeding density was 7500 cell/well for cell lines and hence this concentration was used in the testing of effect of CCCP, Rotenone an Oligomycin.
In this experiment we evaluated the cell viability and the effect of CCCP with different concentration (0.1, 1, 5, 10, 25 and 50 μM of CCCP). Here, I found that CCCP has positive effect on HPAF-II because cell viability decreased when the CCCP concentration increased, with 50 μM CCCP at 48 hours showed the highest cell death.
But Rotenone has negative effect to the pancreatic cancer cell line HPAF-II, because the cell viability increased when the Rotenone concentration increased.
Oligomycin inhibits the ATP synthase, and the cells cannot produce ATP by ATP synthase. Without ATP stop the cells proliferation and growth, as a result the cells die. Based on the experiment that we have done, Oligomycin has positive effect on pancreatic cancer cells, because the cell viability decreased when the Oligomycin concentration increased, with 500 nM Oligomycin at 48 hours showed the highest cell death.
The pancreatic cancer cell line HPAF-II uses both oxidative metabolism or mitochondrial respiration and glycolysis to produce ATP, and cells have very active mitochondria or have a lot of transport electron transport chain complexes and ATP synthase. The cells form a lot of lactates and consume a lot of oxygen.

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7. Appendix:

You can find all data and graphs from the experiments in this part.

Appendix A:

All graphs of experiments to find the optimal seeding cell density are on this Appendix. We had done this experiment three times on three weeks.

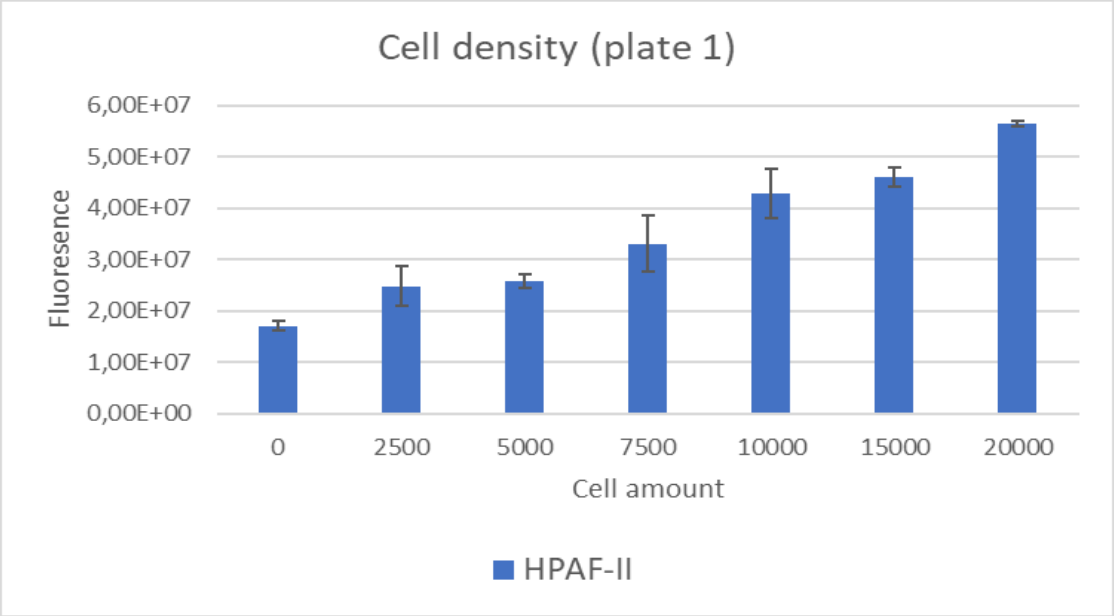

Figure (7.1): Alamar Blue cell viability assay results week 1.

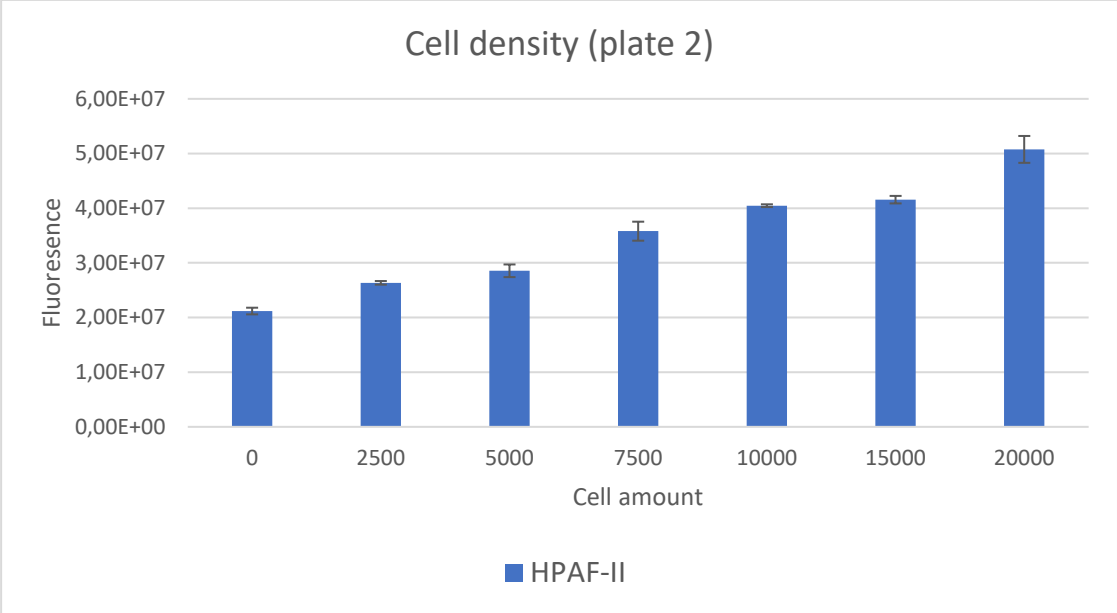

Figure (7.2): Alamar Blue cell viability assay results week 2.

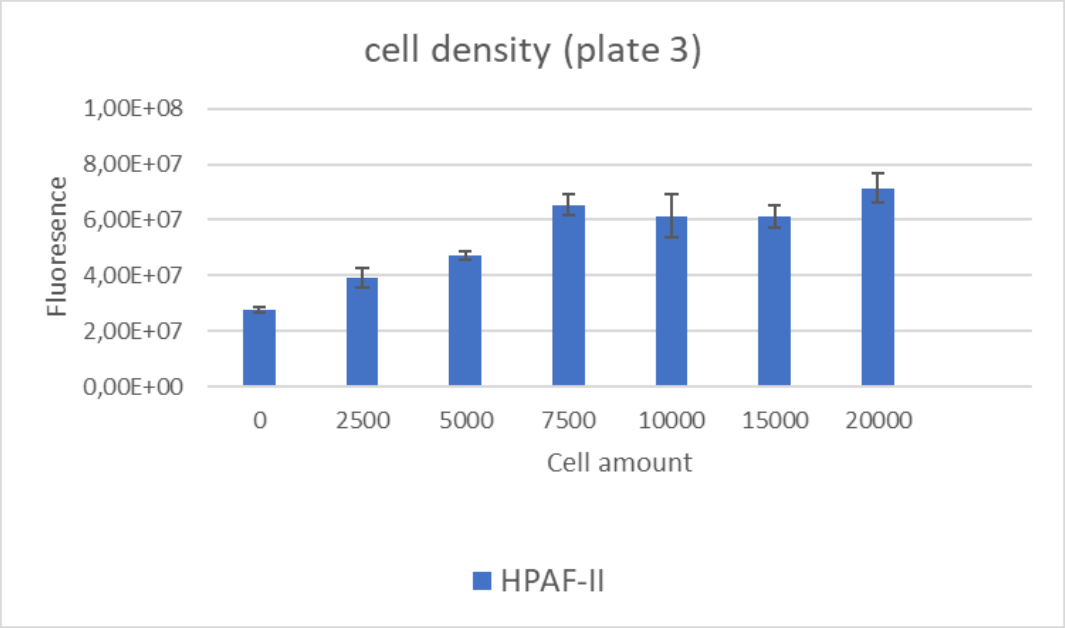

Figure (7.3): Alamar Blue cell viability assay results week 3.

Appendix B:

All data and graphs for CCCP, Rotenone and Oligomycin experiment on different time (24, 48 hours).

- **CCCP (24 hours)**

Plate:	230222_CCC	01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
	Temperature	1	2	3	4	5	6	7	8	9	10	11	12
	22.mai	1,95E+04	1,87E+04	2,02E+04	1,86E+04	2,00E+04	1,85E+04	1,85E+04	2,00E+04	1,84E+04	1,93E+04	1,39E+04	1,55E+04
		1,85E+04	1,76E+07	3,84E+07	3,59E+07	3,53E+07	3,78E+07	4,00E+07	3,05E+07	3,19E+07	1,86E+04	2,38E+08	1,45E+04
		1,99E+04	1,73E+07	3,38E+07	3,84E+07	2,85E+07	3,60E+07	3,97E+07	3,54E+07	2,98E+07	1,87E+04	2,34E+08	1,60E+04
		1,86E+04	2,05E+07	3,17E+07	3,63E+07	3,77E+07	3,51E+07	3,70E+07	3,02E+07	3,19E+07	1,93E+04	2,38E+08	1,51E+04
		1,64E+04	1,96E+07	3,94E+07	4,08E+07	9,25E+07	3,74E+07	3,62E+07	2,97E+07	3,09E+07	1,76E+04	2,41E+08	1,47E+04
		1,89E+04	2,01E+07	1,61E+05	1,62E+05	1,60E+05	1,60E+05	1,60E+05	1,57E+05	1,61E+05	1,83E+04	1,36E+04	1,39E+04
		2,65E+04	1,90E+04	1,79E+04	1,82E+04	1,77E+04	1,83E+04	1,79E+04	1,80E+04	1,84E+04	1,63E+04	1,25E+04	1,37E+04
		1,47E+04	1,35E+04	1,36E+04	1,30E+04	1,39E+04	1,35E+04	1,31E+04	1,37E+04	1,27E+04	1,35E+04	1,28E+04	1,44E+04

Figure (7.4): Data of Spectro Max from CCCP (24 hours) on 22.02.2023.

Concentration	0	0,1	1	5	10	25	50
	3,84E+07	3,59E+07	3,53E+07	3,78E+07	4,00E+07	3,05E+07	3,19E+07
	3,38E+07	3,84E+07	2,85E+07	3,60E+07	3,97E+07	3,54E+07	2,98E+07
	3,17E+07	3,63E+07	3,77E+07	3,51E+07	3,70E+07	3,02E+07	3,19E+07
	3,94E+07	4,08E+07	9,25E+07	3,74E+07	3,62E+07	2,97E+07	3,09E+07
Average	3,58E+07	3,78E+07	4,85E+07	3,66E+07	3,82E+07	3,15E+07	3,11E+07
S.deviation	3,16E+06	1,98E+06	2,56E+07	1,07E+06	1,67E+06	2,28E+06	8,62E+05

Figure (7.5): The average and standard deviation from data of figure (7.4).

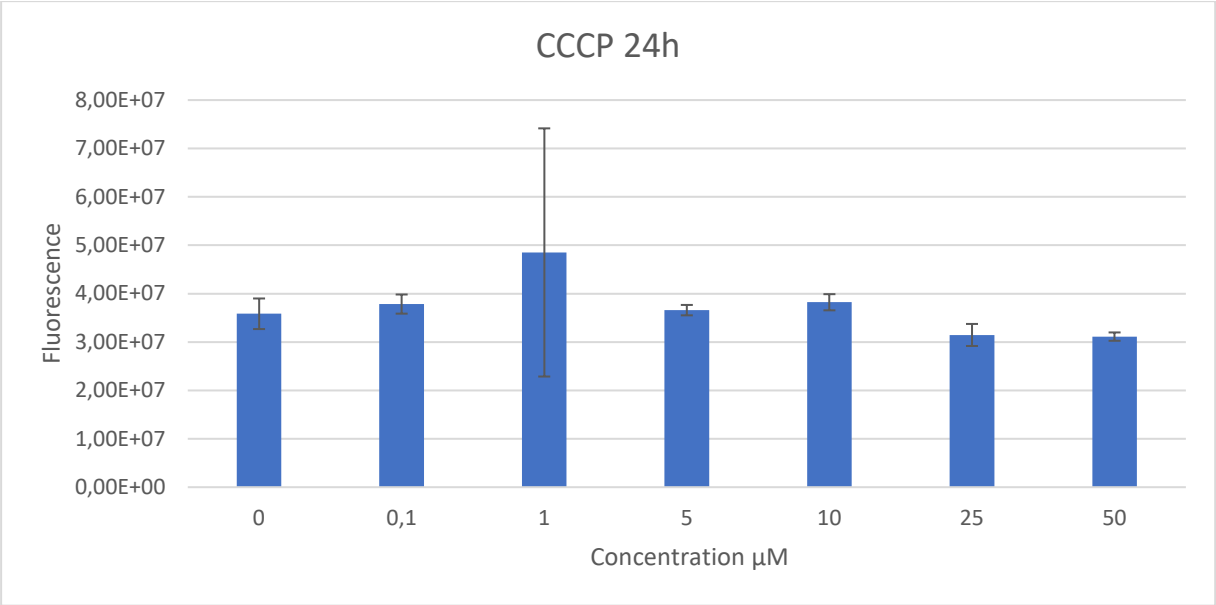

Figure (7.6): The graph of data on the figure (7.5), shows the effect of CCCP on the 24 hours.

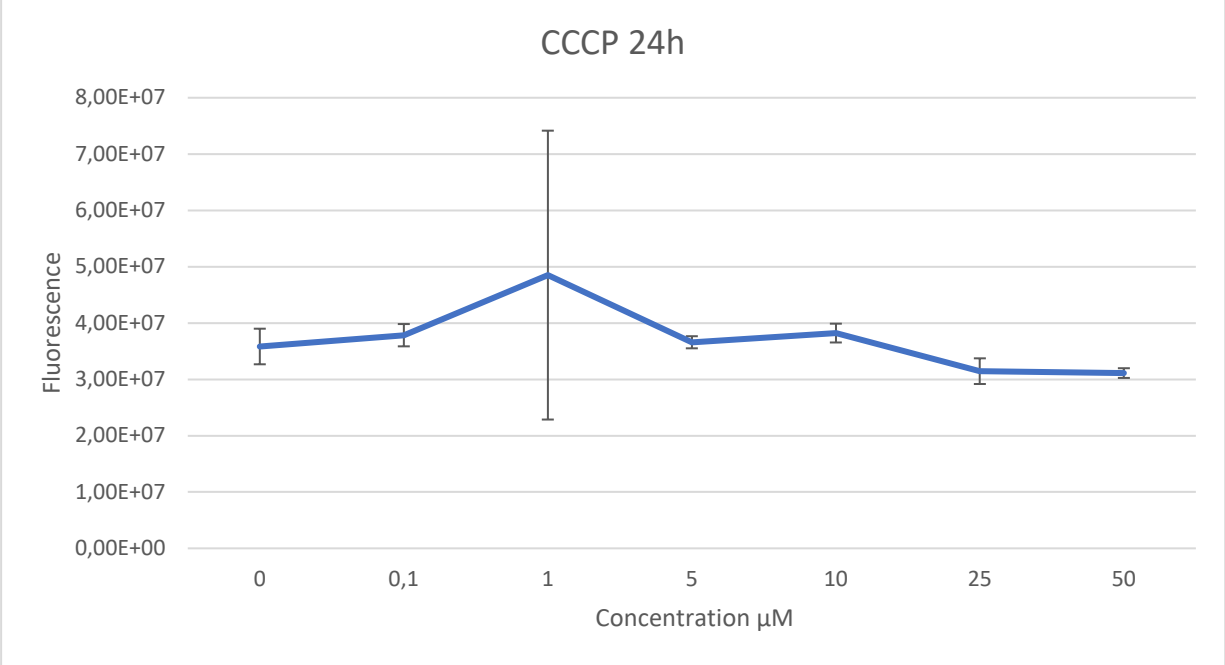

Figure (7.7): The graph of data on the figure (7.5), shows the effect of CCCP on 24 hours.

