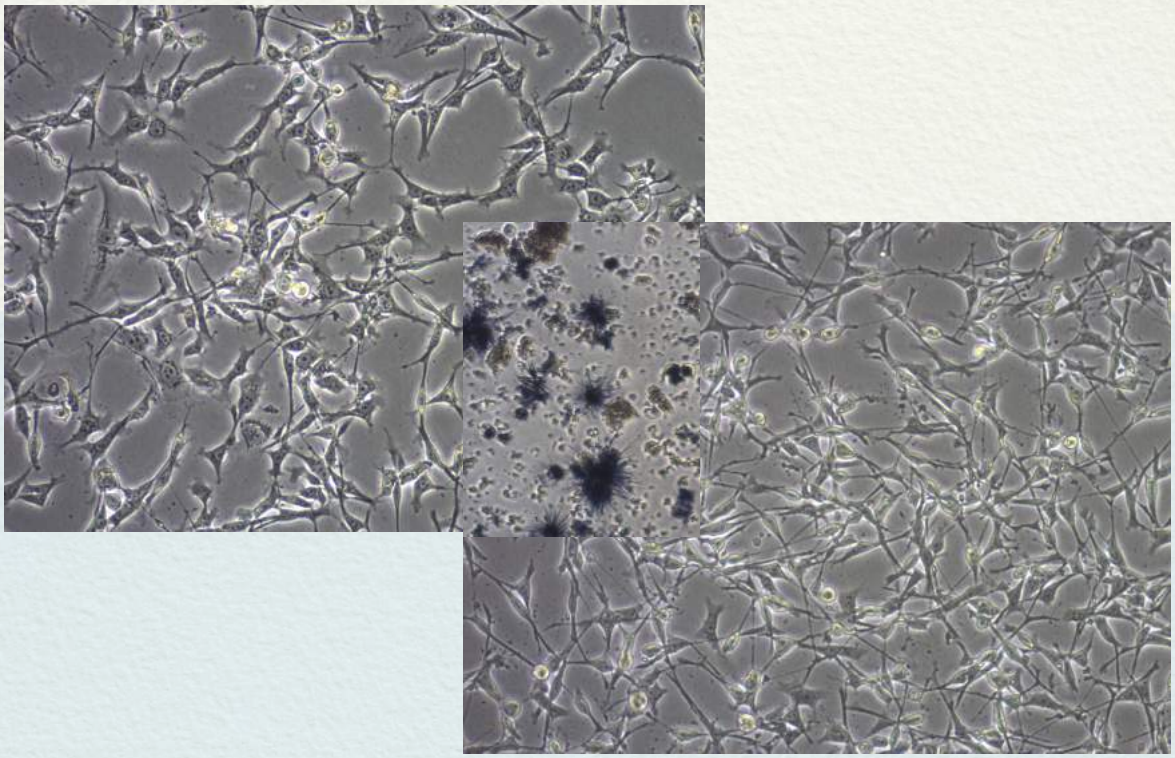
The Use of Oxidases as Triple Negative Breast Cancer Inhibitors



PSEUDÒNIM: **COLORINS**

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TABLE OF CONTENTS

1 INTRODUCTION

1.1 Motives

In modern days, fourteen million people are diagnosed with cancer every year while more than eight million other lose their life to this illness. Cancer is a common subject that surrounds everyone, and as no exception, I have been influenced by this problem as well. My grandparents, my friends' parents, relatives of mine, and even unknown people that have suffered from this disease have caught my attention and have left in me a thrill to investigate about it. Thus, from the beginning I decided to focus this research project on cancer cures.

Lately, research in this field has been focused on new biological therapies that are alternative and/or complementary to the traditional therapies. For my research, I wanted to find out and test a new method for cancer inhibition. The method I came up with was based on oxidases, which had never been explicitly associated with anti-cancer behavior, but have cytotoxic properties. The type of cancer I used for the research was triple negative breast cancer (TNBC) because it is, out of the types that were available for me to experiment with, the one I found the most interesting.

1.2 Purposes and Hypothesis

The purpose of this research project was to investigate about oxidases from two aims, a written-informative one and a practical one.

On the one hand, the first aspect surrounded the bibliographic research of the key subjects of the investigation: triple negative breast cancer and oxidases. The purpose of this section was the documentation and deepening of the following aspects:

- what breast cancer is, which types of breast cancer exist, what characteristics triple negative breast cancer has, how do its cells work and interact, and which possible treatments are used to inhibit it.
- what oxidases are and their characteristics, where they can be found, what are the investigations that have been done with them, what are their applications, and what oxidase is compatible for this project, its characteristics and its applications.

On the other hand, the second part approached the research to try whether oxidases could be applied as a TNBC treatment. My hypothesis was positioned on the idea that oxidases can kill carcinoma cells by oxidizing their components and that the mortality of the cells is higher when the oxidase is applied at higher concentrations.

1.3 Methodology

So as to attain the first aim of the research and carry out the bibliographic research, I collected information on TNBC and oxidases from different resources such as the Internet, books, articles, and professional investigators.

In order to execute the second objective and do the practical investigation, I performed a series of experiments consisting in several tests in which I used increasing concentrations of an oxidase to inhibit cultivated carcinoma cells. To corroborate the results of the experiment, I compared the mortality rates obtained using the oxidase with the results obtained using a valid biological therapy agent, a green-tea polyphenol called (−)−epigallocatechin−3−gallate (EGCG). I executed all these experiments with the oncology unit of the research group *TargetsLab* in the laboratories of El Parc Tecnològic in the University of Girona during my stay in el El Campus Prebat and El Jove Campus de Recerca that the university organizes. There, I learned the appropriate laboratory techniques and had the necessary apparatus. This part allowed me to answer the hypothesis formulated in the beginning.

I started my research on July 2014 and ended it on September 2015.

2 WRITTEN RESEARCH

2.1 Breast Cancer

Breast cancer is a disease of uncontrolled growth generated through a process named carcinogenesis. Carcinogenesis is a multistep mechanism resulting from the accumulation of errors in vital regulatory pathways, something started by the alteration of oncogenes and suppressor genes, which act as accelerators and brakes in cellular growth [1, 2]. Women have the probability of acquiring breast cancer of 1 in 8. In 2009, it was displayed that in the province of Girona, 383 new cases are reported each year, and they represent a 27,5% of all oncologic diagnoses [3].

The classification of breast cancer can vary, but the best approach when studying ways to tackle this disease is the one based on molecular profiling. This profiling concerns three specific receptors in the carcinoma cells: the estrogen receptor (ER), the progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). An estrogen receptor is a group of proteins activated by the hormone estrogen that is able to bind to DNA and regulate the activity of various genes encoded on the sixth and fourteenth chromosome. The activation of these receptors is associated to cancer because it stimulates the proliferation of mammary cells and produces genotoxic waste (agents able to damage genetic information). Meanwhile, a progesterone receptor is also a protein, which is induced by the steroid hormone progesterone, which transcripts genes in chromosome eleven [4]. The role of these receptors in

breast cancer development remains controversial but it is generally attributed to their function as sensors of multiple growth factor inputs in intracellular signaling pathways [9]. Alongside, a HER2 is a protein as well, member of the HER family, which are receptors able to combine and activate downstream signaling pathways. Thus, an overexpression of these receptors is responsible for an excessive cell proliferation, which can ultimately lead to tumor growth [10]. The testing of receptor status in carcinoma cells is a main prognostic factor and a guide to the treatment required. This testing separates breast cancer in three main types: hormone-receptor positive (Luminal A or Luminal B), HER2 positive, and triple-negative. Table 2.1 describes them.

SUBTYPE	RECEPTORS	PREVALENCE	CHARACTERISTICS	TREATMENT
Luminal A	ER and/or PR	40%	High survival, low recurrence	Hormone therapy
Luminal B	ER and/or PR and HER2	20%	Fairly high survival	Hormone therapy
HER2 +	HER2	10-15%	Young age; early recurrences	Trastuzumab (Herceptin)
Triple-Negative	None	15-20%	Young age, black women; aggressive, poor prognosis	Surgery, radiotherapy, chemotherapy

Table 2.1. Types of breast cancer.
Created by the author.

Carcinogenesis begins with a single change in the cell's genetics, which causes the entire regulatory system to modify, and therefore, the cell itself. An altered cell usually acquires additional changes that it uses to invade the surrounding tissues, creating metastases. Metastases are the troublesome part of cancer, given that they are incredibly difficult to contain and treat. As Figure 2.1 represents, their development begins when the primary tumor cells proliferate and by angiogenesis they surround capillaries, detach from the tumor tissue and invade blood vessels. Then, it keeps flourishing as the cells circulate trough the vessels, sometimes adhering to their walls, and get to other organs where they establish new invasions.

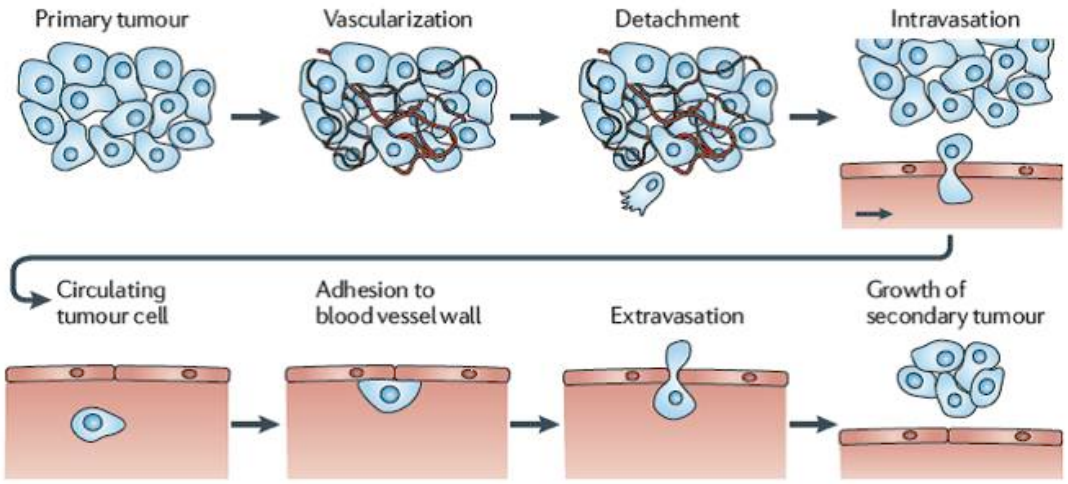


Figure 2.1. The development of metastases.

Source: http://wirtzlab.johnshopkins.edu/research/cancer-metastasis/

In modern days, there are various treatments for breast cancer, but the main ones are chemotherapy, radiotherapy, and biological therapies. Chemotherapy is a common treatment that uses anti-cancer drugs with cytotoxic (toxic to cells) agents that kill cells that divide rapidly, including those in hair follicles, the bone marrow, and the digestive tract. The treatment is administered intravenously, and it is believed that the drugs induce a programmed form of cell death known

as apoptosis, which damages the cells' DNA while preventing mitosis. Some of the chemotherapy drugs used in breast cancer are doxorubicin, cyclophosphamide, methotrexate, and 5-fluorouracil. Even though chemotherapy works, it has adverse effects such as immunosuppression, myelosuppression (destruction of stem cells), gastrointestinal distress, anemia, fatigue, nausea and vomiting, alopecia, and organ damage due to its toxicities [4]. On another side, radiotherapy is a therapy that uses ionizing radiation to damage the DNA of cancerous tissue, provoking cellular death. It can be conducted externally (from the outside) using X-rays from linear accelerator machines, or internally (within the body) by drinking a liquid with radioactive material. Although this treatment is painless, it has many side effects as well, like nausea and vomiting, epithelial damage, swelling, intestinal discomfort, and heart conditions, hair loss, dryness, lymphedema, cancer, and fibrosis [11]. On juxtaposition, biological therapies (or target therapies), are therapies that involve the use of living organisms or substances derived from them to stop cancer cells from dividing/growing, to kill them, and/or to encourage the immune system to attack them. These sorts of treatments often use biological response modifiers (BRMs), substances that the body produces in response to infections and diseases. There are seven types of biological therapies drugs (further explained in Annex 9): *monoclonal antibodies*, *cancer vaccines*, *blood cell growth factors*, *angiogenics*, *immunotherapy*, *gene therapy*, and *cancer growth blockers*. The side effects of these therapies depend on the substance used and the dose taken. They range from nausea, fever, vomiting, muscle pains, fatigue, and swelling to worse effects [11,12], but are considered less adverse than the ones of the other therapies.

2.2 Triple-Negative Breast Cancer

Triple-negative breast cancer (TNBC) is the type of breast cancer that lacks of ER, PR, and HER2. TNBC is known as the most aggressive and difficult to treat given that it has no receptors to block and has transport proteins in the membrane that permit the expulsion of anti-cancer drugs [6]. It constitutes a 15-20% of breast cancer cases, and patients that suffer from it have a higher chance of suffering metastases and relapse as well as a shorter metastasis-free and overall survival [3]. It is thought that the high ratio of recurrence is due to the selection of cancer stem cells (CSC) that chemotherapeutic agents do not target completely. CSC are the cells that initiate tumors and which are able to trigger the development of relapses, as Figure 2.2 shows. New target therapies such as green-tea polyphenols have been used so as to inhibit CSC.