Plate:	230308_CCC	01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
	Temperature	1	2	3	4	5	6	7	8	9	10	11	12
	22.mai	19223	18735	19711	18590	19963	19328	18277	18726	19697	19377	14091	14799
		18650	19203296	46946492	57685440	47593776	48084788	45985216	44084340	45633164	18913	232039600	14511
		18999	18954028	45038524	46188892	45452264	40547580	43534192	39227104	44935592	20016	145172096	14582
		18434	20432052	39369780	49364088	42912088	44788296	44666616	36554944	40770164	18548	151643424	13831
		18668	20426626	47060972	45788676	47929092	44232864	42434324	44313380	37848276	18725	164389552	14099
		19263	160343	160352	159137	171246	158985	159520	161360	160020	17818	13583	13623
		18011	18153	18719	17919	16688	19286	18209	17822	18286	17557	13272	12963
		14655	13380	14528	13051	13930	13637	13462	13665	13621	13499	14589	14336

Figure (7.8): Data of Spectro Max from CCCP (24 hours) on 08.03.2023.

Concentration	0	0.1	1	5	10	25	50
	4,69E+07	5,77E+07	4,76E+07	4,81E+07	4,60E+07	4,41E+07	4,56E+07
	4,50E+07	4,62E+07	4,55E+07	4,05E+07	4,35E+07	3,92E+07	4,49E+07
	3,94E+07	4,94E+07	4,29E+07	4,48E+07	4,47E+07	3,66E+07	4,08E+07
	4,71E+07	4,58E+07	4,79E+07	4,42E+07	4,24E+07	4,43E+07	3,78E+07
Average	4,46E+07	4,98E+07	4,60E+07	4,44E+07	4,42E+07	4,10E+07	4,23E+07
S.deviation	3,13E+06	4,78E+06	2,01E+06	2,67E+06	1,32E+06	3,29E+06	3,17E+06

Figure (7.9): The average and standard deviation from data of figure (7.8).

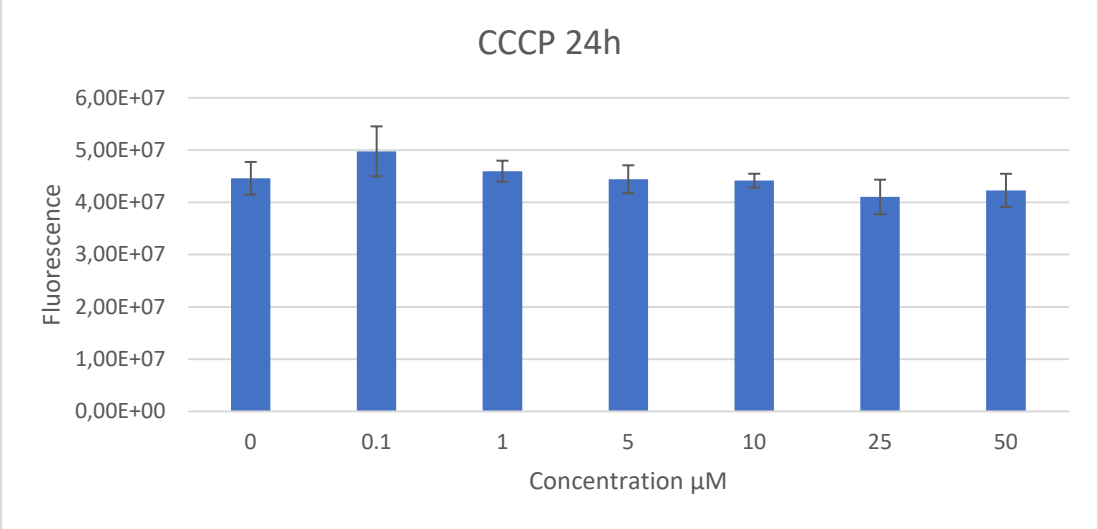

Figure (7.10): The graph of data on the figure (7.9), shows the effect of CCCP on 24 hours.

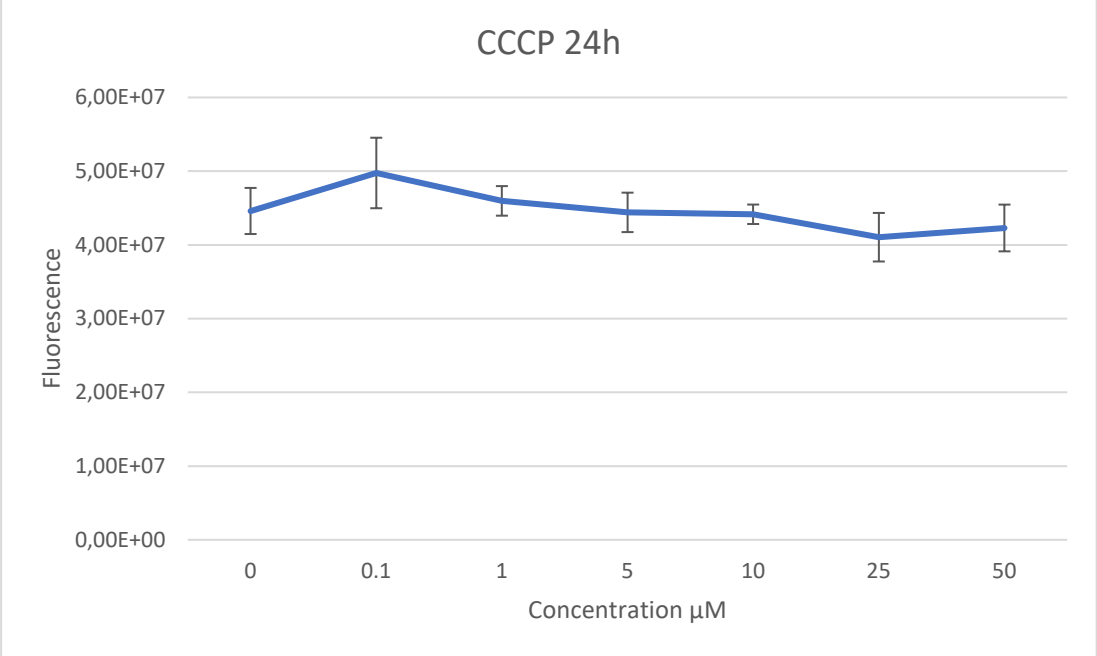

Figure (7.11): The graph of data on the figure (6.9), shows the effect of CCCP on 24 hours.

Three inhibitors in one plate:

Resazurin (positive control) CCCP
PBS Rotenone
Background control (negative control) Oligomycin
Untreatment

230315_CCCI	01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
Temperature	1	2	3	4	5	6	7	8	9	10	11	12
22.mai	2,25E+08	2,00E+04	1,97E+04	1,94E+04	2,03E+04	1,78E+04	1,90E+04	1,97E+04	1,82E+04	2,06E+04	1,95E+04	2,33E+08
	2,02E+04	3,41E+07	3,78E+07	4,16E+07	4,21E+07	4,19E+07	4,10E+07	4,12E+07	4,49E+07	4,00E+07	4,14E+07	1,98E+04
	1,91E+04	3,52E+07	3,87E+07	4,36E+07	4,60E+07	4,49E+07	4,36E+07	4,76E+07	4,16E+07	4,21E+07	4,01E+07	2,01E+04
	1,81E+04	3,80E+07	4,17E+07	4,60E+07	5,03E+07	4,31E+07	4,36E+07	4,20E+07	4,45E+07	4,48E+07	3,99E+07	1,97E+04
	1,84E+04	1,96E+07	3,56E+07	4,42E+07	4,77E+07	4,41E+07	4,18E+07	4,25E+07	4,63E+07	4,68E+07	3,96E+07	1,83E+04
	1,84E+04	1,97E+07	3,62E+07	4,12E+07	4,15E+07	4,54E+07	4,31E+07	4,22E+07	4,66E+07	4,21E+07	3,84E+07	1,89E+04
	1,72E+04	2,05E+07	3,90E+07	4,28E+07	4,64E+07	4,62E+07	4,63E+07	4,26E+07	4,46E+07	4,72E+07	4,06E+07	1,93E+04
	2,29E+08	2,80E+04	1,96E+04	1,86E+04	1,84E+04	1,79E+04	1,83E+04	1,90E+04	1,91E+04	1,87E+04	1,93E+04	2,15E+08

Figure (7.12): Data of Spectro Max from CCCP, Rotenone and oligomycin (24 hours) on 15.03.2023.

Concentratio	0	0.1	1	5	10	25	50
	3,41E+07	3,78E+07	3,87E+07	4,17E+07	3,56E+07	3,62E+07	3,90E+07
	3,52E+07	4,16E+07	4,36E+07	4,60E+07	4,42E+07	4,12E+07	4,28E+07
	3,80E+07	4,21E+07	4,60E+07	5,03E+07	4,77E+07	4,15E+07	4,64E+07
Average	3,57E+07	4,05E+07	4,28E+07	4,60E+07	4,25E+07	3,96E+07	4,28E+07
S.deviation	1,64E+06	1,91E+06	3,02E+06	3,50E+06	5,12E+06	2,45E+06	3,00E+06

Figure (7.13): The average and standard deviation from data of figure (7.12) just for CCCP.

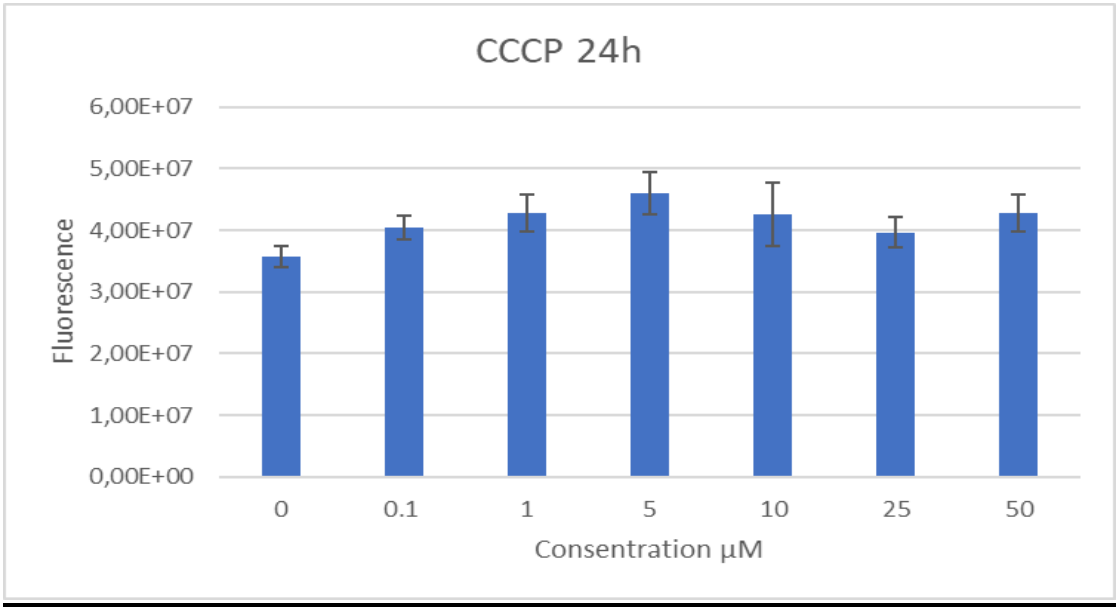

Figure (7.14): The graph of data on the figure (7.13), shows the effect of CCCP on 24 hours.

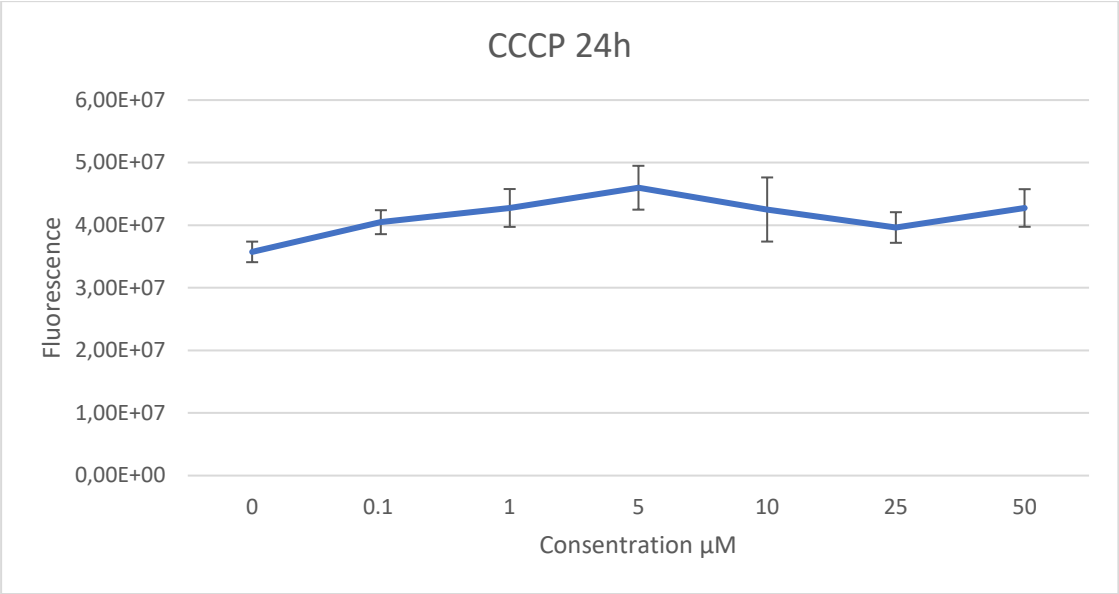

Figure (7.15): The graph of data on the figure (7.13), shows the effect of CCCP on 24 hours.

- **CCCP (48 hours):**

1	2	3	4	5	6	7	8	9	10	11	12
18684	19130	19788	18448	20198	18820	18154	20306	18143	18257	13931	15442
18705	18334060	52144380	48175756	45313876	42895516	47844684	36965952	36099356	19693	2,39E+08	15510
19738	20279692	54741984	40000384	45574704	49302968	38921772	38694376	31484784	19375	2,28E+08	15291
18721	20599982	49661940	48615212	49400892	47595276	42237428	36984956	37312728	18498	2,30E+08	15170
18594	18504604	48870936	42978540	45634672	40664400	44560220	37811356	37730532	17959	2,50E+08	14300
18948	155759	154933	154747	156111	152960	151146	151704	153629	19309	13589	14114
17157	17835	19179	18437	18844	19197	18568	18745	18816	17535	13961	14450
14464	14730	14654	14087	14773	14390	12986	14685	12940	14381	14099	15576

Figure (7.16): Data of Spectro Max from CCCP (48 hours) on 23.02.2023.

Concentration	0	0,1	1	5	10	25	50
	52144380	48175756	45313876	42895516	47844684	36965952	36099356
	54741984	40000384	45574704	49302968	38921772	38694376	31484784
	49661940	48615212	49400892	47595276	42237428	36984956	37312728
	48870936	42978540	45634672	40664400	44560220	37811356	37730532
Average	5,14E+07	4,49E+07	4,65E+07	4,51E+07	4,34E+07	3,76E+07	3,57E+07
S.deviation	2,30E+06	3,61E+06	1,69E+06	3,48E+06	3,26E+06	7,11E+05	2,48E+06

Figure (7.17): The average and standard deviation from data of figure (7.16)

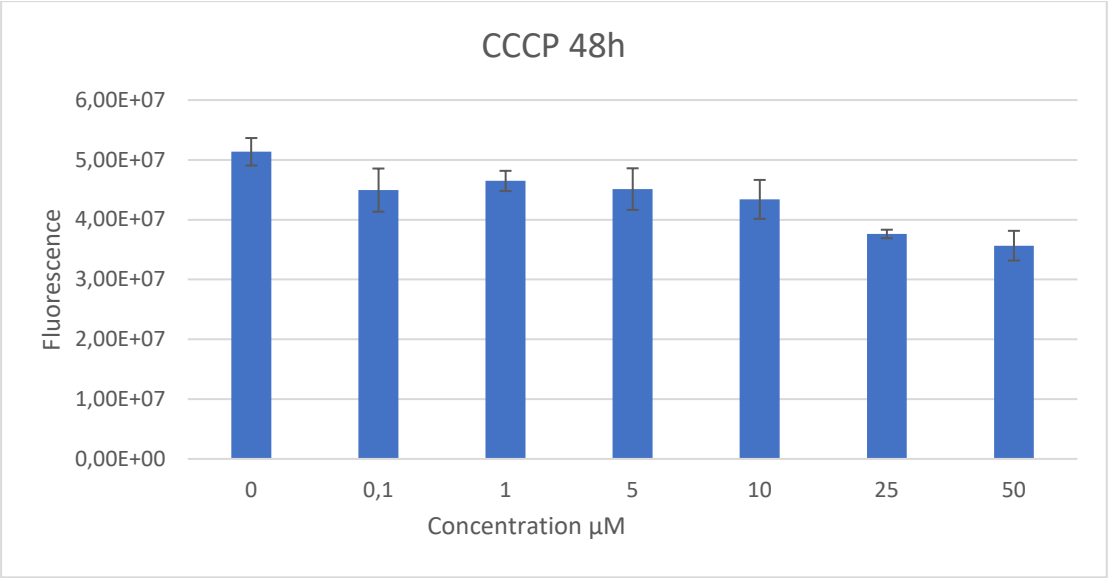

Figure (7.18): The graph of data on the figure (7.17), shows the effect of CCCP on 48 hours.

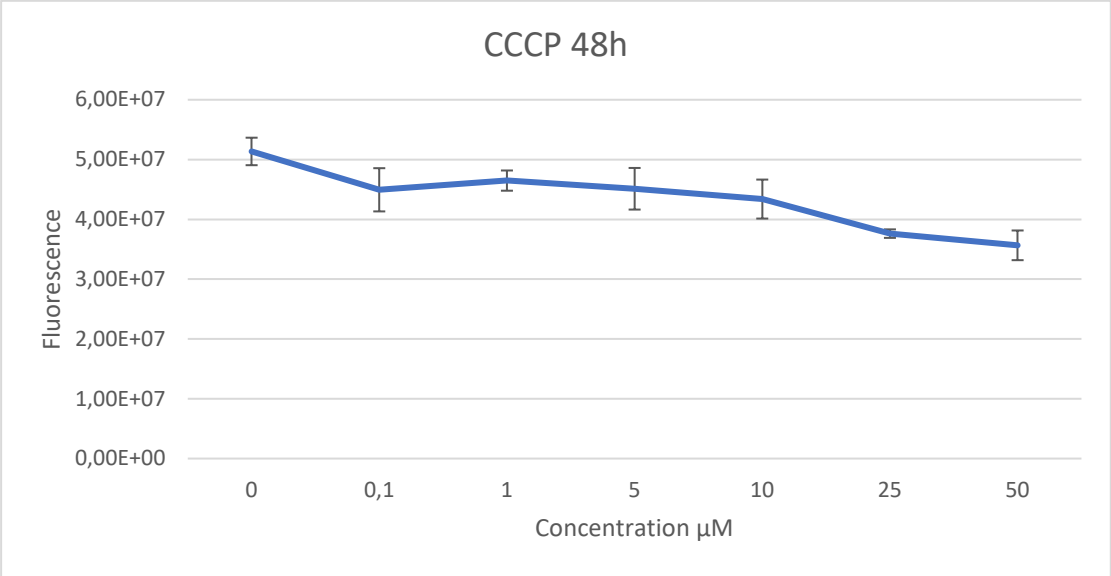

Figure (7.19): The graph of data on the figure (7.17), shows the effect of CCCP on 48 hours.

230309_CCCI	01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
Temperature	1	2	3	4	5	6	7	8	9	10	11	12
22	1,87E+04	1,81E+04	1,96E+04	1,87E+04	1,90E+04	1,99E+04	1,95E+04	1,93E+04	1,89E+04	1,86E+04	1,40E+04	1,46E+04
	1,79E+04	2,11E+07	5,73E+07	6,07E+07	5,93E+07	5,71E+07	7,02E+07	4,83E+07	4,67E+07	1,89E+04	2,12E+08	1,50E+04
	1,87E+04	1,93E+07	5,17E+07	7,09E+07	6,16E+07	5,79E+07	5,62E+07	4,99E+07	4,60E+07	2,02E+04	2,21E+08	1,49E+04
	1,78E+04	2,14E+07	5,68E+07	6,40E+07	5,01E+07	5,13E+07	5,90E+07	5,73E+07	4,95E+07	1,93E+04	2,24E+08	1,44E+04
	1,78E+04	2,17E+07	5,50E+07	6,87E+07	6,73E+07	6,25E+07	6,32E+07	4,66E+07	5,14E+07	1,94E+04	2,19E+08	1,44E+04
	1,77E+04	1,74E+05	1,67E+05	1,68E+05	1,65E+05	1,64E+05	8,03E+04	1,66E+05	1,67E+05	1,80E+04	1,34E+04	1,40E+04
	1,68E+04	1,84E+04	1,79E+04	1,85E+04	1,82E+04	1,89E+04	1,82E+04	1,89E+04	1,84E+04	1,62E+04	1,32E+04	1,38E+04
	1,44E+04	1,34E+04	1,40E+04	1,34E+04	1,39E+04	1,28E+04	1,31E+04	1,39E+04	1,37E+04	1,41E+04	1,39E+04	1,35E+04

Figure (7.20): Data of Spectro Max from CCCP (48 hours) on 09.03.2023.

Concentratio	0	0.1	1	5	10	25	50
	5,73E+07	6,07E+07	5,93E+07	5,71E+07	7,02E+07	4,83E+07	4,67E+07
	5,17E+07	7,09E+07	6,16E+07	5,79E+07	5,62E+07	4,99E+07	4,60E+07
	5,68E+07	6,40E+07	5,01E+07	5,13E+07	5,90E+07	5,73E+07	4,95E+07
	5,50E+07	6,87E+07	6,73E+07	6,25E+07	6,32E+07	4,66E+07	5,14E+07
Average	5,52E+07	6,61E+07	5,96E+07	5,72E+07	6,22E+07	5,06E+07	4,84E+07
S.deviation	2,20E+06	3,99E+06	6,20E+06	3,98E+06	5,27E+06	4,09E+06	2,20E+06

Figure (7.21): The average and standard deviation from data of figure (7.20) and different concertation of CCCP.

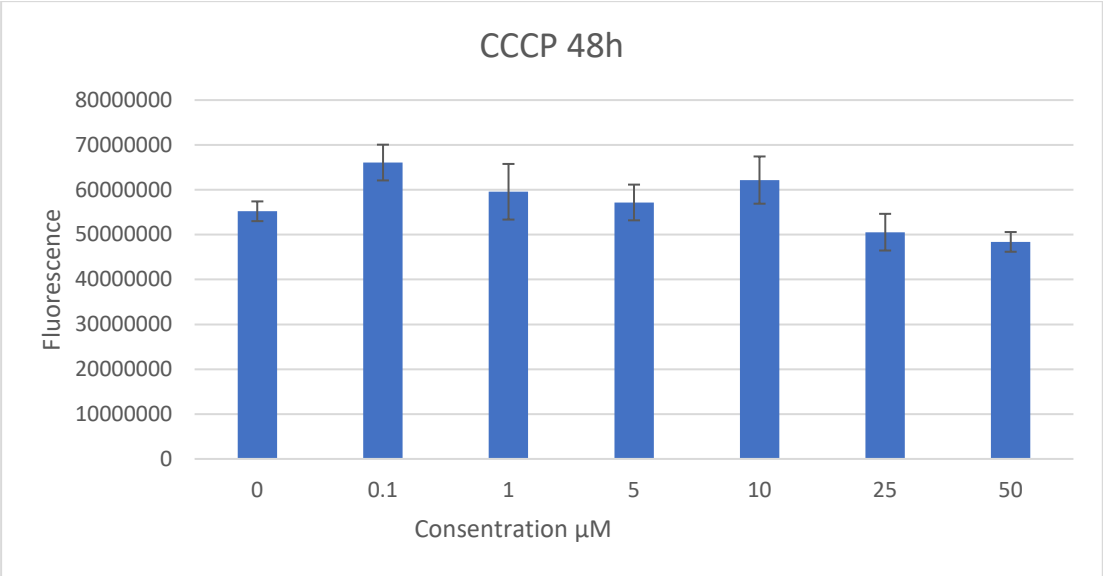

Figure (7.22): The graph of data on the figure (7.21), shows the effect of CCCP on 48 hours.

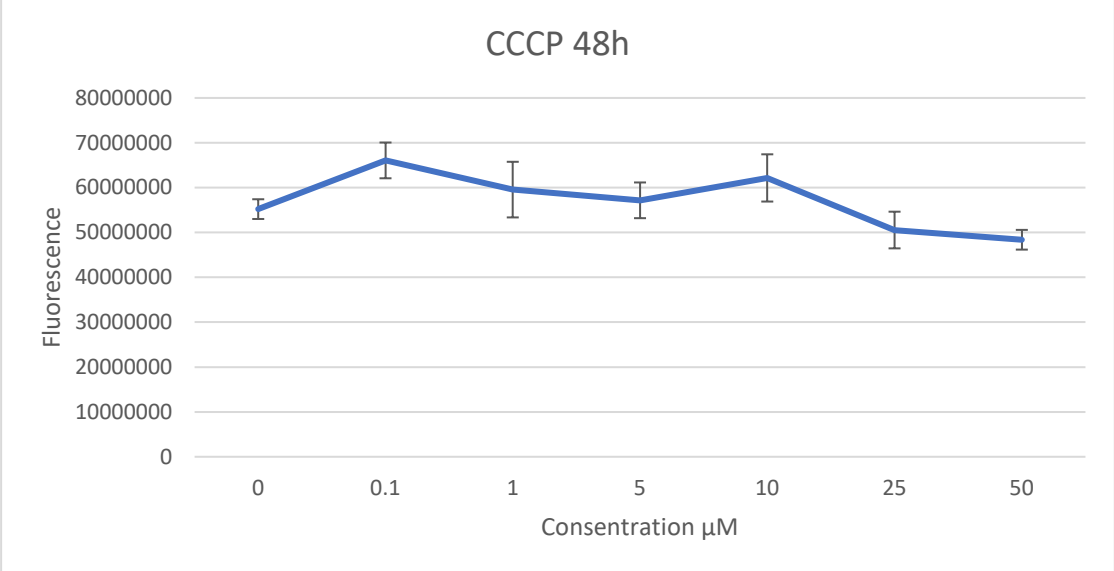

Figure (7.23): The graph of data on the figure (7.21), shows the effect of CCCP on 48 hours.

Three inhibitors in one plate.

Resazurin (positive control) CCCP

PBS Rotenone
Background control (negative control) Oligomycin
Untreatment

230316_CCC	01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
Temperature	1	2	3	4	5	6	7	8	9	10	11	12
21.mai	227094720	20334	19386	18528	18581	19313	18157	19187	17613	19607	17967	233618848
	18666	67582520	64848776	66644396	62631472	65428740	69832760	62532776	61119884	58823840	55065168	18770
	24120	57198340	63937028	65350420	66934652	67936576	68810032	64328040	59907108	55323960	51522424	19376
	18308	62092780	67134520	64704880	70859264	69846432	78287336	63716228	59000388	54075832	49281676	18388
	16689	9127477	70714904	67105824	75651032	69040232	75121984	68112520	56500168	58554980	52604752	18433
	17165	8840911	45899072	44300368	44171824	66863732	73256304	65245860	55648488	50449204	51684112	18560
	16660	9081446	39329100	40666552	37210036	81900584	70843072	67428832	58640276	56952400	57064804	18322
	230438912	18999	18663	17778	18983	17993	17382	19841	17334	18437	17651	242329728

Figure (7.24): Data of Spectro Max from CCCP, Rotenone and oligomycin (48 hours) on 16.03.2023.

Concentration	0	0.1	1	5	10	25	50
	6,76E+07	6,48E+07	6,39E+07	6,71E+07	7,07E+07	4,59E+07	3,93E+07
	5,72E+07	6,66E+07	6,54E+07	6,47E+07	6,71E+07	4,43E+07	4,07E+07
	6,21E+07	6,26E+07	6,69E+07	7,09E+07	7,57E+07	4,42E+07	3,72E+07
Average	6,23E+07	6,47E+07	6,54E+07	6,76E+07	7,12E+07	4,48E+07	3,91E+07
S.deviation	4,24E+06	1,64E+06	1,22E+06	2,53E+06	3,50E+06	7,86E+05	1,42E+06

Figure (7.25): The average and standard deviation from data of figure (7.24) just for CCCP.

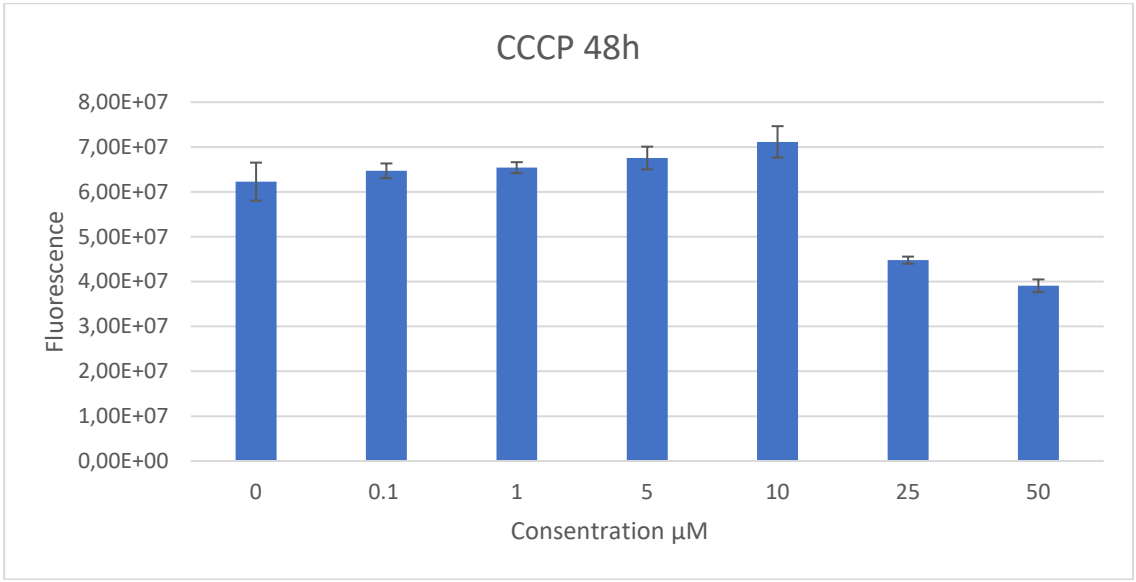

Figure (7.26): The graph of data on the figure (7.25), shows the effect of CCCP on 48 hours.

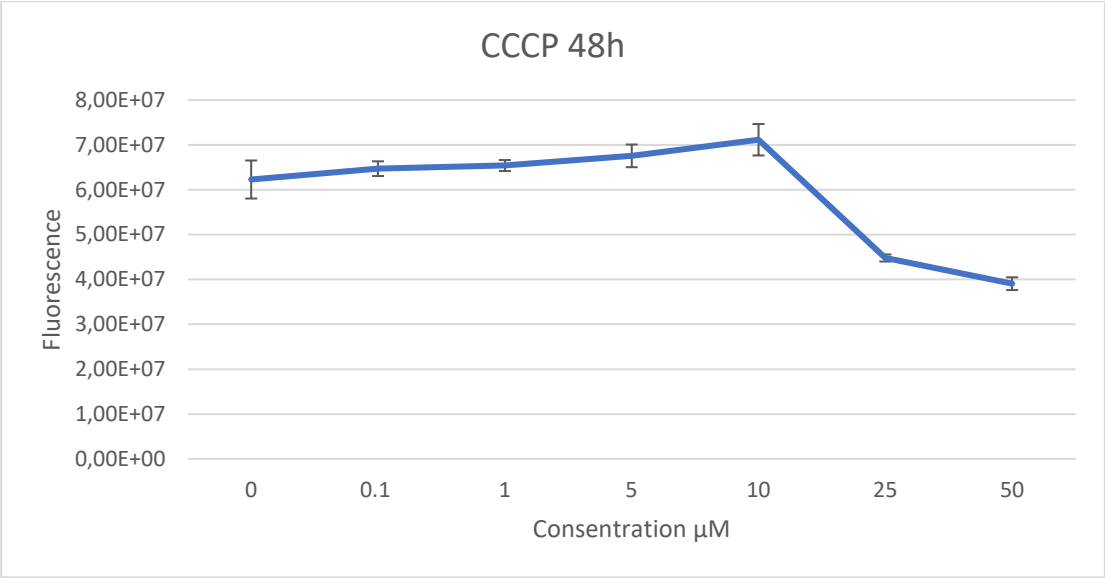

Figure (7.27): The graph of data on the figure (7.25), shows the effect of CCCP on 48 hours.