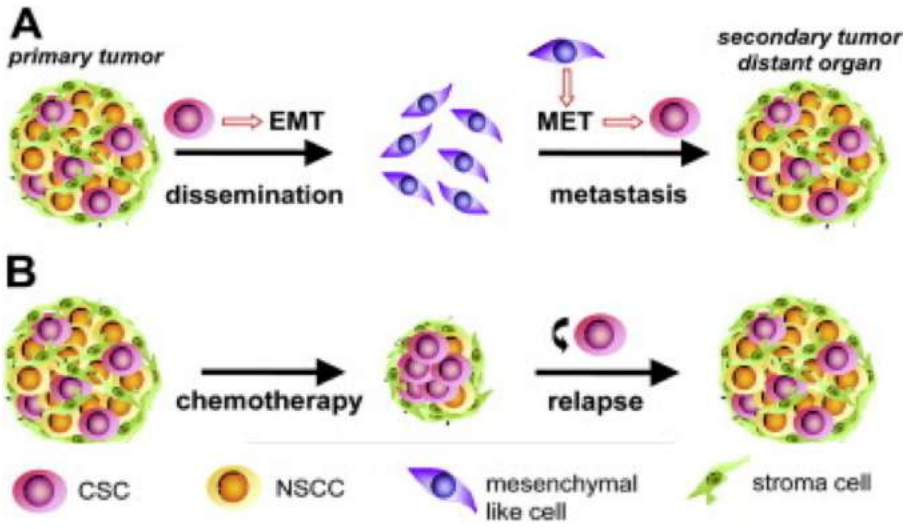


Figure 2.2. The actions of Cancer Stem Cells.
Modified from http://www.sciencedirect.com/science/article/pii/S1574789112001044

2.3 Oxidases

Oxidases are enzymes that catalyze oxidation-reduction reactions, especially those containing oxygen. An oxidation-reduction (redox) reaction is a chemical reaction in which protons and/or electrons are transferred from an atom to another. These reactions are of highly importance in biochemical processes given they can change the structure of molecules.

Oxidases are utilized in different fields. For instance, in microbiology, they are used in the “Oxidase Test”, and in the neurologic system, Monoamine oxidases are involved in the regulation of cognitive processes, while Semicarbazide-Glucose oxidase, which displays antibacterial activity, is used to treat colds.

Some examples of oxidases are Cytochrome P450 oxidase (found in hemoproteins), NADPH oxidase (typical in white blood cells and the plasma membrane), Xanthine oxidase (from uric acid), L-gulonolactone oxidase (found in Vitamin C), Lysyl oxidase (typical in the LOX gene), and Laccase (the one studied in this research project).

Oxidases are able to catalyze the oxidation of molecules such as proteins, carbohydrates, and lipids, which build and fuel cells. At the same time, they drive redox processes such as the one depicted in Figure 2.3. This is an important aspect, given that it gives these enzymes the power to regulate the cell’s metabolism. The image figures the mitochondrial respiratory chain and the oxidative phosphorylation: two parts of the cell’s catabolism that have the responsibility of obtaining ATP (energy to fuel the organism). As the arrows in the image represent, many oxidations happen in this chain, such as lipid peroxidation and protein oxidation, and they are catalyzed by oxidases. These

reactions liberate free radicals, which are molecules that have lost an electron through oxidation and are unstable because of the unpaired electron left. Free radicals are threatening to cells because they start attacking normal molecules so as to gain an electron. The liberation of oxygen free radicals in processes like the one in Figure 2.3 causes mitochondria to be susceptible of receiving oxidative damage, referenced in the picture as “Daño Oxidativo”, which can further damage the mitochondrial DNA [15]. Oxidases are powerful in the sense that they can trigger the liberation of free radicals. If they are unbalanced or found in excess they can be very threatening to cells: their action can result in DNA damage, so the cell itself can be transformed and, most likely, killed.

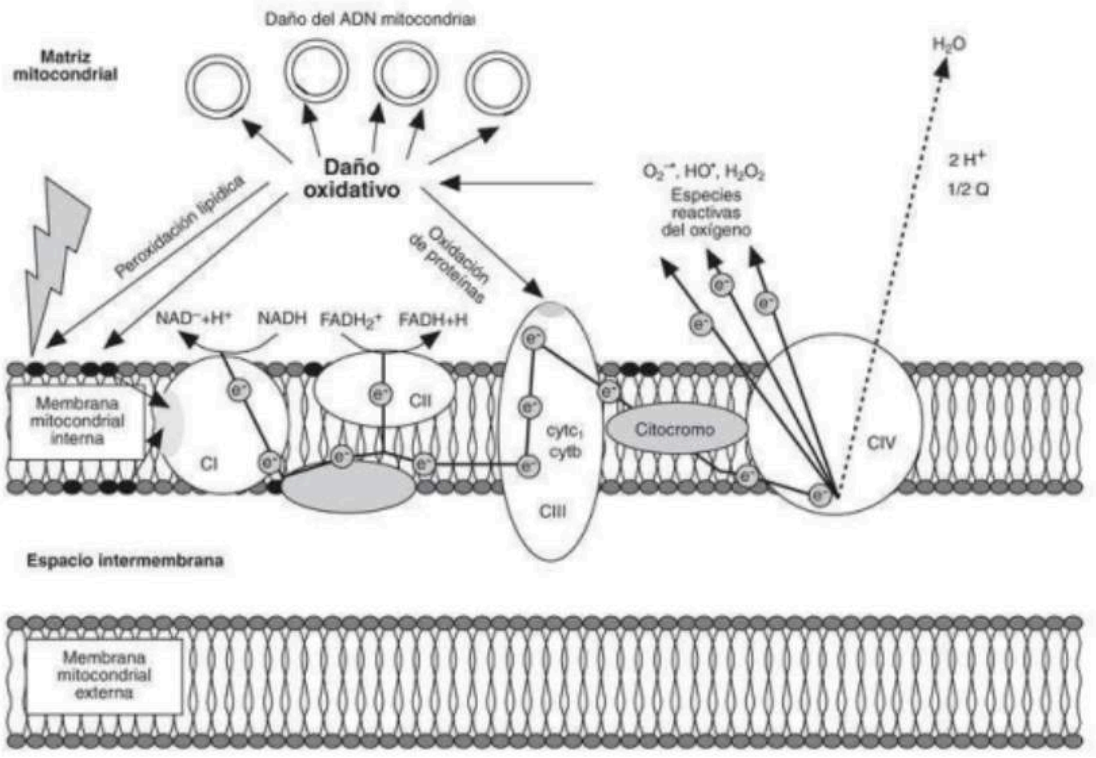


Figure 2.3. Mitochondrial respiratory chain and oxidative phosphorylation.

Source: www.elsevier.es/sites/default/files/elsevier/images/15/15v16n01/grande/15v16n01-13057127fig04.jpg

Several studies have found in oxidases properties for treating diseases. Nishioka et al. (2012) show that a higher expression of Lysyl oxidase is associated with a poor cancer prognosis. Cheuk et al. (2014) argue that Urate oxidase is effective in the prevention and treatment of tumor lysis syndrome in children with malignancies. Mukherjee et al. (2015) expose how apoptosis was inducted in human breast cancer cells by L-amino acid oxidase. Given this evidence, I decided to experiment with Laccase oxidase, which has not been explored much in this field, and find out if it can oxidize and kill malignant cells.

2.4 Laccase

Laccase are multi-copper oxidases that are found mainly in plants, fungi, and microorganisms. They fall in the description of polyphenol oxidases, which are copper proteins able to oxidize aromatic compounds [8]. The characteristics of Laccase depend on their provenance. Mainly, it is the pH, the temperature, and the presence of Laccase inhibitors that influence the activity of this oxidase.

Apart from its natural functions, which include the catalysis of redox processes and the enzymatic work, Laccase has many artificial applications. In the paper industry, it is used to treat wood so the lignin fibers are separated more easily; in waste detoxification and decontamination, Laccase degrades pollutants found in industrial waste and contaminated soil or water; in the textile industry, it is used for decolorizing dyes; in the food industry, it stabilizes wine and beer and processes fruit juice; and in medicine and personal care, Laccase is used to reduce the effect of poison ivy dermatitis, to oxidize iodine for disinfecting, and to manufacture anticancer drugs and cosmetics [8].

Laccase's copper atoms are classified in three copper sites that have different characteristics but, altogether, form a trinuclear center in which dioxygen's reduction to water occurs [8]. The oxidation of a reducing substrate by Laccase typically involves the loss of a single electron and the formation of a free radical. Therefore, Laccase is a great candidate to damage carcinoma cells. The human cells' cytoplasm contains many aromatic molecules like proteins and enzymes that can be oxided by Laccase. Plus, this oxidase may interfere with metabolic processes leaded by other oxidases. Even though Laccase has been used for many things, it has not been explicitly applied in oncology yet. Laccase would be a great option for treating cancer given that it is easy to obtain, abundant, and it has been proven able to catalyze the most resistant molecules. However, it is essential to keep in mind that it would have to be administered with specificity only to the targeted cells.

The Laccase used in the experimental part came from *Agaricus Bisporus* (mushroom). It had a molar mass of 65.000 g/mol. Some works have implicitly related this substance to anti-cancer activity. For instance, a study by Maseko et al. (2014) showed that Selenised *A. Bisporus* increases the expression of gluthathione peroxidase, an enzyme that protects the organism from oxidative damage of normal cells. Also, Elbatrawy et al. (2015) expressed that mushroom extracts possess differential antioxidant activity (which is great for normal cells) and cytotoxicity to cancer cells.

3 PRACTICAL RESEARCH

3.1 Introduction

In order to complete the practical part of the research and find out if oxidases can be a valid treatment for triple negative breast cancer, I carried out several experiments with the research group *TargetsLab* from the University of Girona. These experiments consisted in testing increasing concentrations of the Laccase oxidase as a drug to kill carcinoma cells. The aim of the experiments framed the resolution of the hypothesis formulated in the beginning of the research and the identification of Laccase concentrations able to treat TNBC.

Parts of the execution of the experiments were recorded and can be seen in the following video:

3.2 Experimental Set Up

The below materials, chemicals and cells were used during the practical research. The sterility in the procedures was of highly importance, and therefore, the majority of the steps had to be executed inside a laminar flow cabinet of class 2 biosecurity while all the objects had to be rinsed with ethanol before being used.

CELLS

The cells used came from TNBC human mammary gland tumors from the ATCC cell donation bank. The cells had been cultivated for years at 37ºC with

5% of CO_2 in an incubator. MDA-MB-231_Wild Type (WT) and MDA-MB-231_Doxorubicin Resistant (DxR) were the two cell categories involved. These two categories belong in the cellular line MDA-MB-231, which is characterized for an extraordinarily fast growth. The cells from this line belong to an epithelial cell type, are adherent when cultured, and suffer from adenocarcinoma. The term WT explains that the phenotype of the cells is the typical one (the one in which MDA-MB-231 cells appear in nature). The letters DxR are added to cells that have become resistant to Doxorubicin (chemotherapy drug).

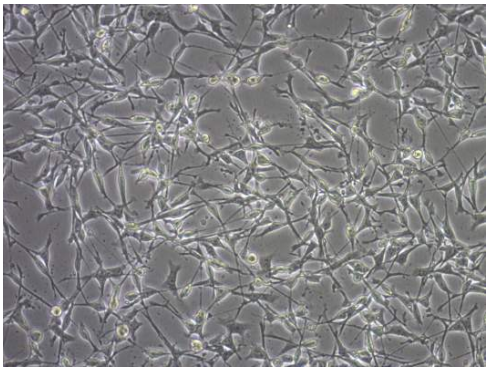


Figure 3.1. MDA-MB-231_WT cells
Created by the author

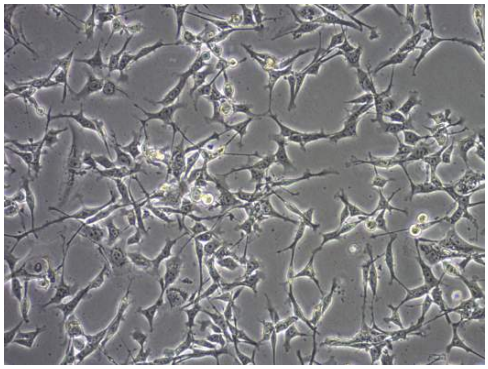


Figure 3.1. MDA-MB-231_DxR cells
Created by the author

DRUG

The oxidase Laccase used in the experiment was bought from Sigma-Aldrich© and it had been extracted from *Agaricus bisporus* (mushroom). It was a deep brown powder that was dissolved with PBS to a concentration of 100 mg/ml. It was stored under a temperature of -20ºC.

MEDIUM

The medium used was basically DMEM *(Dulbecco's modified Eagle's medium)*, which is a basal medium for supporting the growth of cells, with 1% of L-

glutamine (essential amino acid), 1% of sodium pyruvate (tampon), 10% of FBS (serum with growth factors that stops Trypsin's actions), and 100 µg /mL of penicillin and 100 µg/mL of streptomycin (antibiotics to avoid contamination).