Appendix C:

- **Rotenone (24 hours):**

230224 Rote	01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
Temperature	1	2	3	4	5	6	7	8	9	10	11	12
23	1,98E+04	1,94E+04	2,00E+04	1,99E+04	1,97E+04	1,96E+04	1,83E+04	2,05E+04	1,85E+04	1,89E+04	1,44E+04	1,44E+04
	1,88E+04	1,78E+07	3,50E+07	3,90E+07	3,05E+07	3,41E+07	2,84E+07	3,45E+07	4,13E+07	1,95E+04	2,37E+08	1,50E+04
	2,04E+04	1,96E+07	4,41E+07	3,38E+07	3,54E+07	3,86E+07	3,97E+07	4,40E+07	4,81E+07	2,04E+04	2,32E+08	1,49E+04
	1,94E+04	1,73E+07	4,28E+07	4,62E+07	4,12E+07	4,28E+07	4,47E+07	4,71E+07	3,47E+07	2,05E+04	2,24E+08	1,44E+04
	1,98E+04	1,74E+07	4,14E+07	4,75E+07	5,41E+07	4,39E+07	4,49E+07	4,98E+07	5,02E+07	2,02E+04	2,34E+08	1,53E+04
	2,06E+04	1,56E+05	1,58E+05	1,58E+05	1,58E+05	1,57E+05	1,58E+05	1,57E+05	1,59E+05	1,96E+04	1,43E+04	1,38E+04
	1,82E+04	2,05E+04	2,00E+04	1,96E+04	1,93E+04	2,05E+04	1,78E+04	1,98E+04	1,88E+04	2,17E+04	1,41E+04	1,35E+04
	1,41E+04	1,34E+04	1,48E+04	1,37E+04	1,40E+04	1,43E+04	1,40E+04	1,43E+04	1,39E+04	1,38E+04	1,52E+04	1,42E+04

Figure (7.28): Data of Spectro Max from Rotenone (24 hours) on 24.02.2023.

Concentratio	0nM	1nM	10nM	100nM	200nM	1000nM	2000nM
	3,50E+07	3,90E+07	3,05E+07	3,41E+07	2,84E+07	3,45E+07	4,13E+07
	4,41E+07	3,38E+07	3,54E+07	3,86E+07	3,97E+07	4,40E+07	4,81E+07
	4,28E+07	4,62E+07	4,12E+07	4,28E+07	4,47E+07	4,71E+07	3,47E+07
	4,14E+07	4,75E+07	5,41E+07	4,39E+07	4,49E+07	4,98E+07	5,02E+07
Average	4,08E+07	4,16E+07	4,03E+07	3,98E+07	3,94E+07	4,39E+07	4,36E+07
S.deviation	3493029,87	5548795,39	8825122,63	3868107,69	6699006,64	5769614,01	6075127

Figure (7.29): The average and standard deviation from data of figure (7.28) for concertation of Rotenone (24hours).

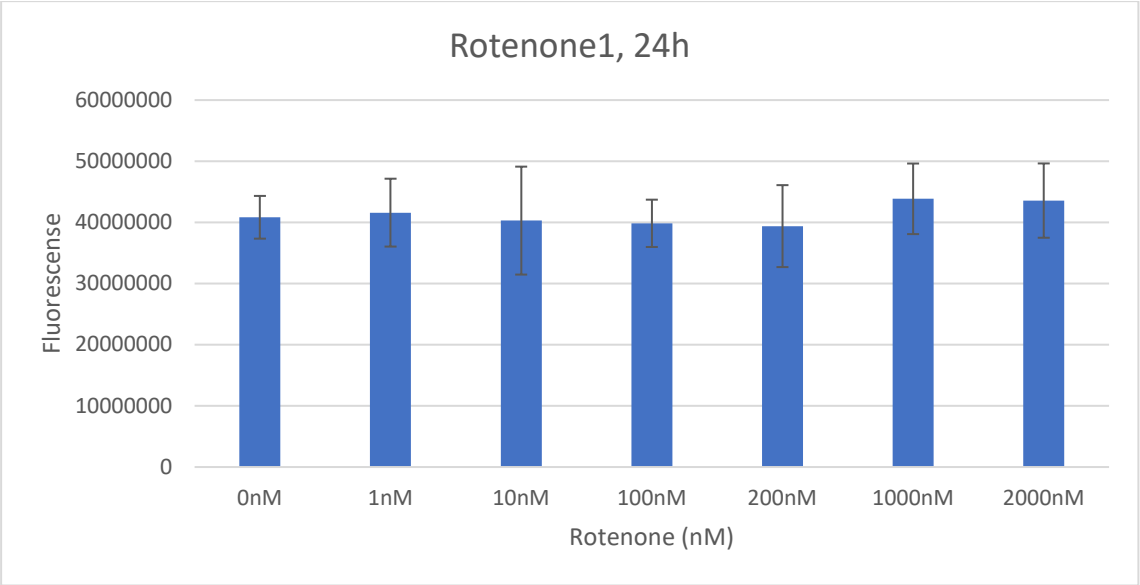

Figure (7.30): The graph of data on the figure (7.29), shows the effect of Rotenone on 24 hours.

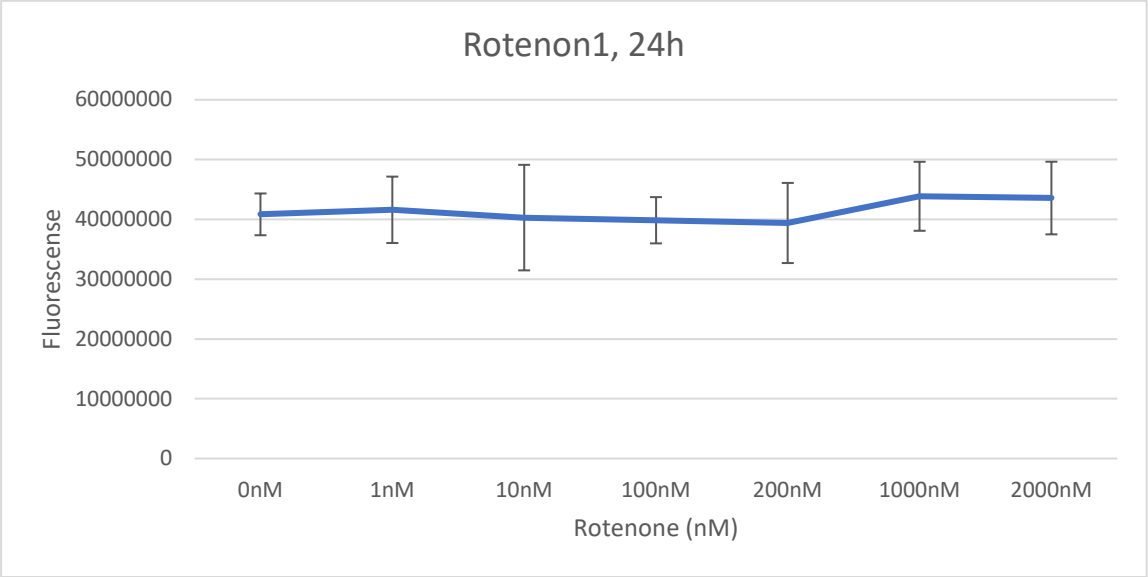

Figure (7.31): The graph of data on the figure (7.29), shows the effect of Rotenone on 24 hours.

230308_Rote	01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
Temperature	1	2	3	4	5	6	7	8	9	10	11	12
23	2,03E+04	1,98E+04	2,06E+04	1,89E+04	2,27E+04	1,85E+04	1,96E+04	1,90E+04	1,93E+04	1,88E+04	1,32E+04	1,42E+04
	1,83E+04	2,07E+07	4,82E+07	4,90E+07	4,91E+07	4,19E+07	4,26E+07	4,48E+07	3,90E+07	2,01E+04	1,76E+08	1,52E+04
	1,99E+04	1,57E+07	3,60E+07	5,31E+07	4,50E+07	4,93E+07	4,78E+07	4,00E+07	4,58E+07	1,90E+04	1,48E+08	1,49E+04
	1,98E+04	1,65E+07	3,56E+07	4,45E+07	4,38E+07	3,97E+07	4,76E+07	3,99E+07	4,56E+07	1,96E+04	1,63E+08	1,43E+04
	1,98E+04	1,70E+07	4,02E+07	5,09E+07	5,31E+07	4,59E+07	4,45E+07	4,28E+07	4,49E+07	1,99E+04	1,84E+08	1,37E+04
	2,01E+04	1,56E+05	1,54E+05	1,59E+05	1,62E+05	1,59E+05	1,60E+05	1,61E+05	1,65E+05	1,81E+04	1,33E+04	1,36E+04
	1,83E+04	1,86E+04	1,92E+04	1,87E+04	1,93E+04	1,86E+04	1,95E+04	1,95E+04	1,88E+04	1,77E+04	1,32E+04	1,49E+04
	1,58E+04	1,41E+04	1,48E+04	1,39E+04	1,50E+04	1,51E+04	1,43E+04	1,44E+04	1,47E+04	1,41E+04	1,45E+04	1,49E+04

Figure (7.32): Data of Spectro Max from Rotenone (24 hours) on 08.03.2023.

Concentratio	0	1	10	100	200	1000	2000
	4,82E+07	3,90E+07	4,48E+07	4,26E+07	4,19E+07	4,91E+07	4,90E+07
	3,60E+07	4,58E+07	4,00E+07	4,78E+07	4,93E+07	4,50E+07	5,31E+07
	3,56E+07	4,56E+07	3,99E+07	4,76E+07	3,97E+07	4,38E+07	4,45E+07
	4,02E+07	4,49E+07	4,28E+07	4,45E+07	4,59E+07	5,31E+07	5,09E+07
Average	4,00E+07	4,38E+07	4,19E+07	4,56E+07	4,42E+07	4,78E+07	4,94E+07
S.deviation	5,04E+06	2,79E+06	2,07E+06	2,19E+06	3,69E+06	3,65E+06	3,18E+06

Figure (7.33): The average and standard deviation from data of figure (7.32) for concertation of Rotenone (24hours).

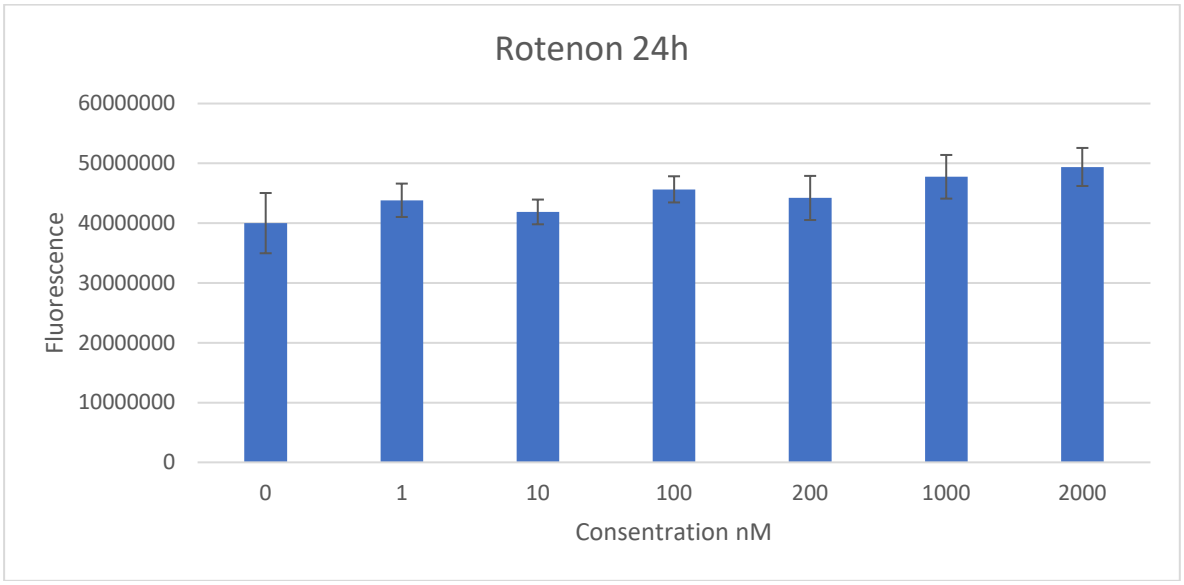

Figure (7.34): The graph of data on the figure (7.33),), shows the effect of Rotenone on 24 hours.

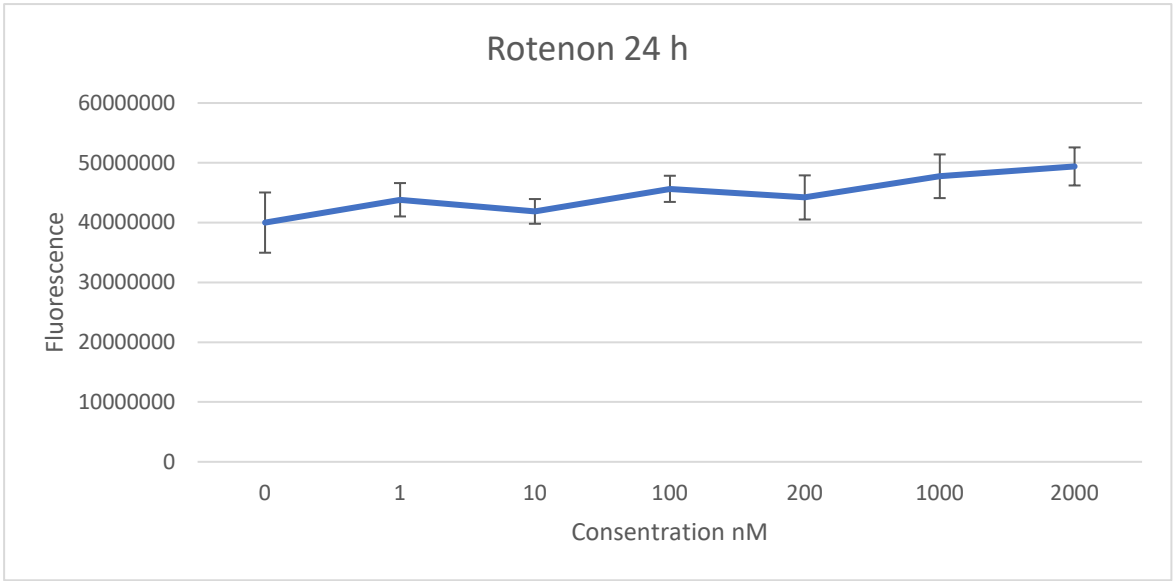

Figure (7.35): The graph of data on the figure (7.33), shows the effect of Rotenone on 24 hours.

Concentration	0	1	10	100	200	1000	2000
	3,41E+07	4,19E+07	4,49E+07	4,31E+07	4,41E+07	4,54E+07	4,62E+07
	3,52E+07	4,10E+07	4,36E+07	4,36E+07	4,18E+07	4,31E+07	4,63E+07
	3,80E+07	4,12E+07	4,76E+07	4,20E+07	4,25E+07	4,22E+07	4,26E+07
Average	3,57E+07	4,14E+07	4,53E+07	4,29E+07	4,28E+07	4,36E+07	4,50E+07
S.deviation	1642791,73	389928,293	1659405,22	665016,935	951868,558	1360443,96	1714658,29

Figure (7.36): The average and standard deviation from data of figure (7.12) just for Rotenone.

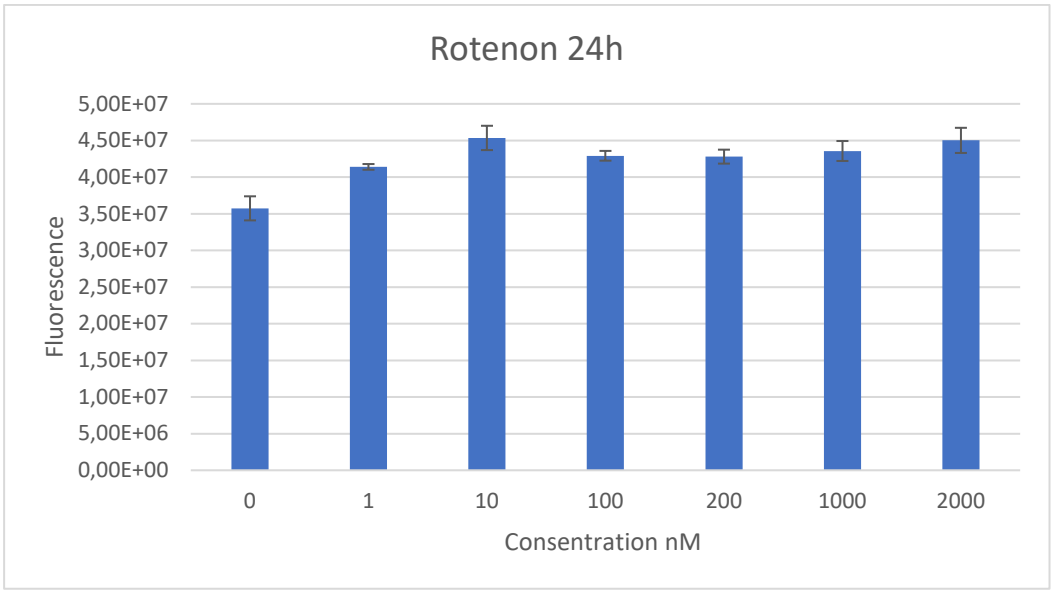

Figure (7.37): The graph of data on the figure (7.36),), shows the effect of Rotenone on 24 hours.

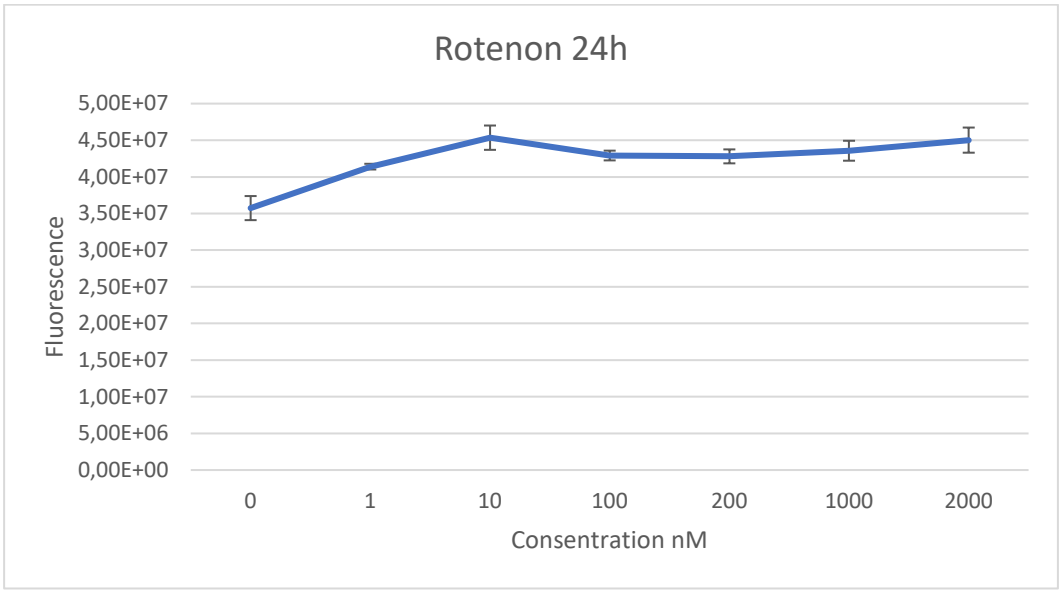

Figure (7.38): The graph of data on the figure (7.36), shows the effect of Rotenone on 24 hours.

Two inhibitors in one plate.

PBS
Background control
Untreatment
Rotenone
Oligomycin
Resazurin

230322_Rot	01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
Temperature	1	2	3	4	5	6	7	8	9	10	11	12
20	328491552	81630	79129	75623	70941	71286	71421	66749	73173	67569	71855	310419712
	65321	9402288	30317220	23349474	30574466	26341702	35185108	28348012	32373564	27471658	37202936	76953
	55962	9062596	21997712	24113776	27895118	23317668	31994592	34478020	30201734	26460834	34950392	60471
	46257	7348866	25942334	24826780	23679326	20693336	32082858	29260108	30765588	29807822	29437332	65017
	43775	150510	23608830	22149468	25157866	21749024	24394334	27699734	25735650	26242422	26937956	50577
	40754	155040	20966268	22549608	28669508	24636428	27320474	34160440	28019808	27250768	32007854	54545
	47808	142005	23710522	21455664	23038240	22965208	28805030	24738494	25809348	24829388	27343626	53006
	229135824	57875	49681	50341	51197	51242	55584	49902	57723	55057	61624	266188016

Figure (7.39): Data of Spectro Max from Rotenone and oligomycin (24 hours) on 22.03.2023.

Concentration	0	1	10	100	200	1000	2000
	2,94E+07	3,03E+07	2,20E+07	2,59E+07	2,36E+07	2,10E+07	2,37E+07
	2,69E+07	2,33E+07	2,41E+07	2,48E+07	2,21E+07	2,25E+07	2,15E+07
	3,20E+07	3,06E+07	2,79E+07	2,37E+07	2,52E+07	2,87E+07	2,30E+07
	2,73E+07	2,63E+07	2,33E+07	2,07E+07	2,17E+07	2,46E+07	2,30E+07
Average	2,89E+07	2,76E+07	2,43E+07	2,38E+07	2,32E+07	2,42E+07	2,28E+07
S.deviation	2,01E+06	2,99E+06	2,19E+06	1,96E+06	1,34E+06	2,89E+06	8,25E+05

Figure (7.40): The average and standard deviation from data of figure (7.39) just for concertation of Rotenone (24 hours).

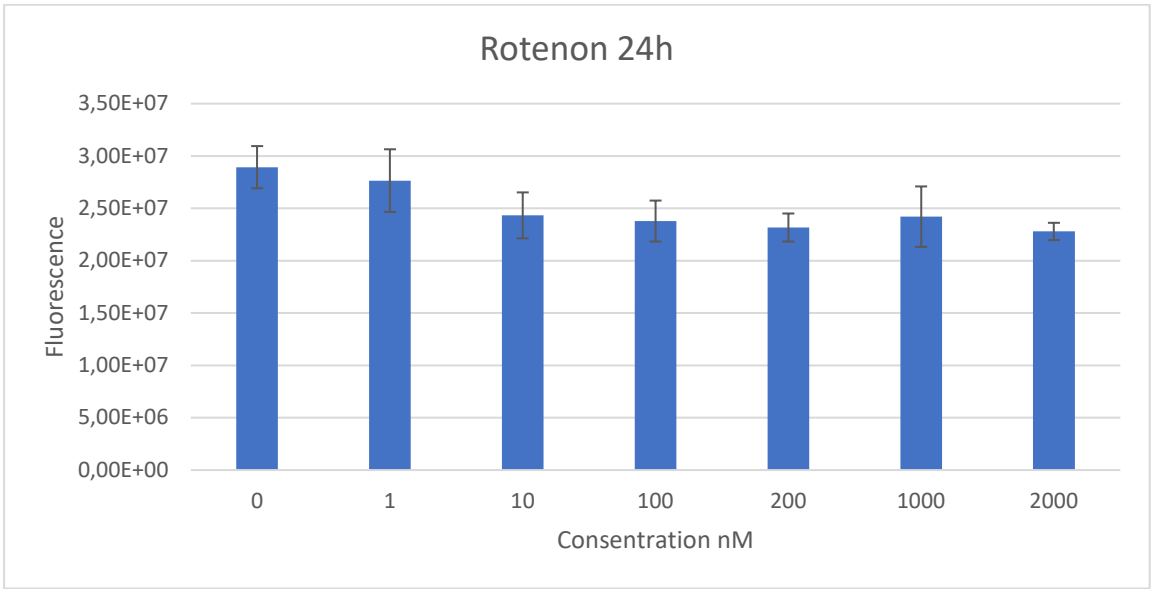

Figure (7.41): The graph of data on the figure (7.40),), shows the effect of Rotenone on 24 hours.

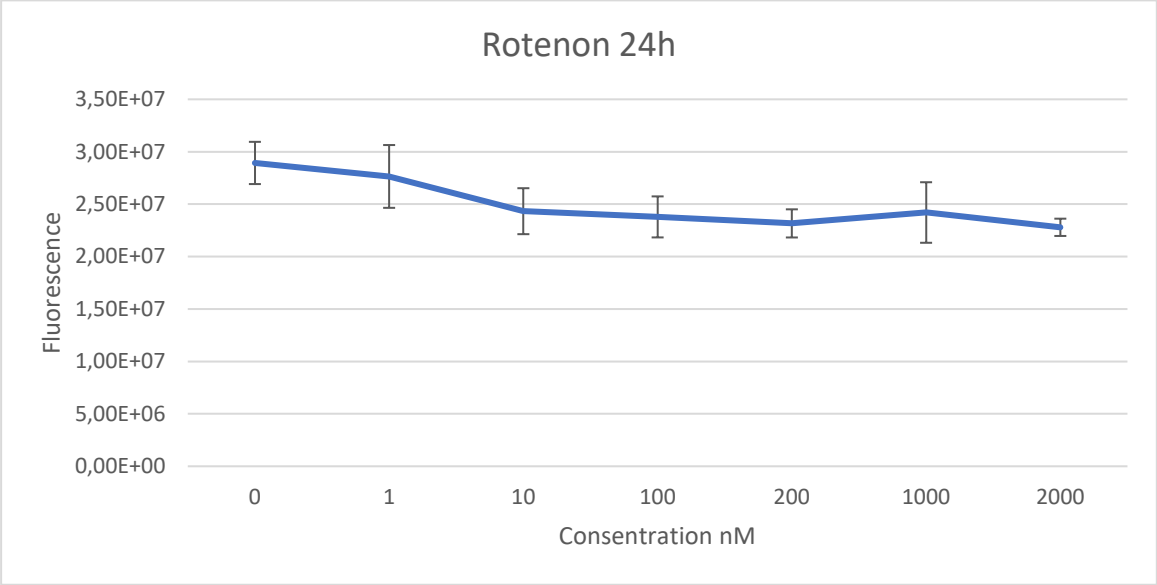

Figure (7.42): The graph of data on the figure (7.40,), shows the effect of Rotenone on 24 hours.

- **Rotenone (48 hours):**

230309_Rote	01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
Temperature	1	2	3	4	5	6	7	8	9	10	11	12
21.mai	1,94E+04	1,81E+04	1,94E+04	1,76E+04	1,86E+04	1,74E+04	1,86E+04	1,88E+04	1,79E+04	1,85E+04	1,40E+04	1,51E+04
	1,85E+04	1,76E+07	5,50E+07	9,32E+07	7,88E+07	5,74E+07	6,26E+07	6,07E+07	6,28E+07	1,81E+04	2,41E+08	1,48E+04
	1,90E+04	2,05E+07	5,60E+07	8,53E+07	9,02E+07	7,87E+07	7,02E+07	7,33E+07	5,56E+07	1,98E+04	2,30E+08	1,48E+04
	1,80E+04	1,90E+07	5,17E+07	9,07E+07	8,45E+07	7,98E+07	6,65E+07	7,21E+07	5,62E+07	1,91E+04	2,15E+08	1,40E+04
	1,72E+04	2,01E+07	5,64E+07	8,90E+07	7,86E+07	8,51E+07	7,72E+07	6,87E+07	6,26E+07	1,74E+04	2,19E+08	1,37E+04
	1,85E+04	1,68E+05	1,64E+05	1,62E+05	1,97E+05	1,58E+05	1,62E+05	1,64E+05	1,67E+05	1,79E+04	1,36E+04	1,42E+04
	1,74E+04	1,91E+04	1,76E+04	1,74E+04	1,77E+04	1,82E+04	1,74E+04	1,79E+04	1,81E+04	1,58E+04	1,34E+04	1,35E+04
	1,43E+04	1,36E+04	1,36E+04	1,30E+04	1,34E+04	1,36E+04	1,29E+04	1,39E+04	1,32E+04	1,35E+04	1,36E+04	1,35E+04

Figure (7.43): Data of Spectro Max from Rotenone (48 hours) on 09.03.2023.

Concentratio	0	1	10	100	200	1000	2000
	5,50E+07	6,28E+07	6,07E+07	6,26E+07	5,74E+07	7,88E+07	9,32E+07
	5,60E+07	5,56E+07	7,33E+07	7,02E+07	7,87E+07	9,02E+07	8,53E+07
	5,17E+07	5,62E+07	7,21E+07	6,65E+07	7,98E+07	8,45E+07	9,07E+07
	5,64E+07	6,26E+07	6,87E+07	7,72E+07	8,51E+07	7,86E+07	8,90E+07
Average	5,48E+07	5,93E+07	6,87E+07	6,91E+07	7,53E+07	8,30E+07	8,96E+07
S.deviation	1,84E+06	3,41E+06	4,92E+06	5,38E+06	1,06E+07	4,76E+06	2,88E+06

Figure (7.44): The average and standard deviation from data of figure (7.43) and concertation of Rotenone (24 hours).

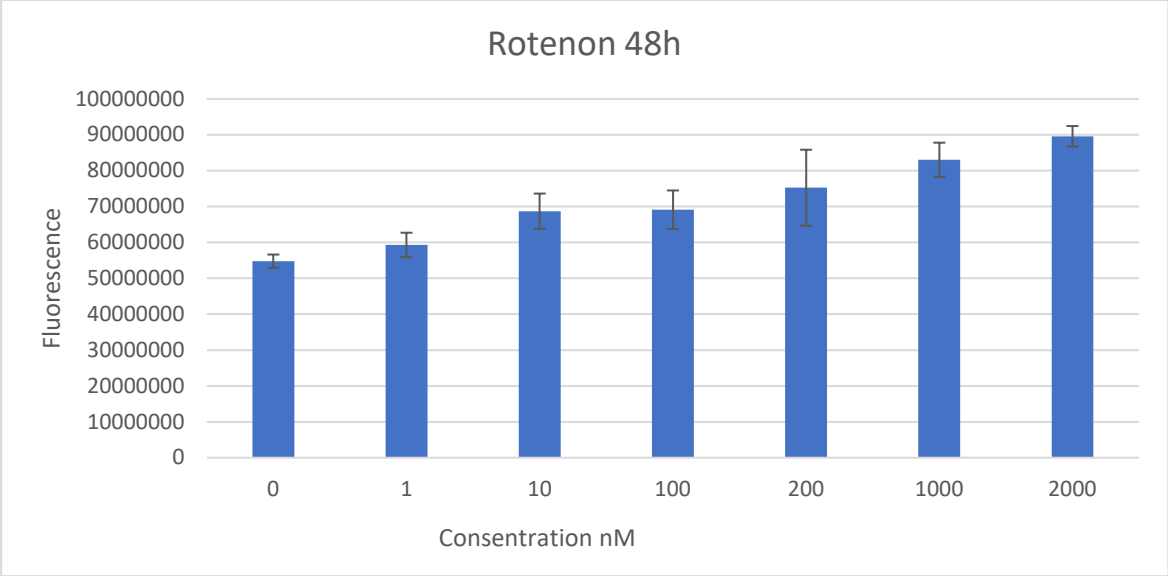

Figure (7.45): The graph of data on the figure (7.44), shows the effect of Rotenone on 48 hours.