CHEMICALS

• PBS (phosphate-buffered saline): a buffer solution containing sodium phosphate and sodium chloride, used for diluting and cell container rinsing.

• DMSO (dimethyl sulfoxide): a polar aprotic solvent able to dissolve both polar and nonpolar compounds, sometimes used as a mild oxidant.

• MTT: a colorimetric reagent-based, used to assess cell viability as a function of redox potential.

• Trypan Blue: a vital stain that selectively colors dead tissues or cells blue.

• Trypsin: a serine protease (enzyme that splits peptide bonds in proteins) found in the digestive system used to detach and suspend cells in a solution.

APPARATUS

• Neubauer Chamber: a small object used for cell counting.

• Microplate reader machine: reads the viability of the cells through their MTT absorbance. Its model was Benchmark Plus by the brand Bio-Rad. The wavelength of the waves it emitted was 570 nm.

• Centrifuge machine: its model was Universal 320 R by the brand Hettich.

• Microscope: its model was Eclipse T5100 by the brand Nikon. The magnification was of 1000.

• Incubator: its model was Nu-8500 by the brand Nuaire.

3.3 Methodology

All the experiments carried out during the practical research of this project were done along the margins of the following procedure, which features three parts.

SOWING OF THE CELLS

This first section of the procedure consists in the sowing of TNBC MDA-MB-231_WT and MDA-MB-231_DxR cells onto microwell plates. The experiment is begun with the extraction of the carcinoma cells from the Petri dishes in which they have been cultivated. To start, the medium inside the Petri dish is sucked with a glass Pasteur that is pressed to the edge of the wall of the plate to avoid the crushing of the cells. Then, 3 mL of PBS are poured into the dish and dissolved using the “eight” technique so as to completely wash the medium. 3mL of Trypsin are added thereafter with the purpose of detaching the cells from the plate. The Petri dish is put into the incubator for 3 minutes so that the substance does its work. In the meantime, a 15 mL centrifuge tube is prepared inside the cabin. Once the 3 minutes have passed, the dish is taken out of the incubator and observed with a microscope so as to rule if the cells have been dissembled from the dish. At that point, twice the Trypsin’s volume of medium is added to inhibit the Trypsin, using the “flushing” technique. The substances are then sucked with a micropipette and poured into the centrifuge tube. Next, the centrifuge tube is centrifuged at 20 ºC and 1500 rpm for 5 minutes. In the meantime, a microwell plate is prepared as well as a microcentrifuge tube with 40 µL of Trypan blue. Once the centrifuge tube is centrifuged, the medium (liquid on the top) is sucked and eliminated so the cells are separated lying on the bottom. 10 µL of this cell substance left in the tube are then poured with a

micropipette and mixed into the microcentrifuge tube. Thereupon, 10 µL of the new mixture (cells and Trypan blue) are put onto a Neubauer Chamber so as to count the amount of cells present. With the microscope, the number of cells per quadrant is determined, which is then used to calculate the volume of medium and cell solution (centrifuge tube substance) needed. Following, the substances in the corresponding volumes are mixed in a tray, and 100 µL are taken with a multichannel pipette. From the left to the right of the microwell plate, the substance is poured into the wells so that each well is left with 100 µL. The wells in the external frame are filled up with 100 µL of PBS. Once finished, the plate is put inside the incubator and the working area is cleaned up.

TREATMENT OF THE CELLS

This second part of the procedure consists in the application of the Laccase drug into the wells. It has to be carried out the day after part one is completed. In the first place, various calculations have to be done in order to know the amount of volume needed from each substance. So that the concentrations are the desired ones, dilutions have to be prepared.

In the lab, the concentrations are prepared in different microcentrifuge tubes. Some medium is put into a plastic tray and the corresponding volume of this medium (depending on the calculations) is poured into the microcentrifuge tubes. Then, the dilutions are made by the addition of PBS to the regular Laccase. Once everything is ready, the oxidase is put inside the initial microcentrifuge tubes that contain the medium, changing the tip of the micropipette every time the substance is poured. When the microcentrifuge tubes are all finished, the microwell plate that was cultivated in part one is taken from the incubator and its medium is sucked. 50 µL of new medium are then put

into each well. At that point, the substances in the microcentrifuge tubes have to be applied to the different wells. One by one, each microcentrifuge tube is used for a column or half a column, depending on the experiment. Once the process is completed, the plate is put into the incubator for two days and the working area is cleaned up.

LECTURE OF ABSORBANCE

This last phase of the procedure is about the lecture and analysis of the treatment's results. To initiate it, the liquid from the wells in the mircowell plate is sucked. Then, 50 µL of new medium are put into each well along with 50 µL of MTT. The plate is then stored inside the incubator for two hours. After the time passes, crystals appear in the wells depending on the mortality of the carcinoma cells. So, the liquid remaining is sucked and the plate is brought to a microplate reader machine. The working area is cleaned up. Data is obtained from the machine, which is then projected on a computer and analyzed through statistics.

3.4 Results and Discussion

A. Observations

When I started working with Laccase, there were no guidelines about this oxidase so I decided to work with a large range of concentrations, which varied from 10 to 1000 ug/mL. While administering the drug to the cells, the substance changed its color, a sign that it is easily oxidized by air or irradiated by light. When observing the mircowell plate after 48h of incubation, I saw that large crystals had appeared because of the precipitation of Laccase. This captured

the fact that the oxidase was too concentrated, so I tired using it in low concentrations. The low concentrations had no effect on the cells and I had to go back to using high concentrations. In order to do avoid the formation of crystals, I diluted the Laccase with 5-6% of DMSO and 40% of DMSO. DMSO has a high toxicity when its final concentration in cells is more than 0,1%, so the maximum DMSO that I diluted with Laccase was a 40%. The experiments with DMSO improved the issue with crystals but still, there were some present. Thus, I did a series of qualitative experiments with Laccase dissolved in other substances to check whether the oxidase would precipitate less when mixed to other compounds. These experiments are explained in Annex 7, but since their results weren't favorable, I stuck with DMSO.

B. Laccase in high concentrations in medium

The first and second trials consisted in the treatment of the cells with Laccase concentrated from 10 to 1000 ug/mL in medium only. A large number of crystals appeared in the substance when the experiment was conducted. Wild Type cells had a 26% of average mortality and Doxo-resistant cells had that of 15%. The mortality of the cells increased as the concentration of the oxidase increased as well. These low cell viability rates, captured in Table 3.1, were mainly due to the presence of Laccase crystals, which makes this condition a non-viable treatment.

AVERAGE % CELL VIABILITY								
µg/ml		**10**	**25**	**50**	**100**	**250**	**500**	**1000**
WT	Trial 1	75,43	70,46	79,30	69,41	58,15	56,79	48,96
	Trial 2	87,27	86,92	97,41	94,35	103,29	96,62	93,91
DxR	Trial 1	115,95	14,48	46,40	47,28	55,37	15,55	11,48
	Trial 2	82,86	110,88	87,67	166,57	237,07	122,33	79,41

Table 3.1. Percentage of cell viability with Laccase in high concentrations in medium only

C. Laccase in low concentrations in medium

In the third trial, the oxidase was concentrated from 0,001 to 10 ug/mL in medium only. The average mortality of the cells in this case was very low and it even helped the cells grow. Its average was a -13% in WT cells and -9% in DxR cells. The lower the concentration of Laccase was, the higher the viability of the cells turned to be, as depicted in Table 3.2.

AVERAGE % CELL VIABILITY								
µg/ml		**0,001**	**0,01**	**0,1**	**1**	**2**	**5**	**10**
WT	Trial 3	39,55	143,07	137,33	119,95	130,05	122,52	105,97
DxR	Trial 3	125,10	128,23	103,80	115,18	121,92	110,87	85,89

Table 3.2. Percentage of cell viability with Laccase in low concentration with medium only

D. Laccase in high concentrations with 40% DMSO

In the fourth trial, Laccase was used concentrated from 10 to 1000 ug/mL dissolved with 40% DMSO and medium. The carcinoma cells had a 40% of average mortality in WT cells and a -110% in DxR cells. However, crystals were present. The difference of viability between the two cell types was exaggerated, which shows that probably the line with DxR cells had errors and the results where altered. Table 3.3. portrays this fact.

AVERAGE % CELL VIABILITY								
µg/ml		**10**	**25**	**50**	**100**	**250**	**500**	**1000**
WT	Trial 4	71,03	65,95	59,77	62,47	58,92	53,98	46,22
DxR	Trial 4	82,99	103,91	343,43	173,78	255,01	248,84	260,88

Table 3.3. Percentage of cell viability with Laccase in high concentrations with medium and 40% DMSO.

E. Laccase in high concentrations with 5% DMSO

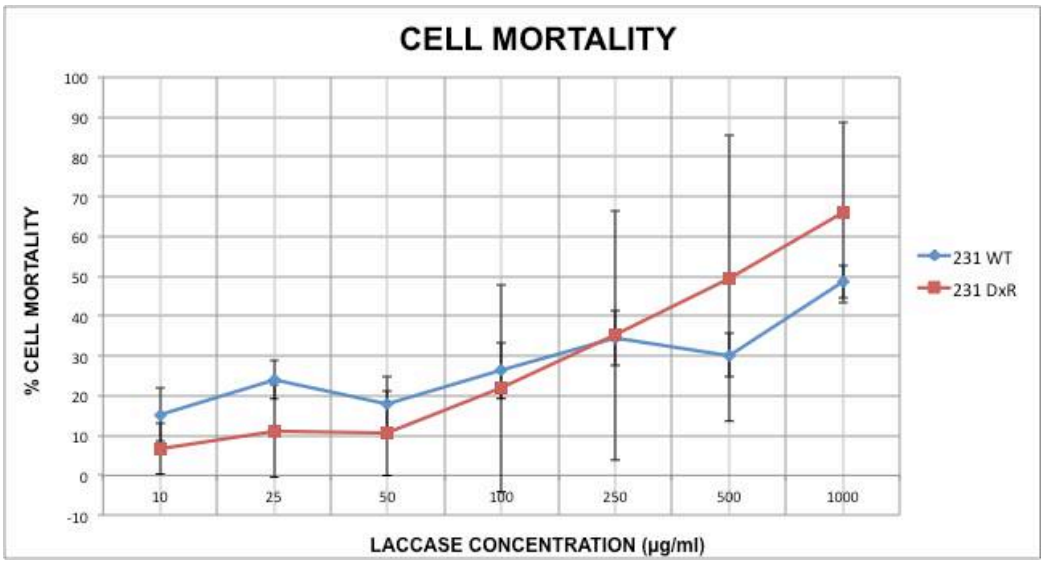
Trials number 5 and 6 were based on the treatment of the cells with Laccase concentrations from 10 to 1000 ug/mL dissolved with 5% DMSO and medium. The average mortality of the carcinoma cells in these cases was of 48% in Wild Type cells and 23% in Doxo-resistant cells. With the exception of the results in DxR cells from trial 5, the viability of the cells was smaller as the concentration of the oxidase was lower, showed in Table 3.4. Only a few crystals were present, thus the agent responsible for the high mortality was the drug, not the crystals.

AVERAGE % CELL VIABILITY								
µg/ml		**10**	**25**	**50**	**100**	**250**	**500**	**1000**
WT	Trial 5	80,89	80,69	78,19	56,56	12,29	11,27	11,42
	Trial 6	117,83	103,19	96,00	85,44	79,28	71,72	59,29
DxR	Trial 5	86,60	80,66	72,26	57,20	15,91	13,43	11,28
	Trial 6	22,85	59,97	108,61	129,84	137,64	155,76	135,40

Table 3.4. Percentage of cell viability with Laccase in high concentrations with medium and 5% DMSO.

F. Overall discussion

Graph 3.1 shows the combination of the data from all the trials. It describes the mortality of the carcinoma cells in various Laccase concentrations. At lower concentrations (from 10 to 100 µg/ml), the WT cells have a higher mortality compared to the DxR ones, but at higher concentrations (from 250 to 1.000 µg/ml) it is the opposite. From 50 to 1.000 µg/ml, the variables for the DxR values have a direct relationship given that the graph line is straight. Differently, the slope for WT cells stays more irregular. Overall, the higher Laccase concentrations resulted in a higher mortality of the carcinoma cells. Both cell lines reached, at least, a mortality of 50%.



Graph 3.1. Cell mortality based on Laccase concentrations.

G. Comparison between Laccase and EGCG

So as to corroborate as valid all the results, I compared the mortality rates obtained using the oxidase with the values obtained using a new biological therapy agent, a green-tea polyphenol called (−)−epigallocatechin−3−gallate (EGCG). The green-tea polyphenol therapy is considered a valid treatment, and I particularly choose it to validate Laccase because their mechanisms of action have some similarities in common and are both based on the oxidation of cell molecules. Green-tea polyphenols focus their anti-cancer action on their antioxidant activity, which is further explained in Annex 10.