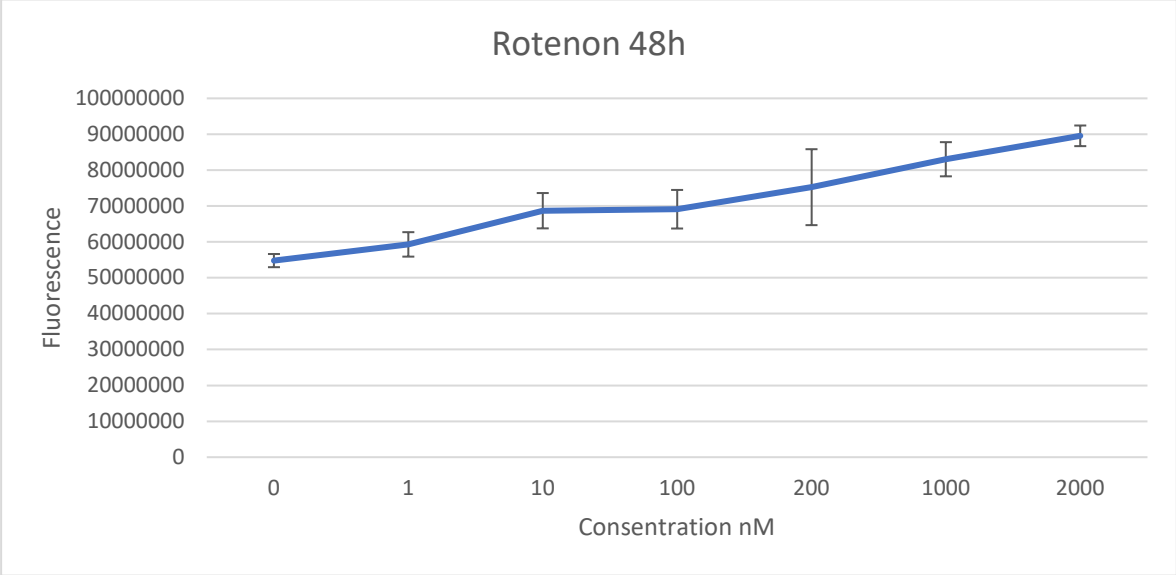

Figure (7.46): The graph of data on the figure (7.44), shows the effect of Rotenone on 48 hours.

Concentration	0	1	10	100	200	1000	2000
	6,76E+07	6,54E+07	6,79E+07	6,98E+07	6,90E+07	6,69E+07	8,19E+07
	5,72E+07	6,98E+07	6,88E+07	7,83E+07	7,51E+07	7,33E+07	7,08E+07
	6,21E+07	6,25E+07	6,43E+07	6,37E+07	6,81E+07	6,52E+07	6,74E+07
Average	6,23E+07	6,59E+07	6,70E+07	7,06E+07	7,08E+07	6,85E+07	7,34E+07
S.deviation	4,24E+06	3,00E+06	1,94E+06	5,97E+06	3,11E+06	3,46E+06	6,18E+06

Figure (7.47): The average and standard deviation from data of figure (7.24) just for concertation of Rotenone 48 hours).

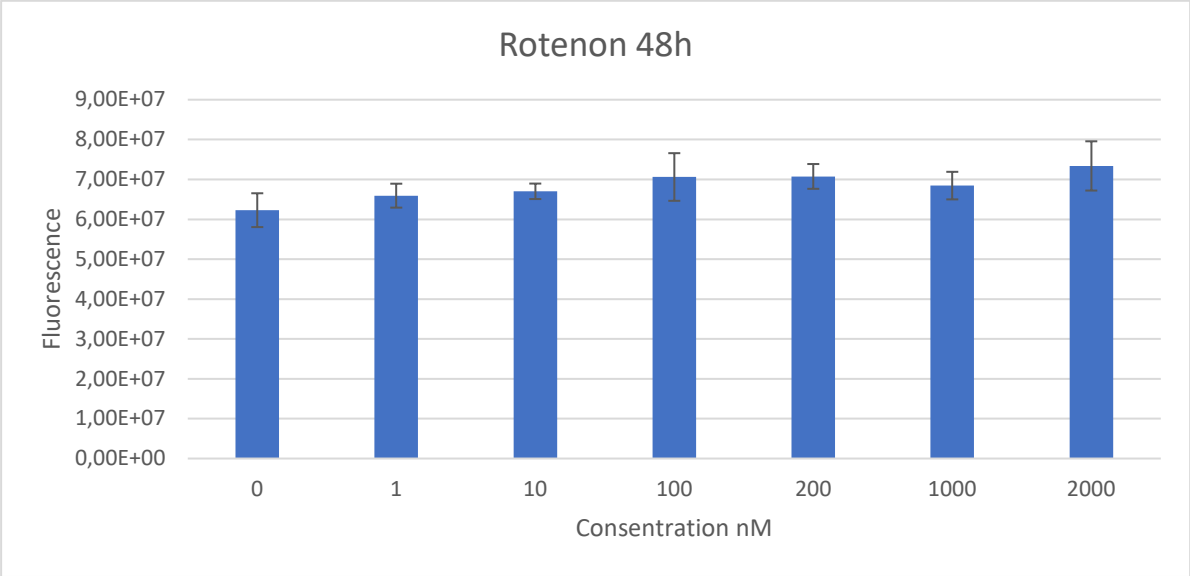

Figure (7.48): The graph of data on the figure (7.47), shows the effect of Rotenone on 48 hours.

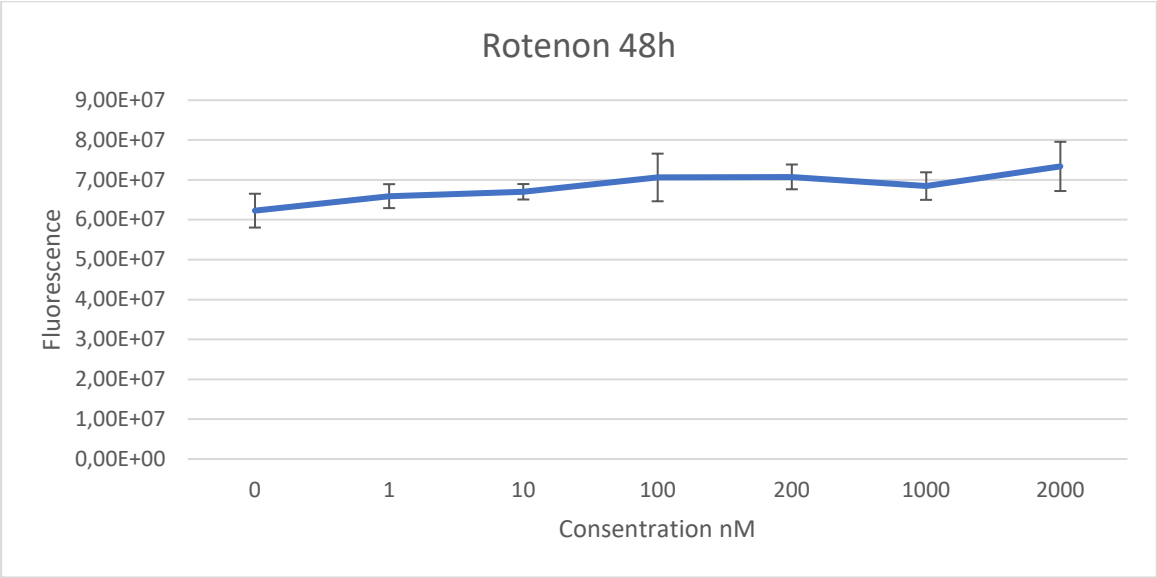

Figure (7.49): The graph of data on the figure (7.47), shows the effect of Rotenone on 48 hours.

Two inhibitors in one plate.

PBS
Background control
Untreatment
Rotenone
Oligomycin
Resazurin

230223 Rot/	01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
Temperature	1	2	3	4	5	6	7	8	9	10	11	12
22.mai	1,77E+08	1,51E+04	8,14E+03	7,70E+03	8,37E+03	8,68E+03	8,08E+03	8,54E+03	7,34E+03	7,81E+03	1,13E+04	1,93E+08
	1,50E+04	5,12E+04	3,35E+07	3,89E+07	1,67E+07	3,37E+07	1,53E+07	1,69E+07	1,69E+07	1,70E+07	3,96E+07	1,44E+04
	7,52E+03	4,90E+04	1,44E+07	3,48E+07	1,82E+07	1,61E+07	3,68E+07	1,65E+07	3,41E+07	1,82E+07	1,80E+07	8,81E+03
	7,51E+03	4,90E+04	1,74E+07	1,86E+07	1,63E+07	1,63E+07	1,52E+07	1,58E+07	1,92E+07	1,56E+07	1,59E+07	9,06E+03
	8,04E+03	3,06E+06	1,59E+07	1,75E+07	1,72E+07	1,50E+07	1,47E+07	1,48E+07	1,56E+07	1,56E+07	1,57E+07	8,46E+03
	7,69E+03	3,11E+06	1,33E+07	1,47E+07	1,55E+07	1,28E+07	1,41E+07	1,77E+07	1,94E+07	1,45E+07	1,56E+07	8,21E+03
	1,12E+04	2,99E+06	1,28E+07	1,24E+07	1,39E+07	1,36E+07	1,37E+07	1,62E+07	1,51E+07	1,75E+07	1,52E+07	1,10E+04
	8,40E+07	1,50E+04	8,47E+03	7,55E+03	8,81E+03	8,47E+03	8,18E+03	8,17E+03	7,75E+03	8,90E+03	1,12E+04	8,80E+07

Figure (7.50): Data of Spectro Max from Rotenone and oligomycin (48 hours) on 23.03.2023.

Concentratio	0	1	10	100	200	1000	2000
	1,33E+07	3,89E+07	3,48E+07	1,86E+07	1,75E+07	1,47E+07	1,24E+07
	1,28E+07	1,67E+07	1,82E+07	1,63E+07	1,72E+07	1,55E+07	1,39E+07
	1,44E+07	3,37E+07	1,61E+07	1,63E+07	1,50E+07	1,28E+07	1,36E+07
	1,59E+07	1,53E+07	3,68E+07	1,52E+07	1,47E+07	1,41E+07	1,37E+07
Average	1,41E+07	2,61E+07	2,65E+07	1,66E+07	1,61E+07	1,43E+07	1,34E+07
S.deviation	1171429,03	10313557,5	9393779,14	1221070,71	1244089,69	981417,825	572399,071

Figure (7.51): The average and standard deviation from data of figure (7.50) and concertation of Rotenone (48 hours).

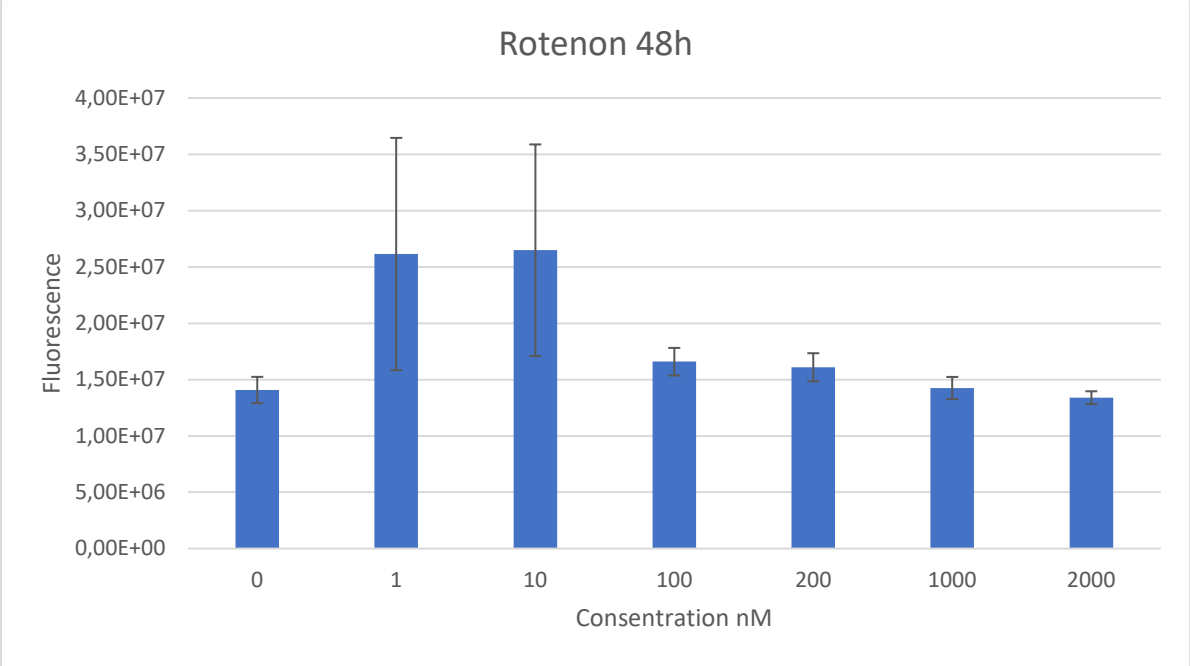

Figure (7.52): The graph of data on the figure (7.51), shows the effect of Rotenone on 48 hours.

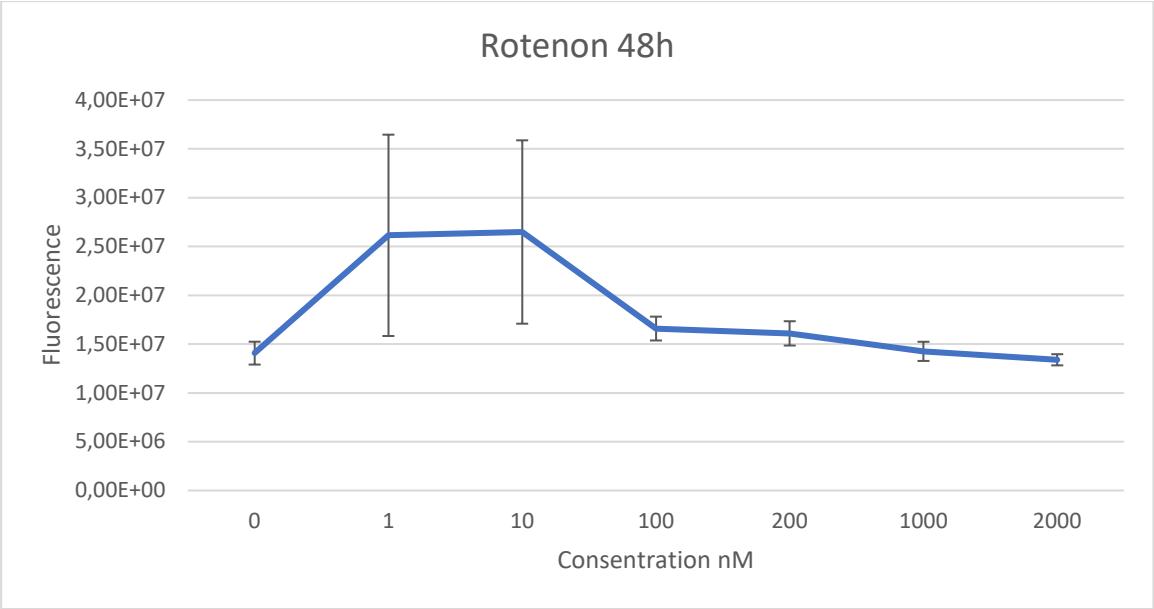

Figure (7.53): The graph of data on the figure (7.51), shows the effect of Rotenone on 48 hours.

Appendix D:

- **Oligomycin (24)**

Concentration	0	1	10	50	100	250	500
	3,41E+07	4,49E+07	4,16E+07	4,45E+07	4,63E+07	4,66E+07	4,46E+07
	3,52E+07	4,00E+07	4,21E+07	4,48E+07	4,68E+07	4,21E+07	4,72E+07
	3,80E+07	4,14E+07	4,01E+07	3,99E+07	3,96E+07	3,84E+07	4,06E+07
Average	3,57E+07	4,21E+07	4,12E+07	4,30E+07	4,42E+07	4,24E+07	4,41E+07
S.deviation	1,64E+06	2,05E+06	8,50E+05	2,26E+06	3,28E+06	3,36E+06	2,71E+06

Figure (7.54): The average and standard deviation from data of figure (7.12) just for Oligomycin.

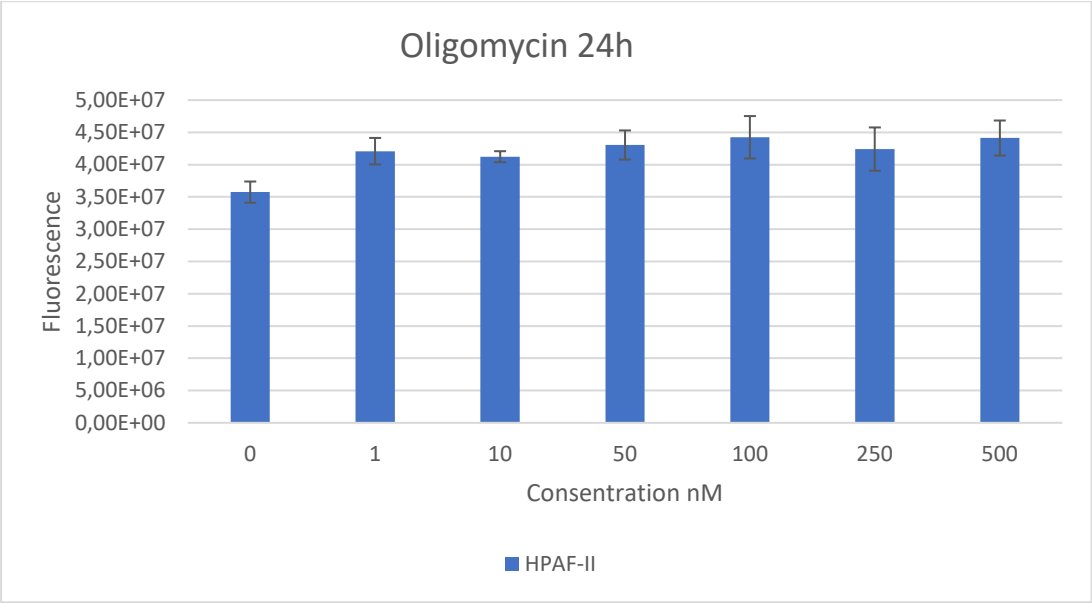

Figure (7.55): The graph of data on the figure (7.54), shows the effect of Oligomycin on 24 hours.

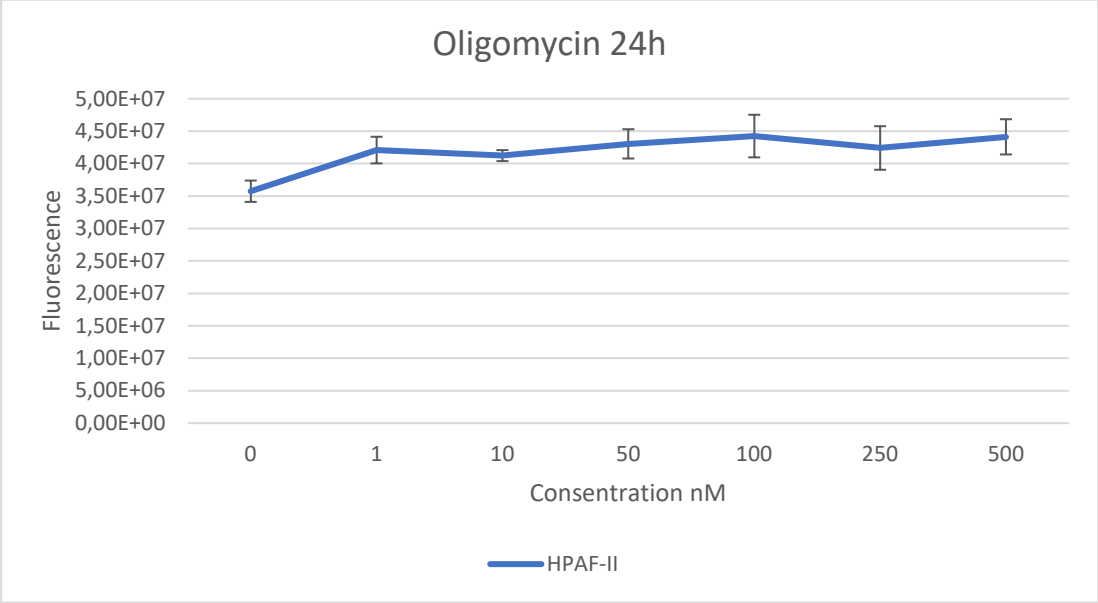

Figure (7.56): The graph of data on the figure (7.54), shows the effect of Oligomycin on 24 hours.

Concentratio	0	1	10	50	100	250	500
	4,68E+07	3,89E+07	4,42E+07	4,54E+07	4,25E+07	4,24E+07	3,76E+07
	4,38E+07	5,88E+07	4,87E+07	5,06E+07	5,39E+07	4,92E+07	4,37E+07
	4,45E+07	4,65E+07	4,86E+07	5,34E+07	4,96E+07	5,15E+07	4,00E+07
	4,63E+07	4,30E+07	4,78E+07	4,44E+07	4,42E+07	4,46E+07	4,54E+07
Average	4,54E+07	4,68E+07	4,73E+07	4,84E+07	4,75E+07	4,69E+07	4,17E+07
S.deviation	1,23E+06	7,43E+06	1,83E+06	3,69E+06	4,52E+06	3,60E+06	3,07E+06

Figure (7.57): The average and standard deviation from data of figure (7.39) just for concertation of Oligomycin (24 hours).

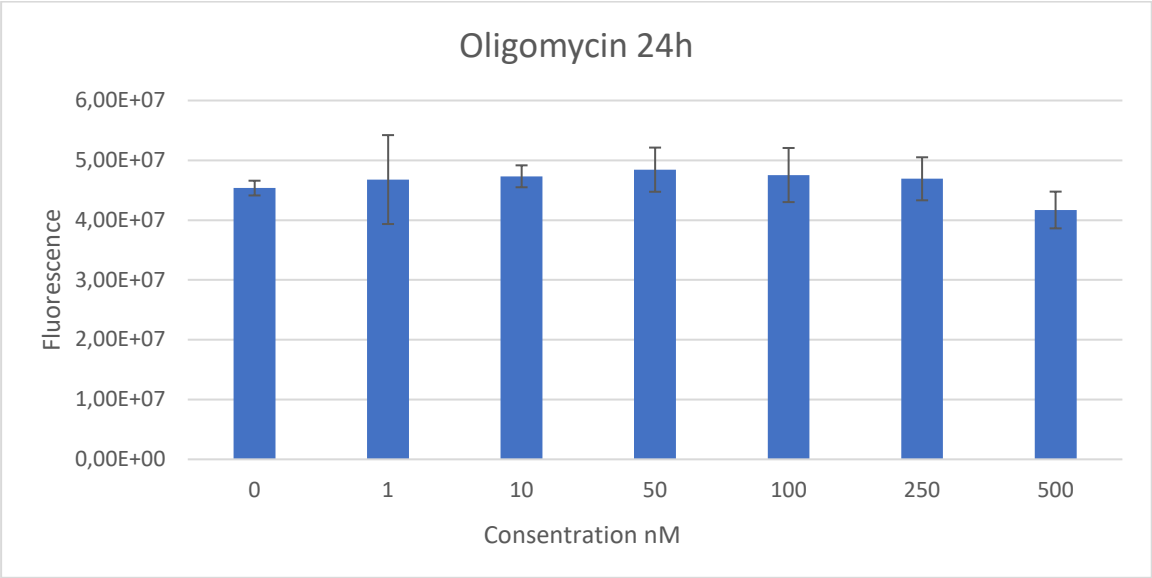

Figure (7.58): The graph of data on the figure (7.57),), shows the effect of Oligomycin on 24 hours.

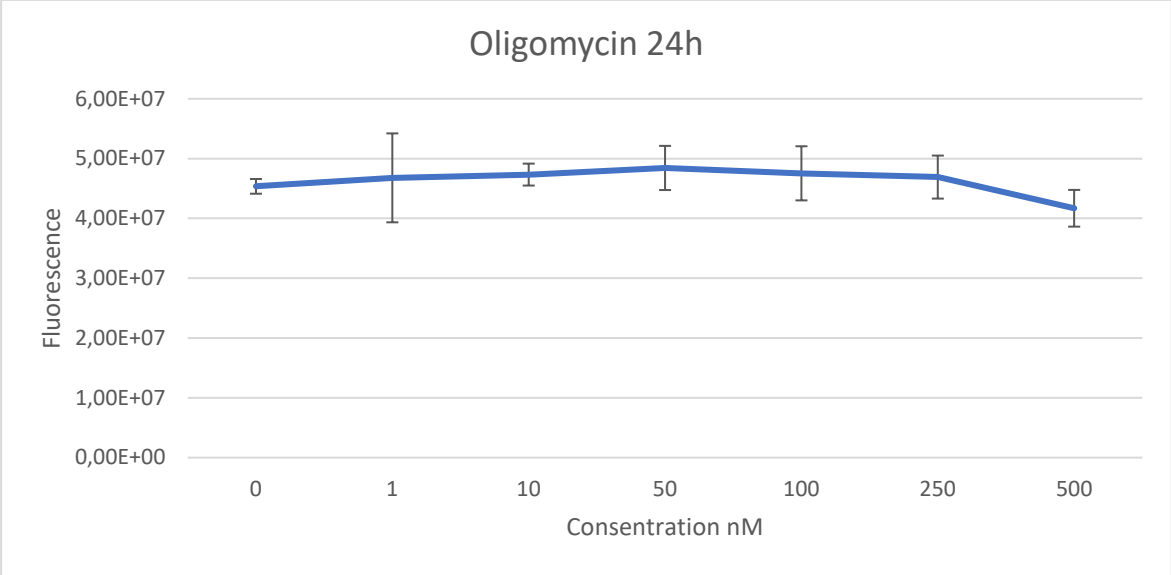

Figure (7.59): The graph of data on the figure (7.57), shows the effect of Oligomycin on 24 hours.

230322_Olig,	01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
Temperature	1	2	3	4	5	6	7	8	9	10	11	12
20	69471	84537	80198	75889	68570	71286	71242	70877	67379	71114	68689	90926
	46005	14701881	46797080	38860956	44212840	45419492	42454912	42405628	37615160	310071552	67495	83959
	76237	13934731	43824124	58777976	48726724	50577628	53912192	49210160	43730572	299727840	68260	83355
	74925	14158892	44500640	46495524	48588996	53371840	49594500	51457768	39991040	84162	72221	84958
	72680	14555866	46316504	42989420	47777908	44373392	44193664	44566472	45442120	82623	67951	89486
	69615	276364	312755	309274	296739	315809	323120	317486	295047	80263	69190	87037
	73296	75628	76079	75713	77096	72267	54492	74101	73497	70376	74162	88009
	85511	83297	85708	83193	84728	84400	79574	84390	88378	86629	85037	89155

Figure (7.60): Data of Spectro Max from oligomycin (24 hours) on 22.03.2023.

Concentratio	0	1	10	50	100	250	500
	4,68E+07	3,89E+07	4,42E+07	4,54E+07	4,25E+07	4,24E+07	3,76E+07
	4,38E+07	5,88E+07	4,87E+07	5,06E+07	5,39E+07	4,92E+07	4,37E+07
	4,45E+07	4,65E+07	4,86E+07	5,34E+07	4,96E+07	5,15E+07	4,00E+07
	4,63E+07	4,30E+07	4,78E+07	4,44E+07	4,42E+07	4,46E+07	4,54E+07
Average	4,54E+07	4,68E+07	4,73E+07	4,84E+07	4,75E+07	4,69E+07	4,17E+07
Average	1,23E+06	7,43E+06	1,83E+06	3,69E+06	4,52E+06	3,60E+06	3,07E+06

Figure (7.61): The average and standard deviation from data of figure (7.60) and concertation of Oligomycin (24 hours).

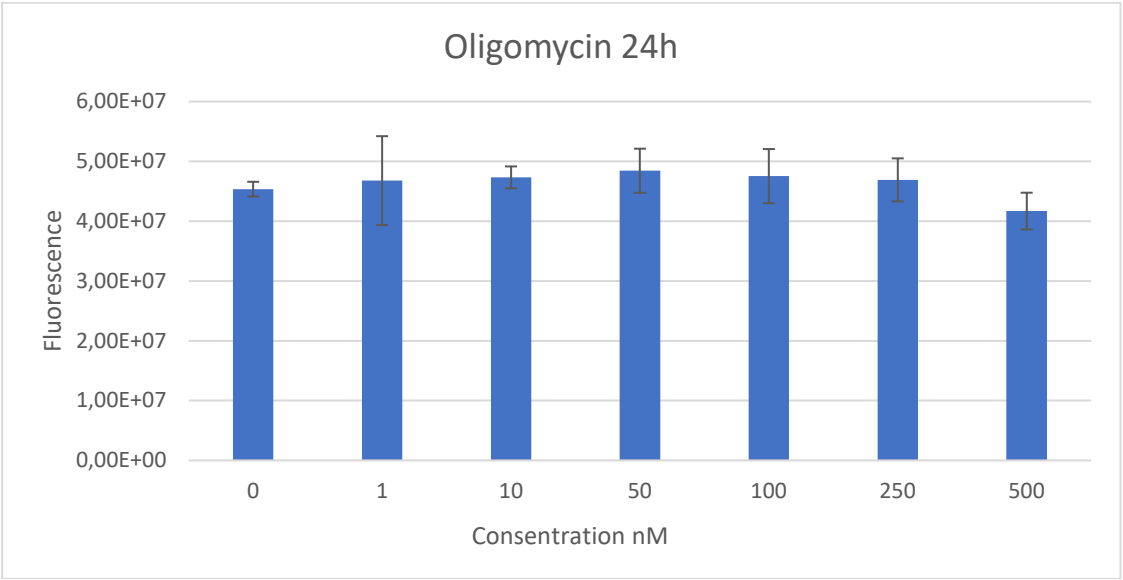

Figure (7.62): The graph of data on the figure (7.61), shows the effect of Oligomycin on 24 hours.

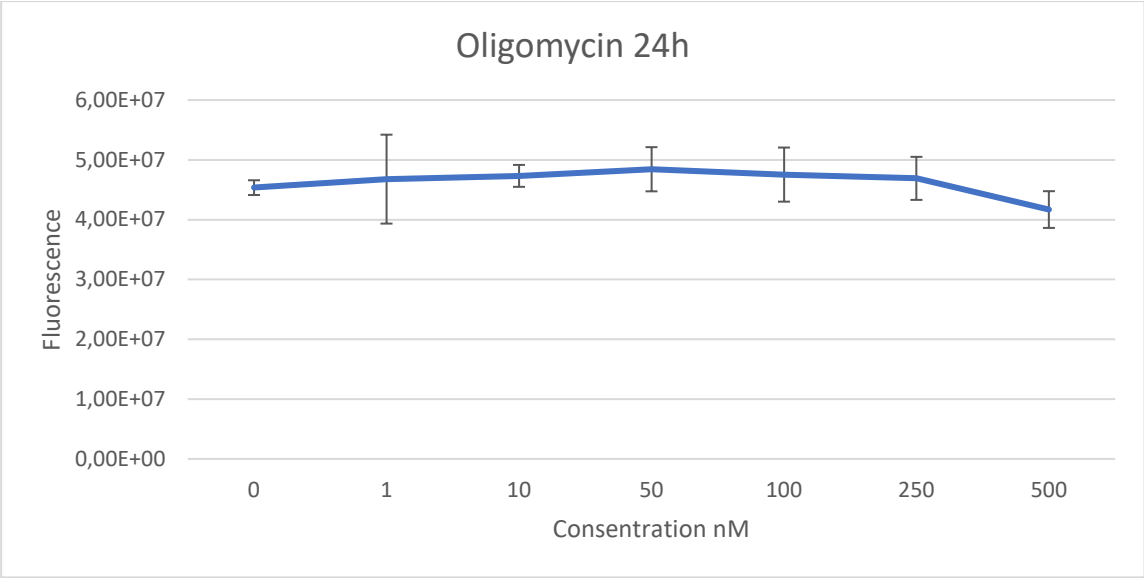

Figure (7.63): The graph of data on the figure (7.61), shows the effect of Oligomycin on 24 hours.

- **Oligomycin (48 hours)**

01.mar	PlateFormat	Endpoint	Fluorescence	FALSE	Raw	FALSE	1				
1	2	3	4	5	6	7	8	9	10	11	12
14097	18859	19009	16642	19352	19301	19069	19078	16978	19501	18330	12171
18132	8079094	39994272	37447460	35395604	33816636	32506764	33996688	34352060	163443408	21310	14411
19451	7506138	36795844	38542240	42741200	32092968	35234740	38050356	36518688	153747936	19200	13505
19313	6338956	34389056	29297960	32715032	28037330	29619304	27228876	26645044	150083904	20367	14565
19474	6860426	32346022	36119572	34861216	32021524	20218756	27539496	21984624	153478560	23982	13998
19768	147003	142378	137573	141017	135230	133152	142432	138432	14175	15941	15190
17825	19474	19652	20075	19617	20015	20437	19406	19233	18936	19344	13538
13127	14800	15254	12494	15578	15210	16706	15360	12360	14946	15051	12799

Figure (7.64): Data of Spectro Max from oligomycin (48 hours) on 23.03.2023.