The values that appear in Table 3.5 are Laccase's and EGCG's inhibitory concentrations, quantitative measures that indicate the concentration of a particular drug needed to inhibit a given biological process by the percentage indicated. If these values are compared, it can be noticed that Laccase has much lower IC's. This means that, related to EGCG, a lower amount of Laccase is needed to kill the same amount of cells, something that vaguely points out that Laccase is more effective. Despite this, as the graphs in Annex 8 delineate,

EGCG has much lower standard error, which is a very important aspect to consider when judging the previous statement. All in all, the mortality in both graphs rises to similar values, which are fine ones. This gives certainty of the validity of Laccase and corroborates that it can kill carcinoma cells.

	LACCASE		EGCG	
	IC_{30}	IC_{50}	IC_{30}	IC_{50}
MDA-MB-231	2,7 µM	15 µM	145 µM	150 µM
MDA-MB-231_DxR	1,5 µM	7,7 µM	180 µM	195 µM

Table 3.5. Laccase and EGCG's inhibitory concentrations.

4 CONCLUSIONS

The purpose of this study was to develop and test a new method of cell inhibition that involved the use of living organisms or substances derived from them. The new method this research project suggests takes advantage of oxidases, enzymes that are able to oxidize the cells' components and liberate free radicals while creating oxidative stress on DNA and essential molecules such ATP, which leads to cell damage.

The oxidase method was tested using an oxidase, Laccase, to directly treat TNBC cells cultivated in a laboratory. The results obtained proved Laccase capable of damaging carcinoma cells. They depicted that the oxidase in high concentrations, the highest being 1.000 μg/ml, achieved a mortality up to 50% in Wild Type cells and 65% in Doxorubicin Resistant Type cells. These results were compared to the ones obtained using a valid treatment, the green-tea polyphenol (EGCG) therapy, to check if the new procedure would be competent. The inhibitory concentrations from the oxidase treatment were much lower than the polyphenol's ones. Laccase's IC_{50} was 15 μM in WT cells and 7,7μM in DxR cells, while EGCG's was 195μM in WT cells and 150μM in DxR cells, which is a favorable characteristic for oxidases. Nevertheless, the first method is not as reliable as the second.

Laccase formed crystals when applied to the cells. Out of the different combinations of concentrations and solvents used, the dilution that worked best, in which the oxidase presented the least crystals, was when it was diluted in

medium with 5% of DMSO. The mortality of the cells in this condition was moderated but the agent responsible for the high mortality was the drug, not the crystals.

Concluding, the best way to use Laccase as a treatment would be at high concentrations (100-1.000 μg/ml) diluted with medium with 5% of DMSO.

Despite the fact that the results show that the oxidase cell inhibitor method can work, they must be considered preliminary for three main reasons. The first one is that only six replicas were done, given that there was not enough time to carry out a longer, deeper study. The second one is that the results gathered have a large standard error, which should be much smaller if these results were to be considered secure. The third one is that the qualitative results (observations) explain that Laccase formed crystals in most of the concentrations, which is a characteristic that a substance for cancer treatment cannot have.

In conclusion, the inhibition of TNBC cells using oxidases is possible and opens a new path in cancer research. Oxidases are presented as non-toxic, abundant and common agents with many advantages. Therefore, a great future option to further exploit these substance's anti-cancer activity and to perfection the method is to create a synergism between oxidases and green-tea polyphenols, given that the second ones are substrates to the first ones' reactions.

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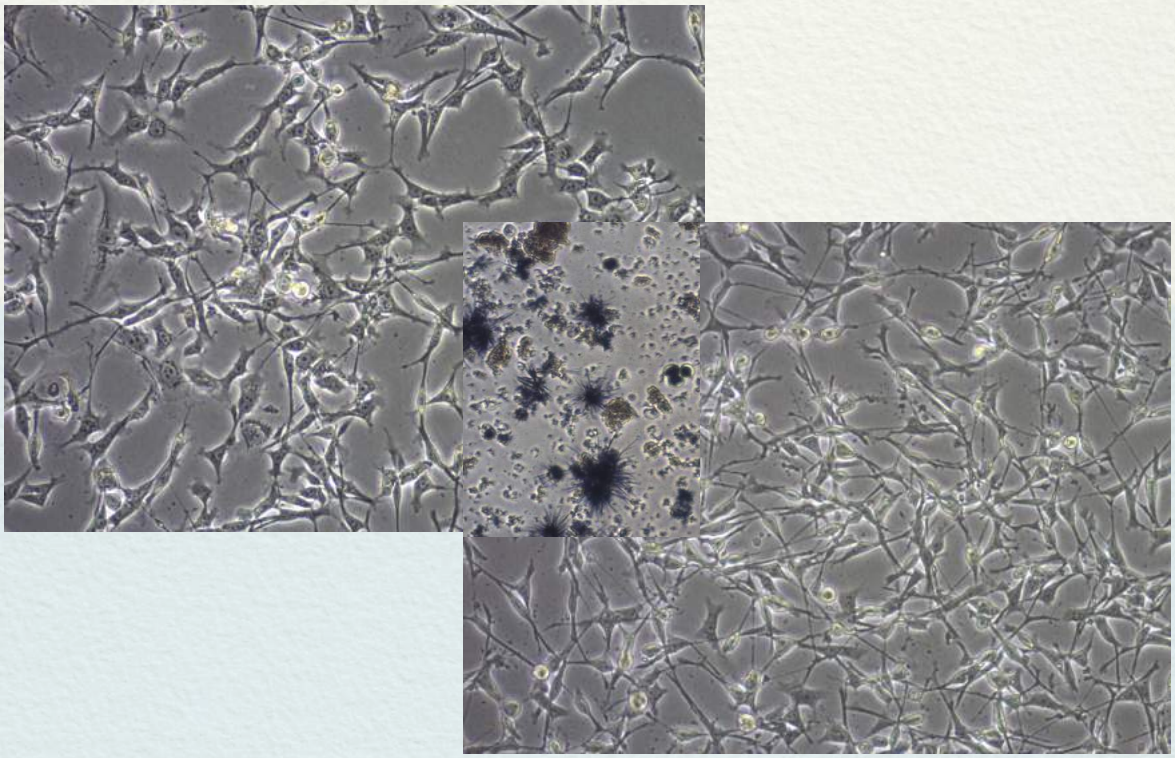
The Use of Oxidases as Triple Negative Breast Cancer Inhibitors



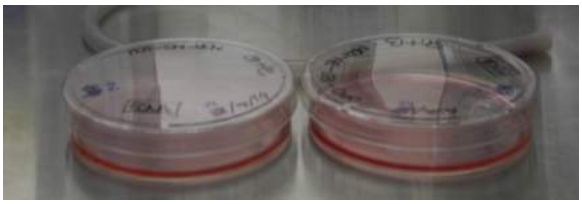
ANNEX

TABLE OF CONTENTS

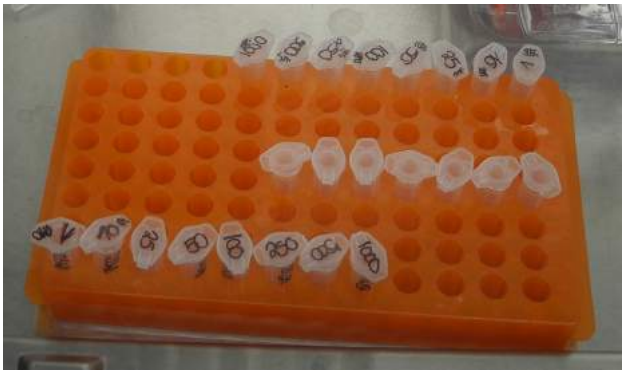
ANNEX 1.1. Images of the Experimental Set Up

Common laboratory objects used.

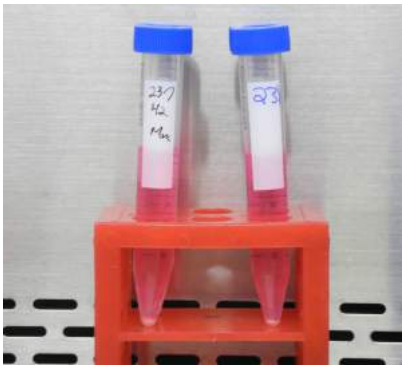
Microwell plates



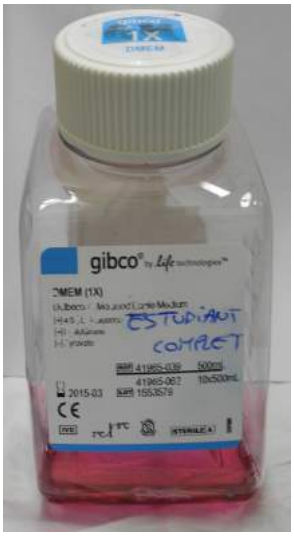
Petri capsules



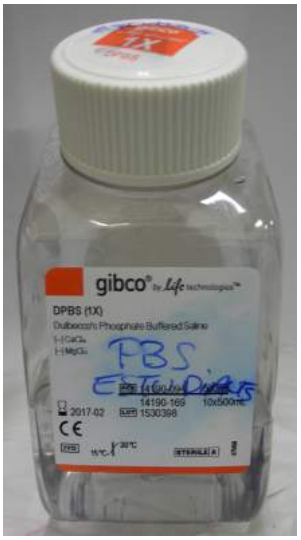
Microcentrifuge tubes containing different concentrations of Laccase



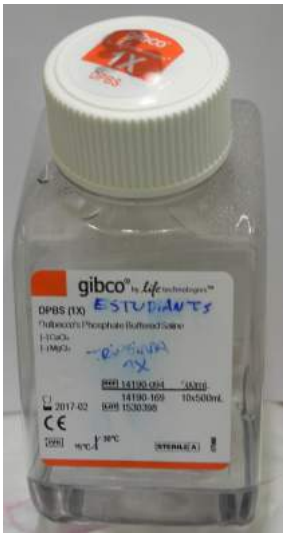
Centrifuge tubes with cell solutions



DMEM (medium)

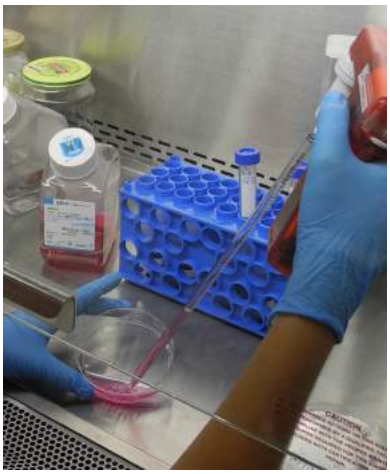


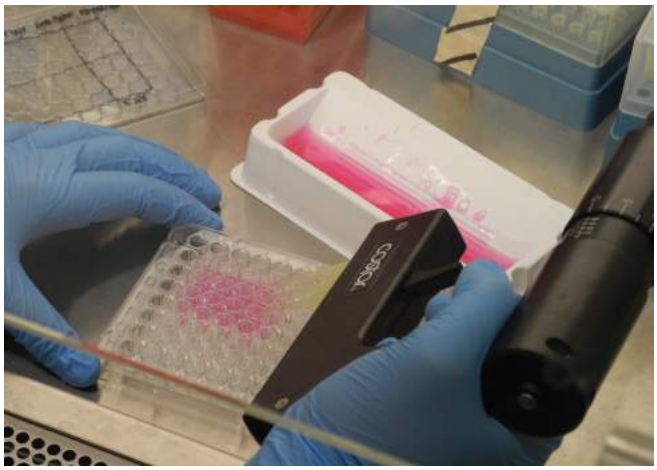
PBS



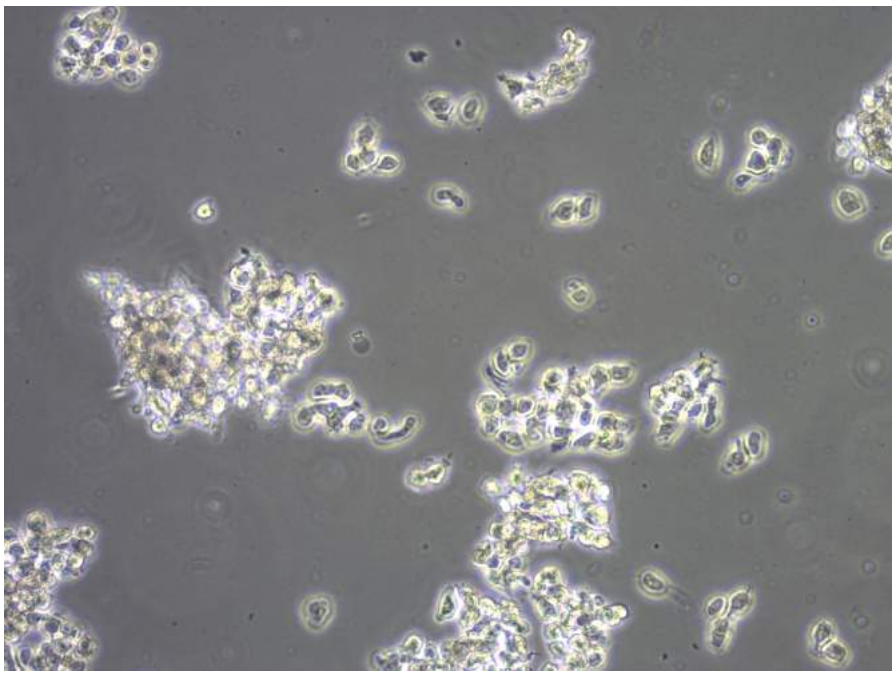
Trypsin

ANNEX 1.2. Images of the Experimental Procedure

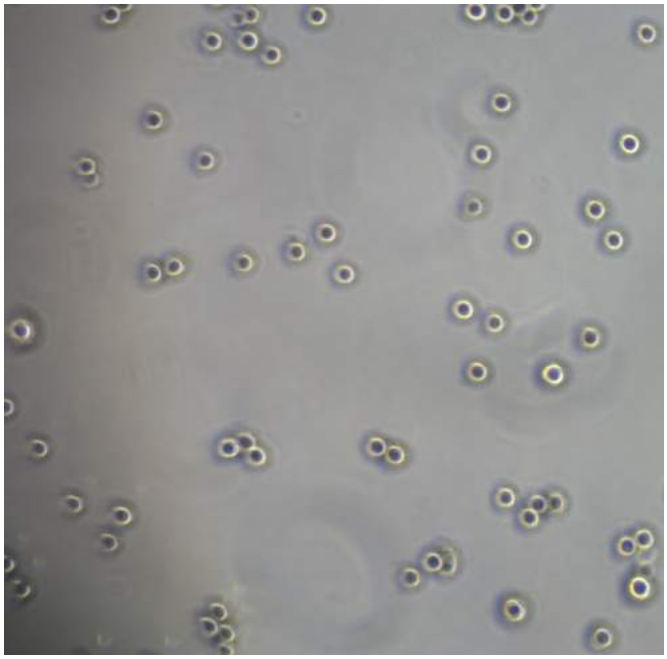

The Flushing Technique


Sowing of the cell solution to the plates

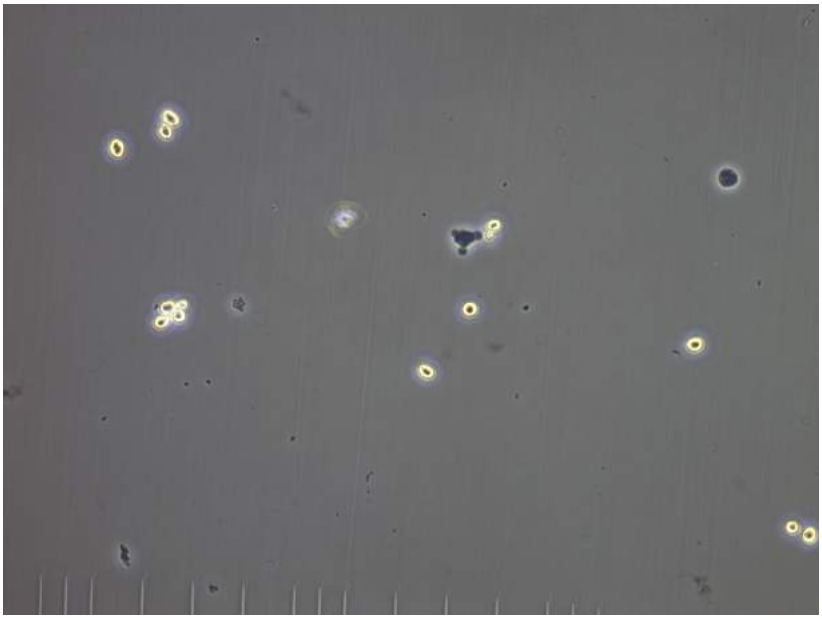
ANNEX 2. Images of the Cells in Different Stages of the Procedure



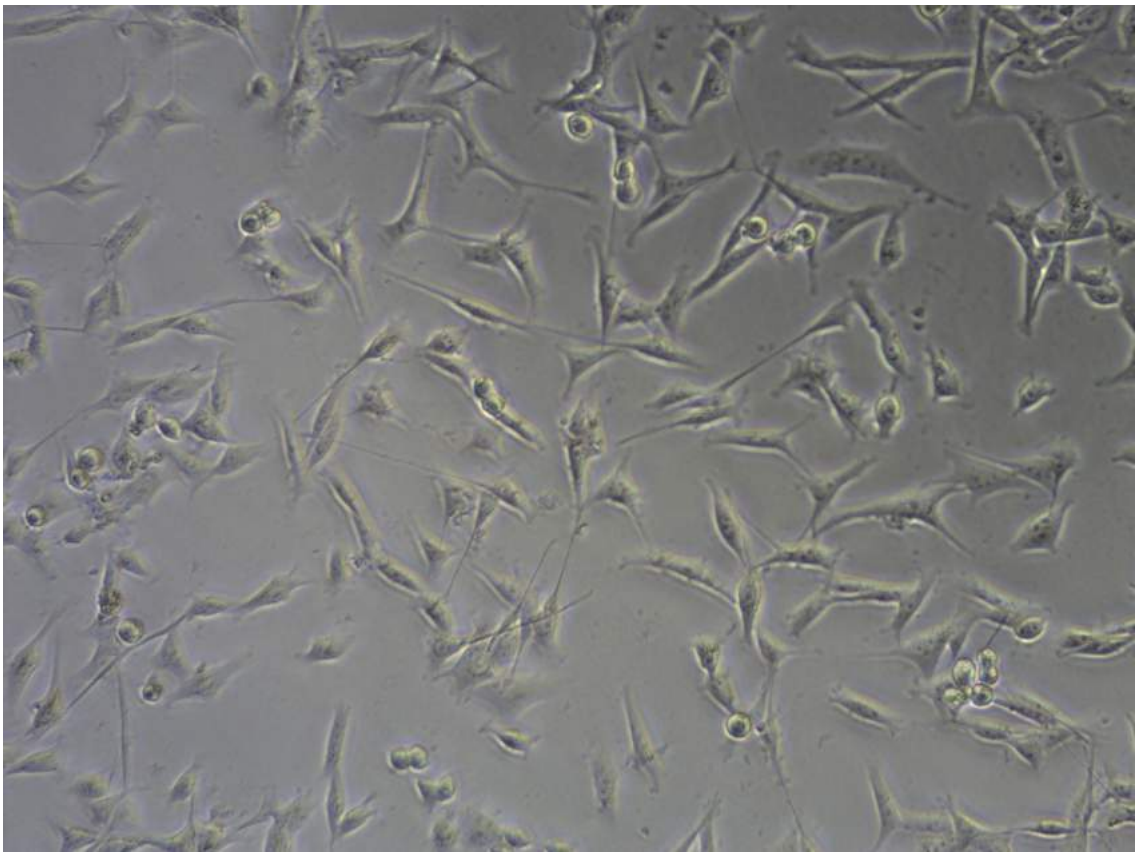
MDA-MB-231_WT cells suspended after treated with Trypsin



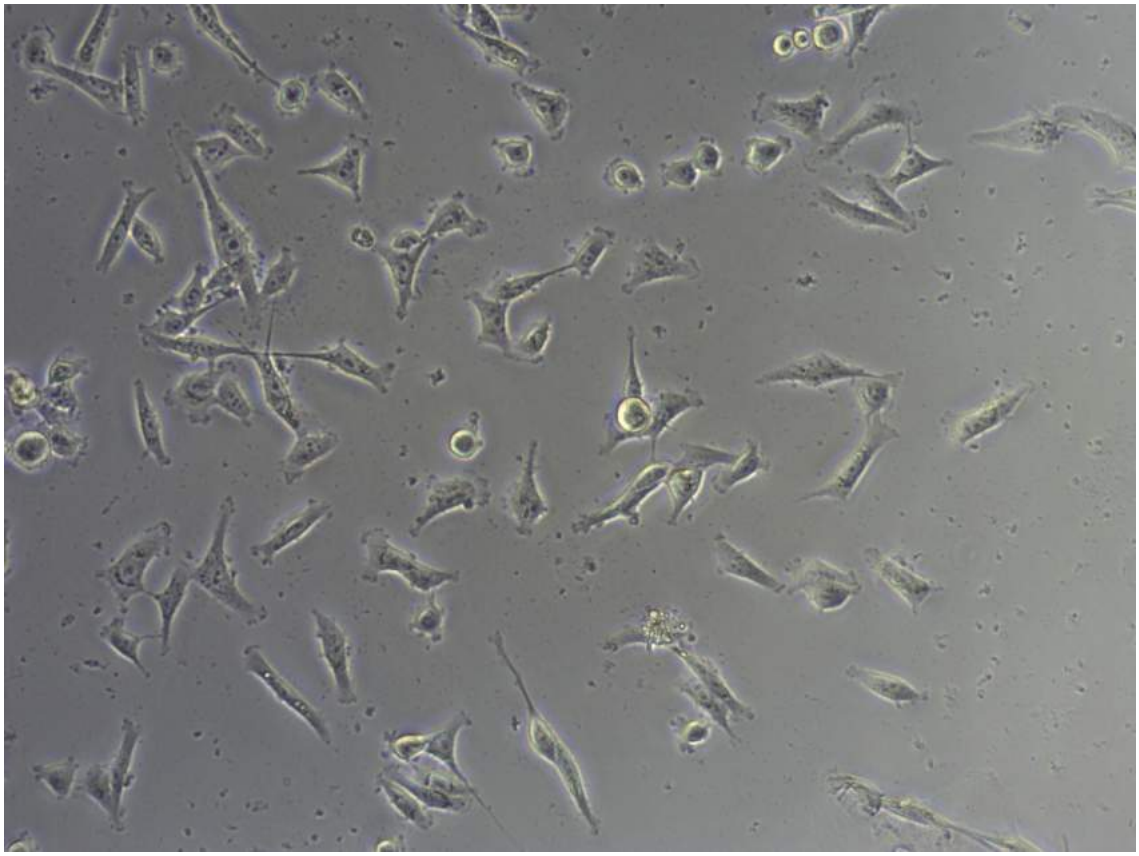
Cells after being sowed to the plate



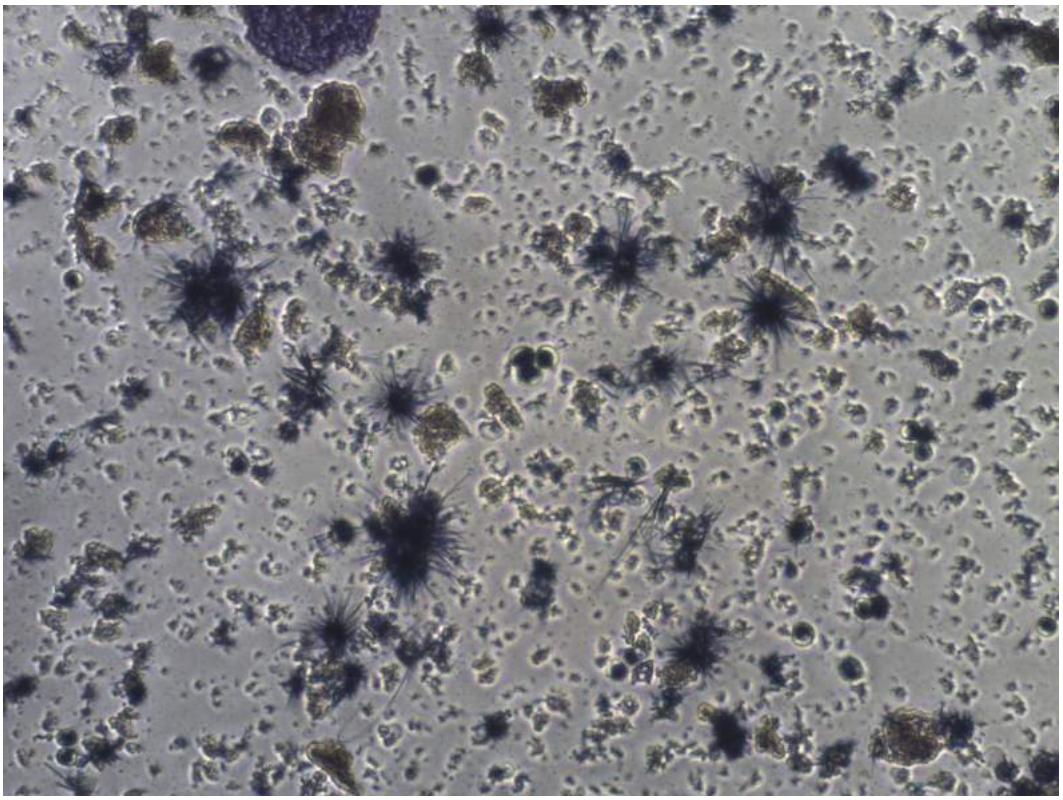
Alive (white) and dead (blue) cells in the Neubauer chamber after being treated with Trypan Blue