Concentratio	0	1	10	50	100	250	500
	4,00E+07	3,74E+07	3,54E+07	3,38E+07	3,25E+07	3,40E+07	3,44E+07
	3,68E+07	3,85E+07	4,27E+07	3,21E+07	3,52E+07	3,81E+07	3,65E+07
	3,44E+07	2,93E+07	3,27E+07	2,80E+07	2,96E+07	2,72E+07	2,66E+07
	3,23E+07	3,61E+07	3,49E+07	3,20E+07	2,02E+07	2,75E+07	2,20E+07
Average	3,59E+07	3,54E+07	3,64E+07	3,15E+07	2,94E+07	3,17E+07	2,99E+07
S.deviation	2,85E+06	3,60E+06	3,78E+06	2,12E+06	5,66E+06	4,55E+06	5,85E+06

Figure (7.65): The average and standard deviation from data of figure (7.64) and concertation of Oligomycin (48 hours).

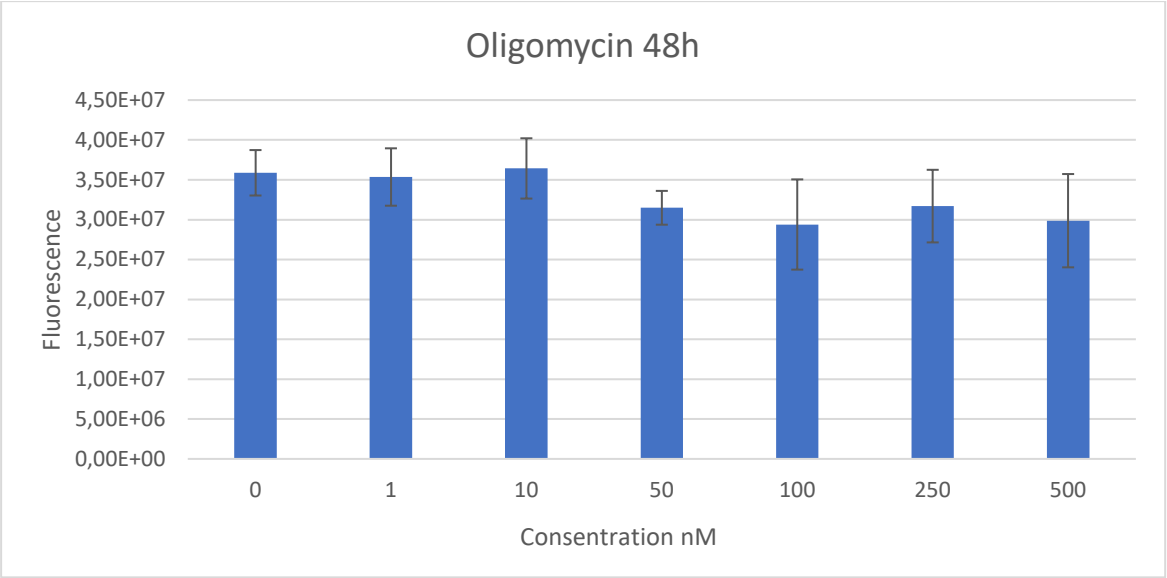

Figure (7.66): The graph of data on the figure (7.65), shows the effect of Oligomycin on 48 hours.

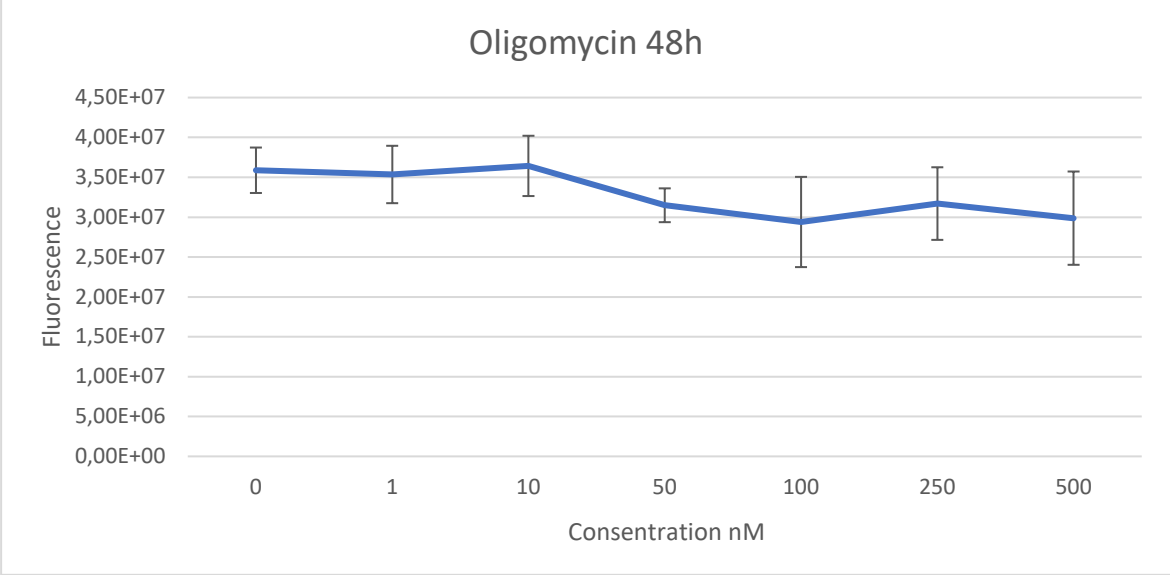

Figure (7.67): The graph of data on the figure (7.65), shows the effect of Oligomycin on 48 hours.

Concentratio	0	1	10	50	100	250	500
	6,76E+07	6,11E+07	5,99E+07	5,90E+07	5,65E+07	5,56E+07	5,86E+07
	5,72E+07	5,88E+07	5,53E+07	5,41E+07	5,86E+07	5,04E+07	5,70E+07
	6,21E+07	5,51E+07	5,15E+07	4,93E+07	5,26E+07	5,17E+07	5,71E+07
Average	6,23E+07	5,83E+07	5,56E+07	5,41E+07	5,59E+07	5,26E+07	5,76E+07
S.deviation	4,24E+06	2,50E+06	3,43E+06	3,97E+06	2,47E+06	2,22E+06	7,71E+05

Figure (7.68): The average and standard deviation from data of figure (7.24) just for concertation of Oligomycin 48 hours).

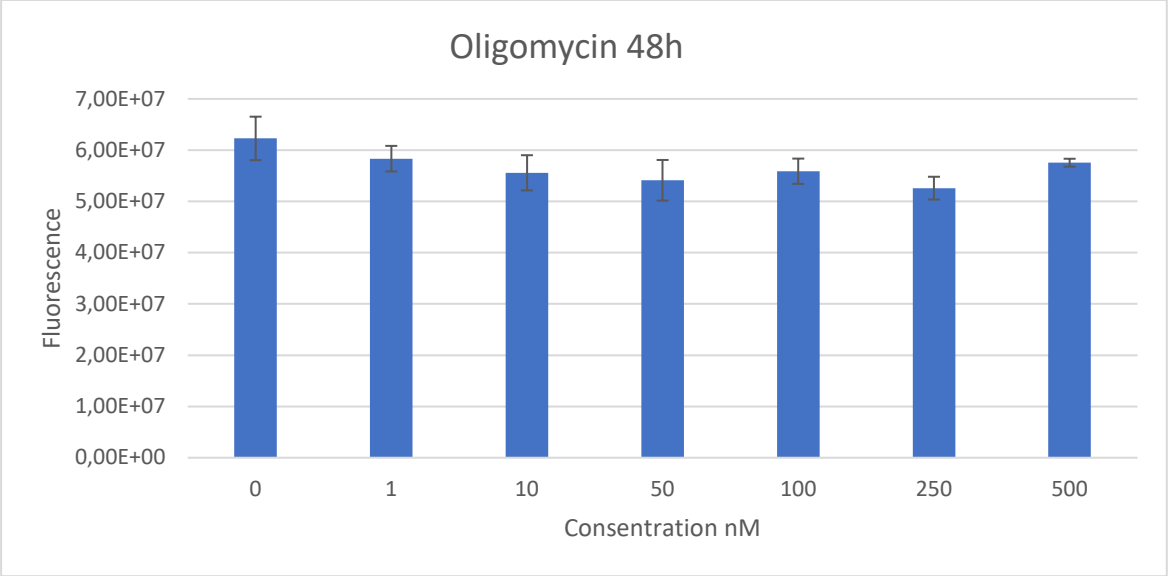

Figure (7.69): The graph of data on the figure (7.68), shows the effect of Oligomycin on 48 hours.

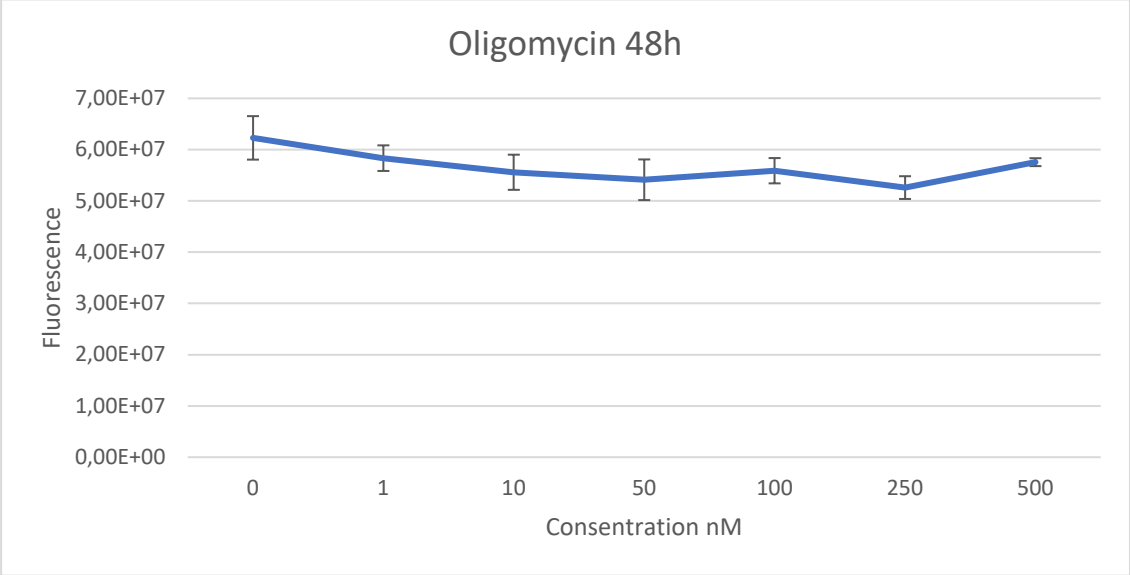

Figure (7.70): The graph of data on the figure (7.69), shows the effect of Oligomycin on 48 hours.

Concentratio	0	1	10	50	100	250	500
	1,59E+07	1,69E+07	1,65E+07	1,58E+07	1,48E+07	1,77E+07	1,62E+07
	1,33E+07	1,69E+07	3,41E+07	1,92E+07	1,56E+07	1,94E+07	1,51E+07
	1,28E+07	1,70E+07	1,82E+07	1,56E+07	1,56E+07	1,45E+07	1,75E+07
	1,44E+07	3,96E+07	1,80E+07	1,59E+07	1,57E+07	1,56E+07	1,52E+07
Average	1,41E+07	2,26E+07	2,17E+07	1,66E+07	1,54E+07	1,68E+07	1,60E+07
Average	1,17E+06	9,79E+06	7,20E+06	1,49E+06	3,37E+05	1,91E+06	9,51E+05

Figure (7.71): The average and standard deviation from data of figure (7.50) and concertation of Oligomycin (48 hours).

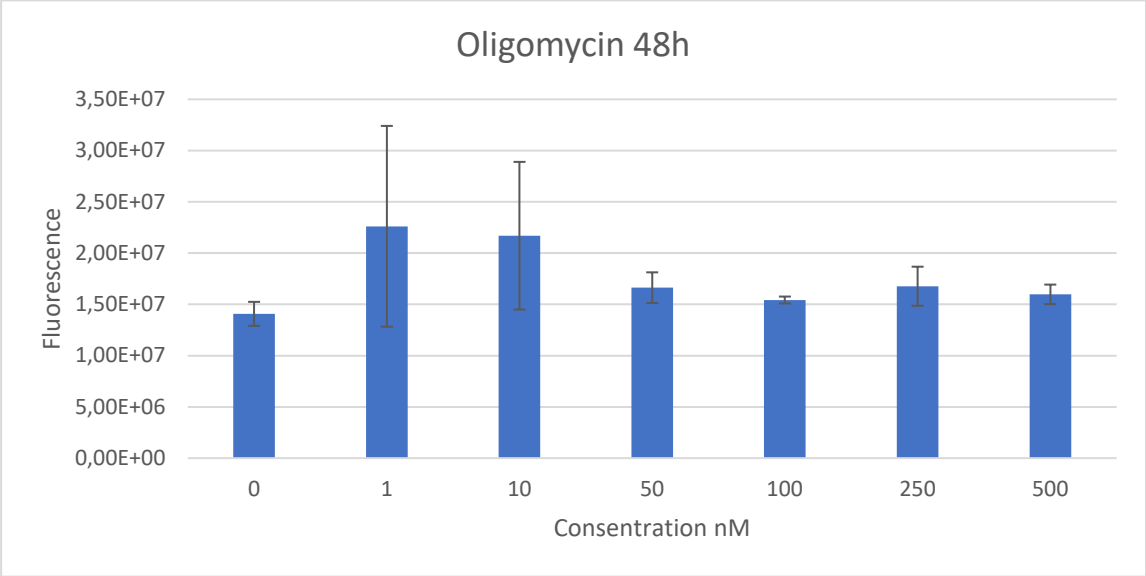

Figure (7.72): The graph of data on the figure (7.71), shows the effect of Oligomycin on 48 hours.

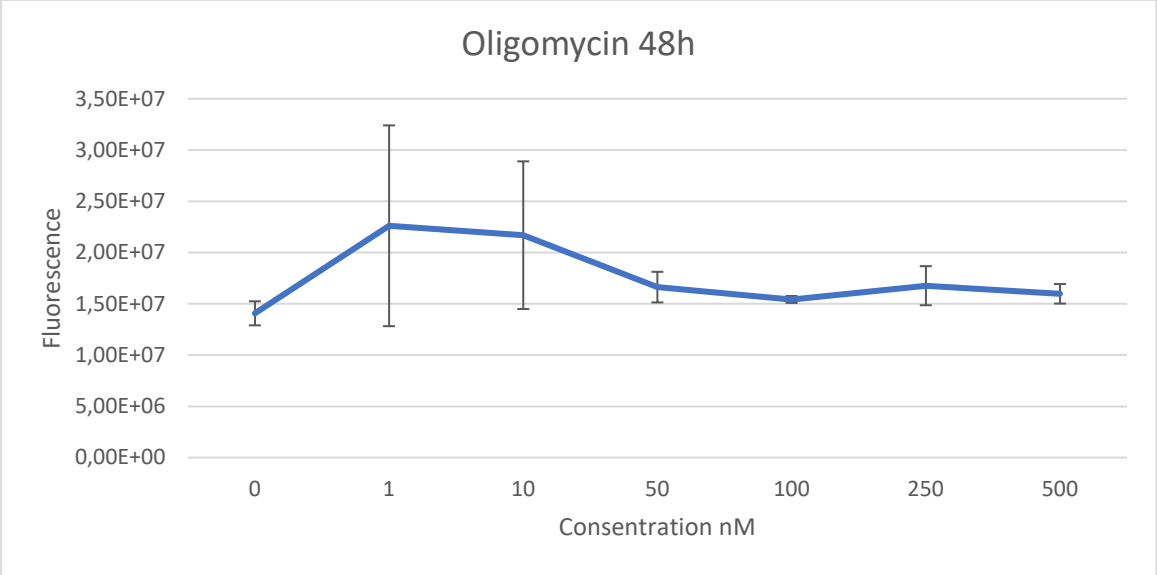

Figure (7.73): The graph of data on the figure (7.71), shows the effect of Oligomycin on 48 hours.