MDA-MB-231_WT cells before being treated with the drug



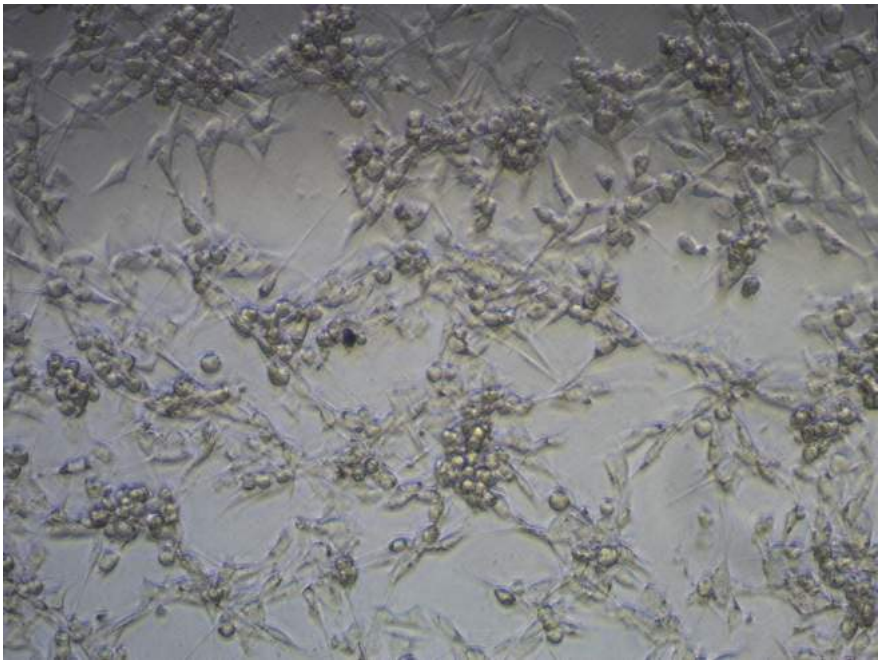
MDA-MB-231_DxR cells before being treated with the drug



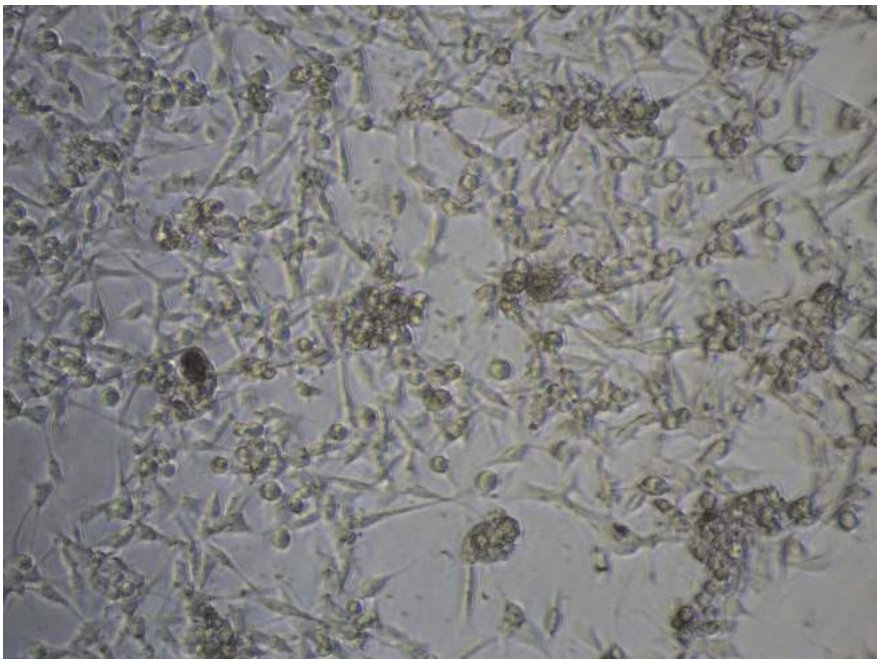
Crystals formed by MTT

ANNEX 3. Images from Trials 1 and 2

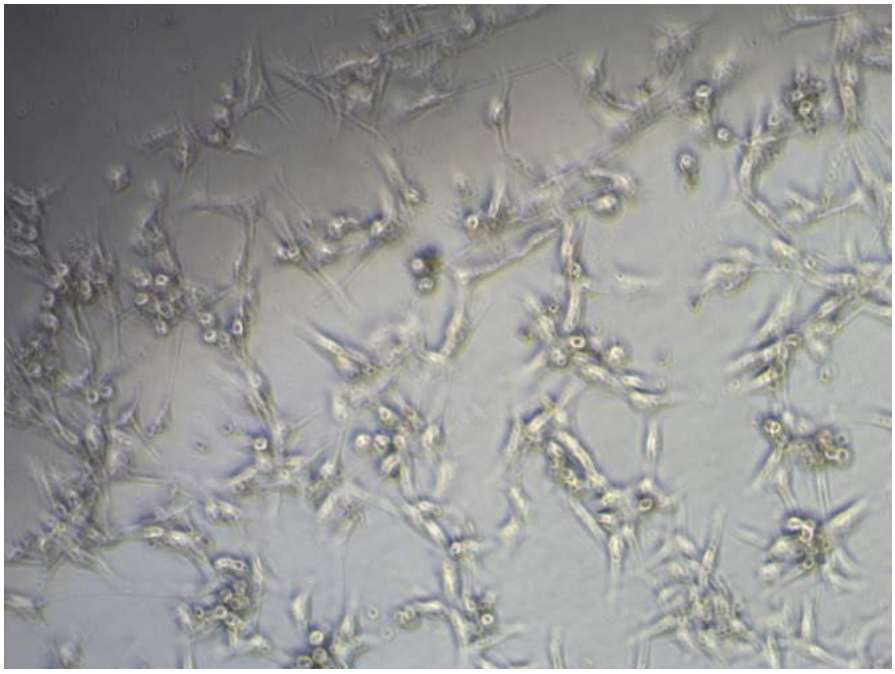
- **MDA-MB-231_WT cells**



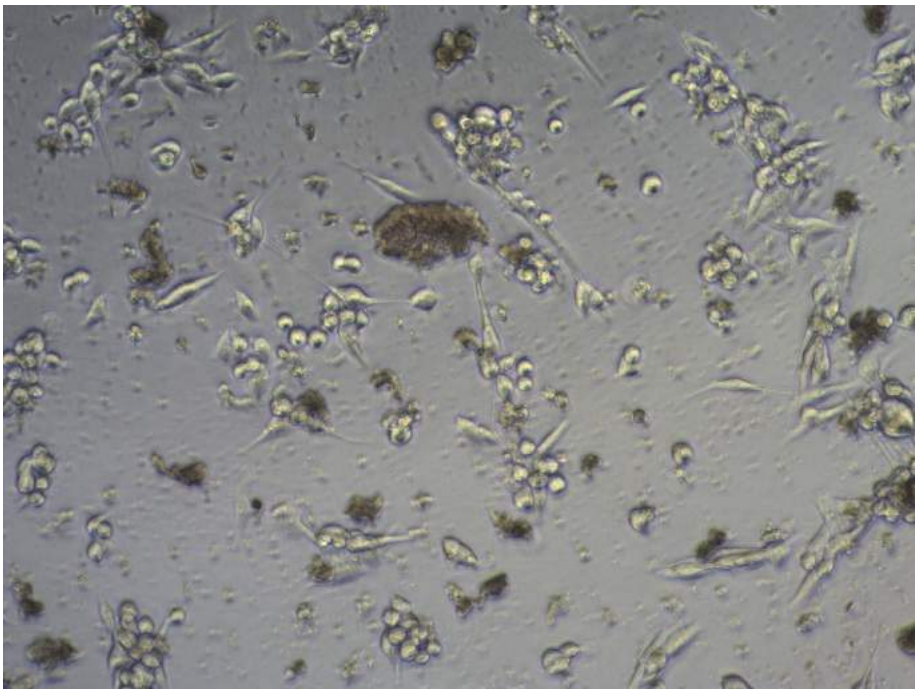
Cells treated with Laccase concentrated to 1 µg/ml



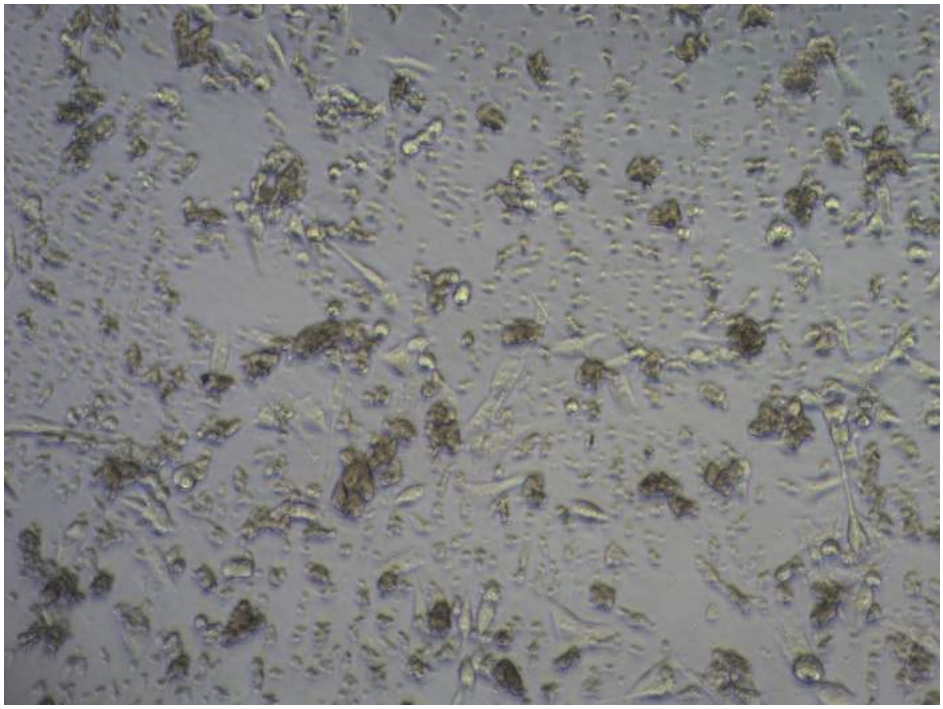
Cells treated with Laccase concentrated to 10 µg/ml



Cells treated with Laccase concentrated to 50 µg/ml

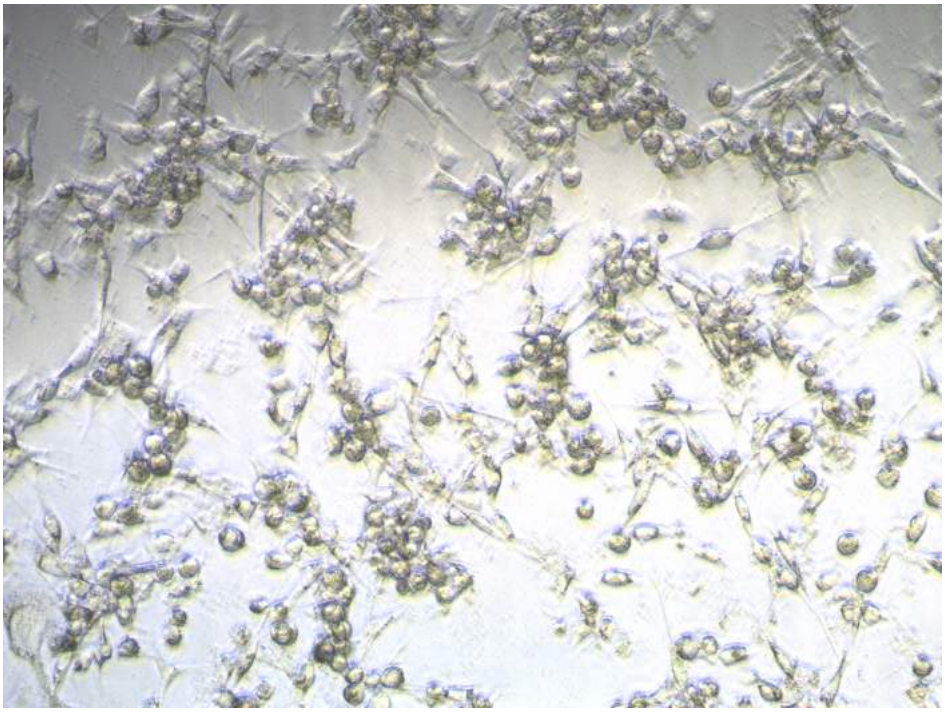


Cells treated with Laccase concentrated to 250 µg/ml

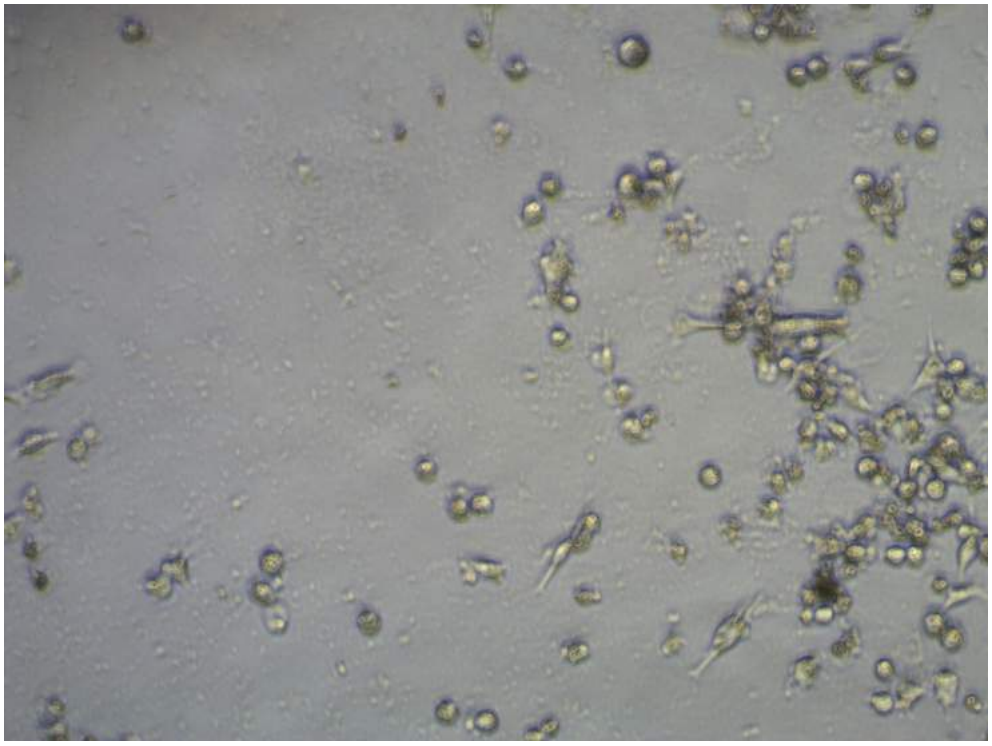


Cells treated with Laccase concentrated to 1.000 µg/ml

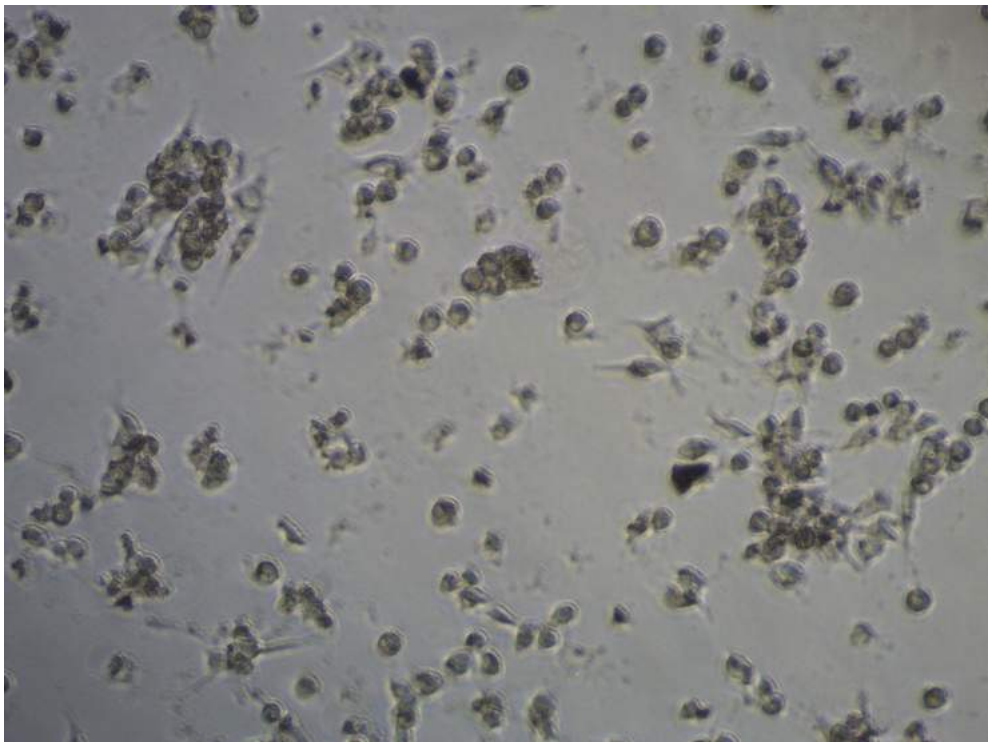
- **MDA-MB-231_DxR cells**



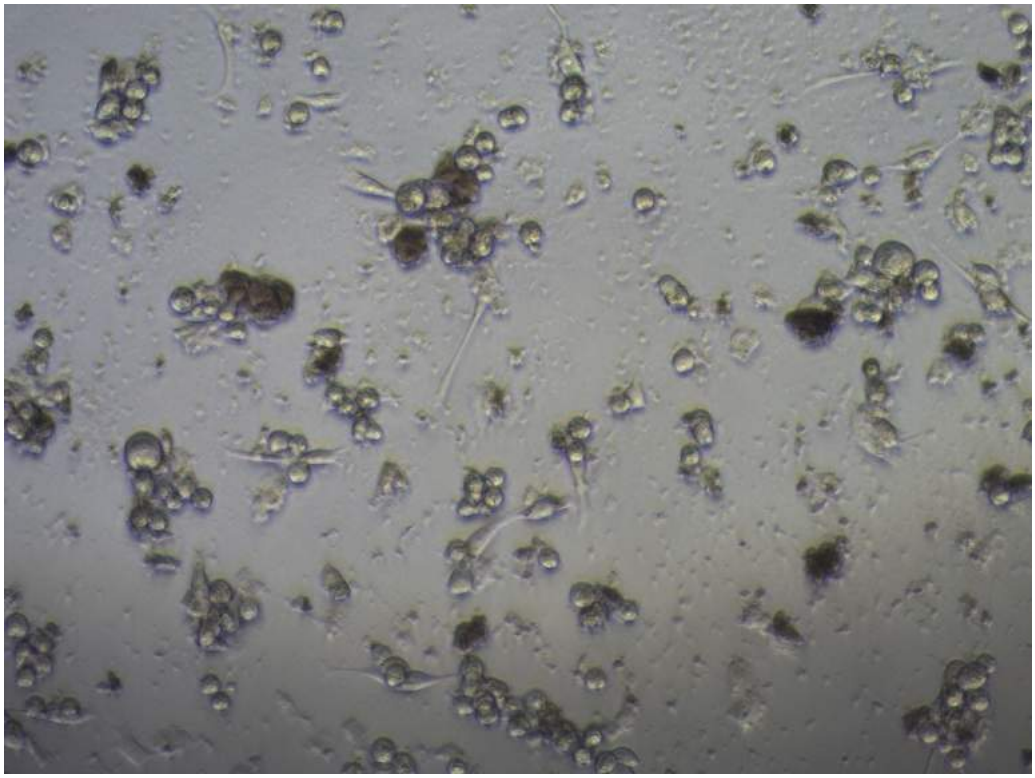
Cells treated with Laccase concentrated to 1 µg/ml



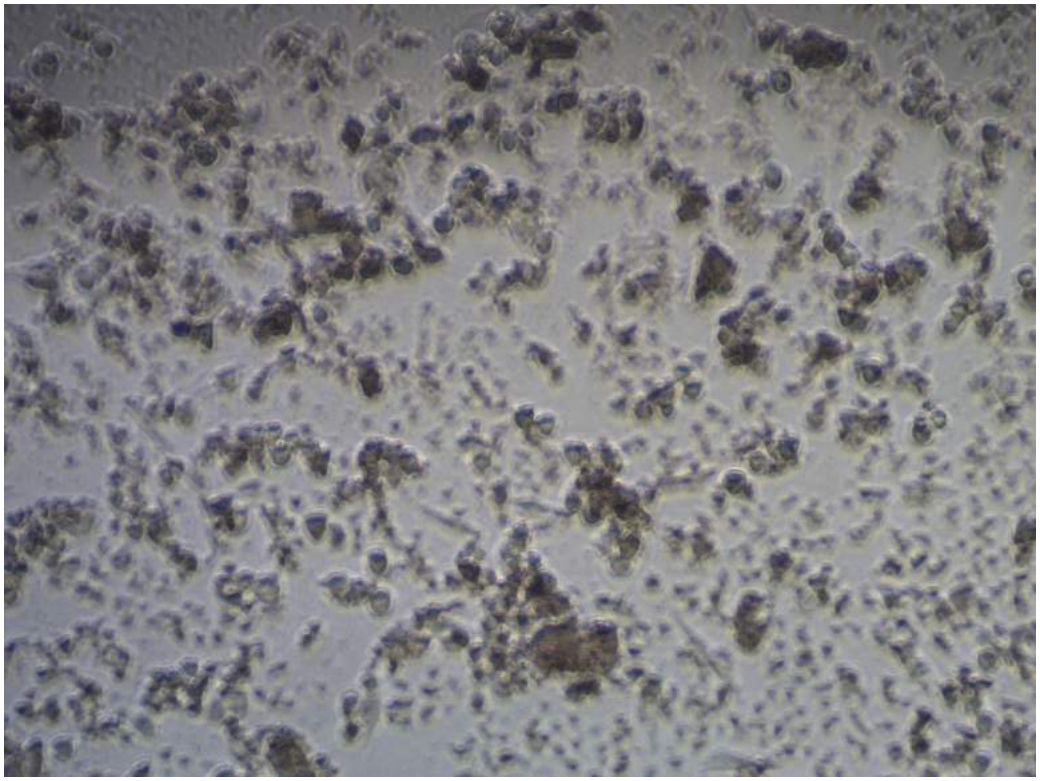
Cells treated with Laccase concentrated to 10 µg/ml



Cells treated with Laccase concentrated to 50 µg/ml

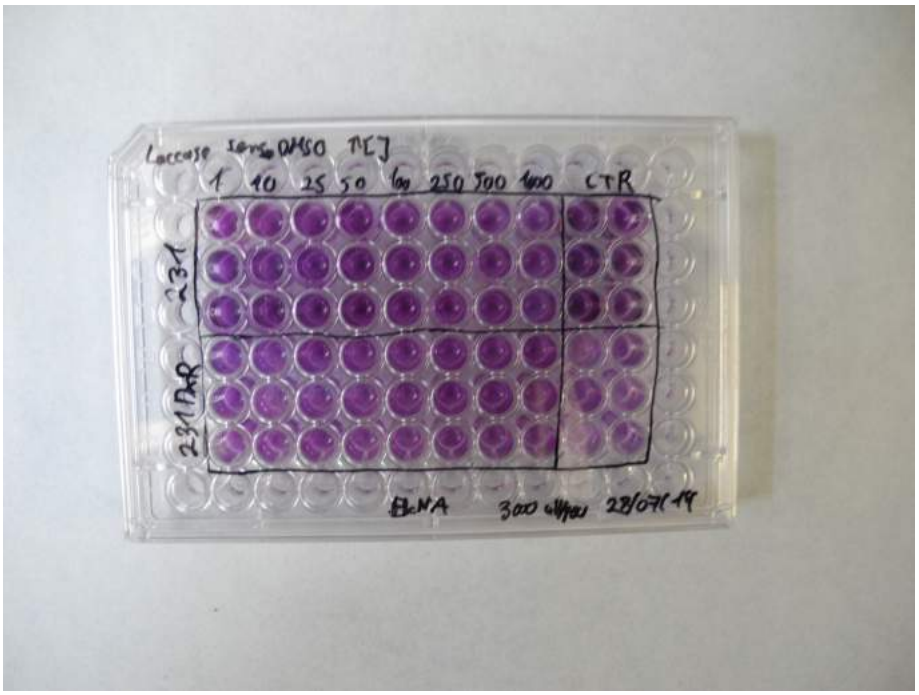


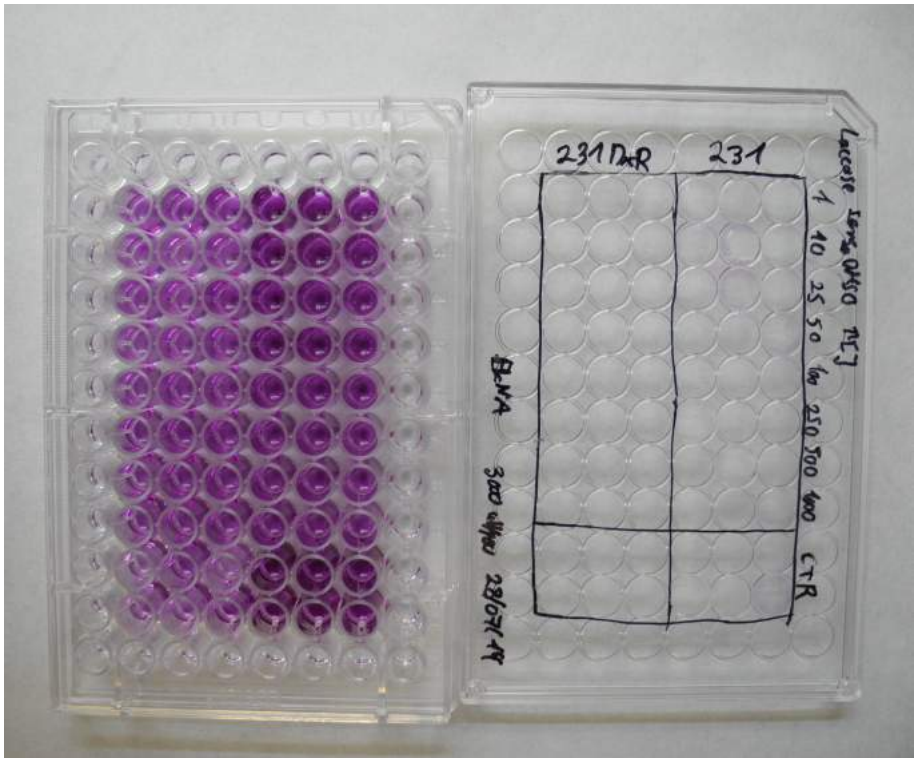
Cells treated with Laccase concentrated to 250 µg/ml



Cells treated with Laccase concentrated to 1.000 µg/ml

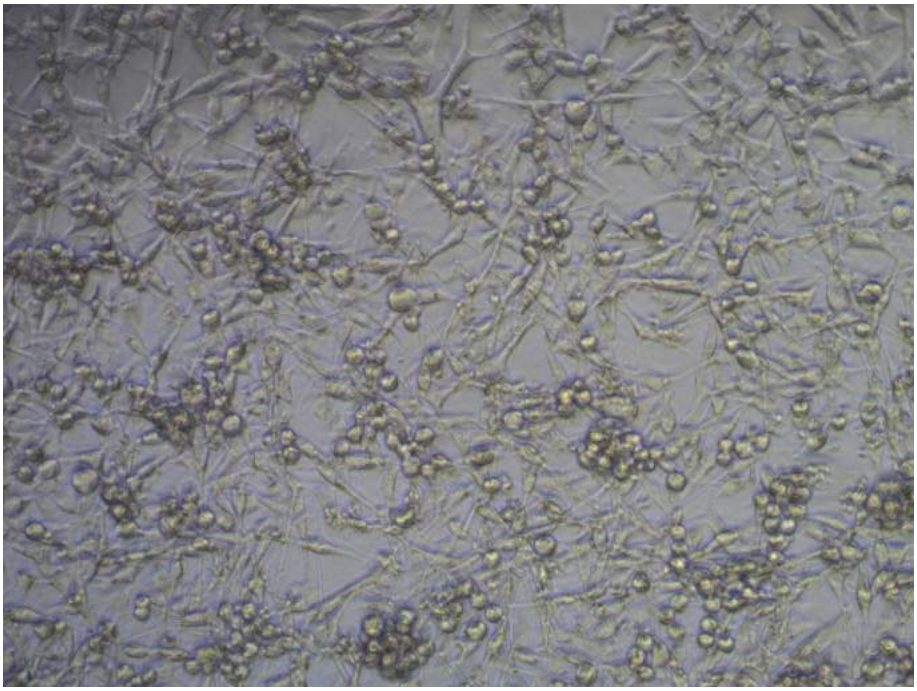
- **MICROWELL PLATE**



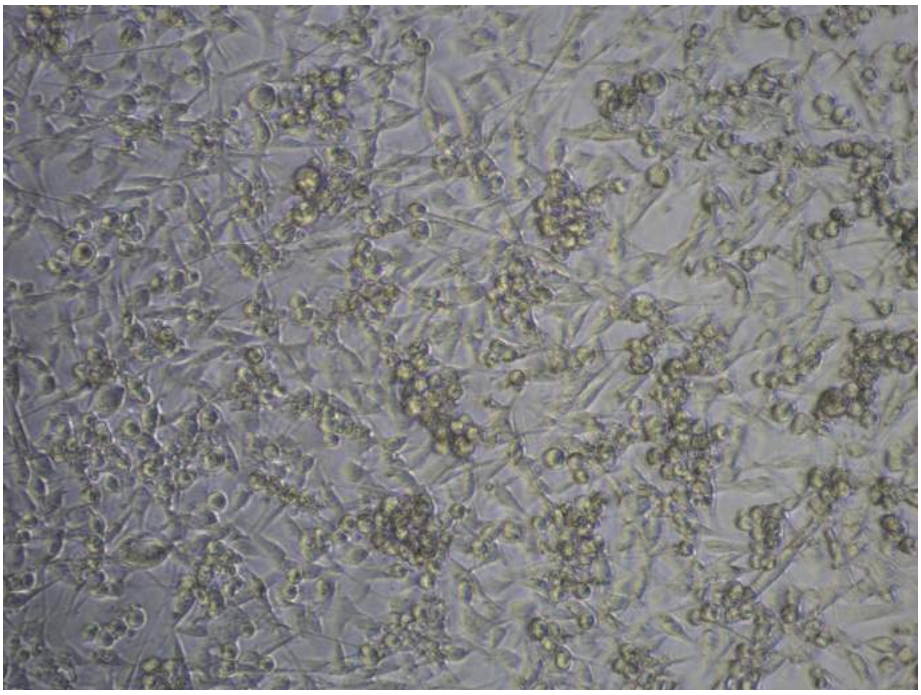


ANNEX 4. Images from Trial 3

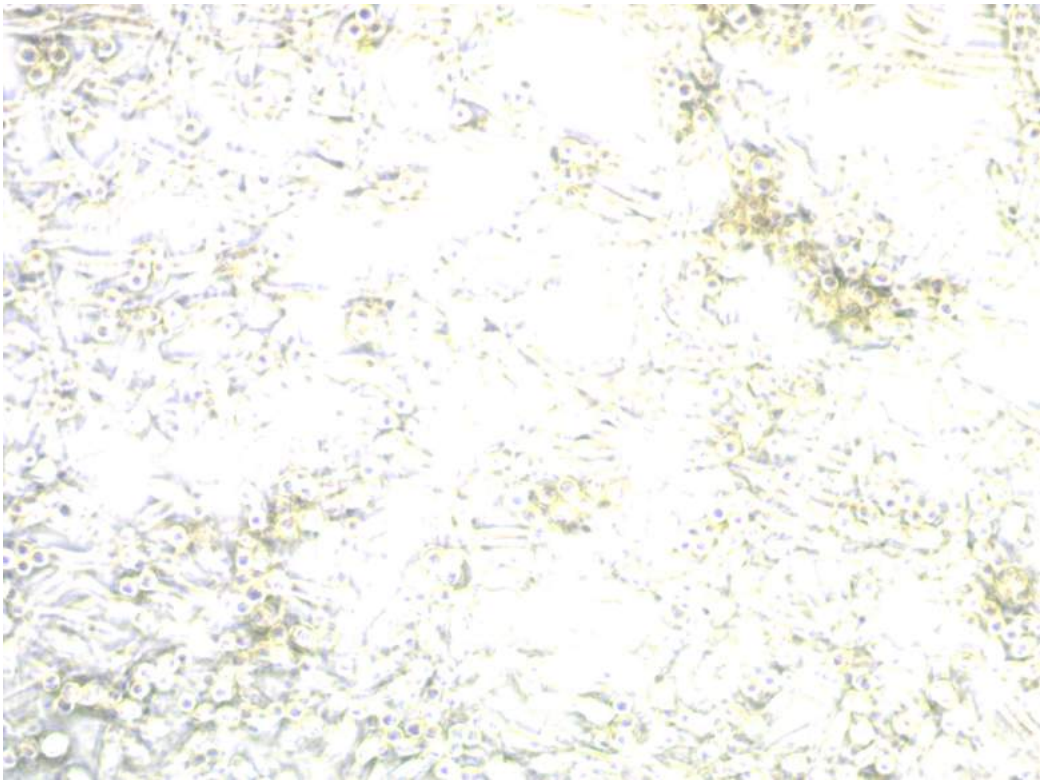
- **MDA-MB-231_WT cells**



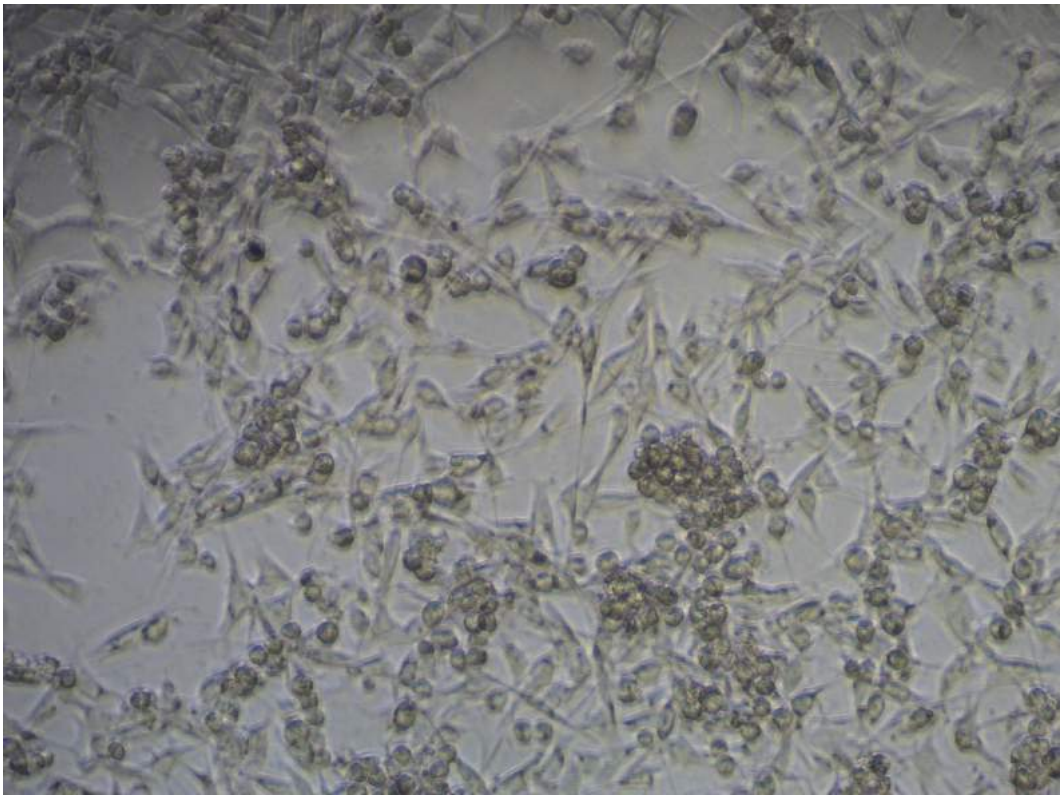
Cells treated with Laccase concentrated to 0.001 µg/ml



Cells treated with Laccase concentrated to 0.1 µg/ml

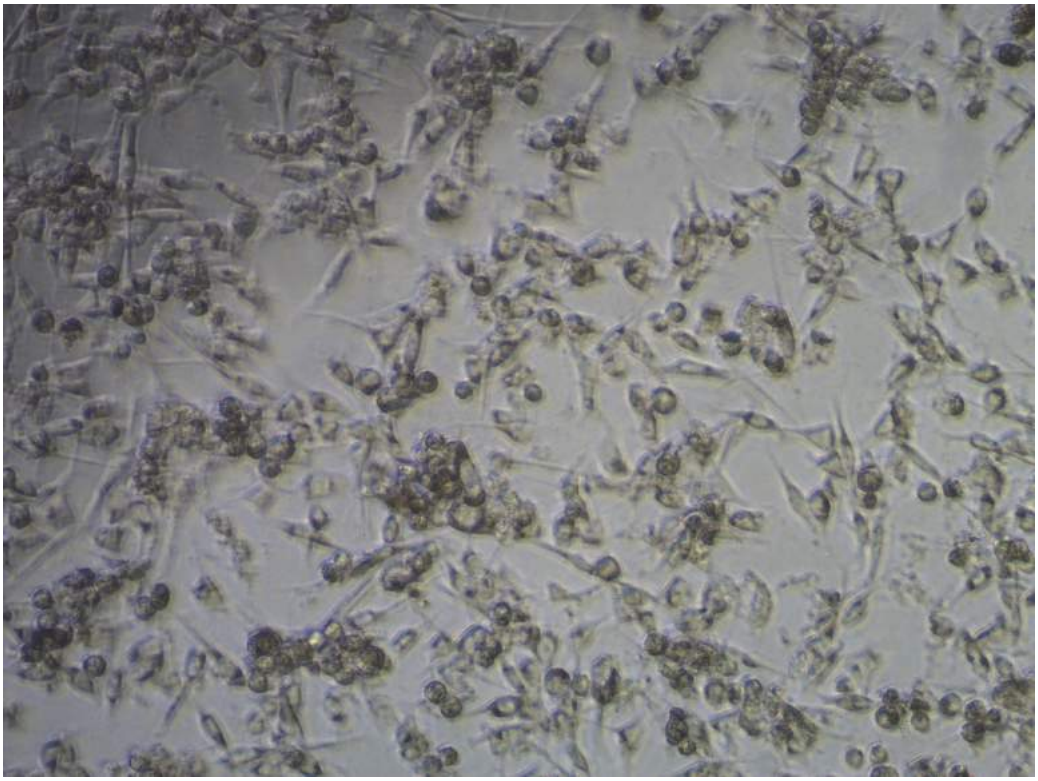


Cells treated with Laccase concentrated to 1 µg/ml

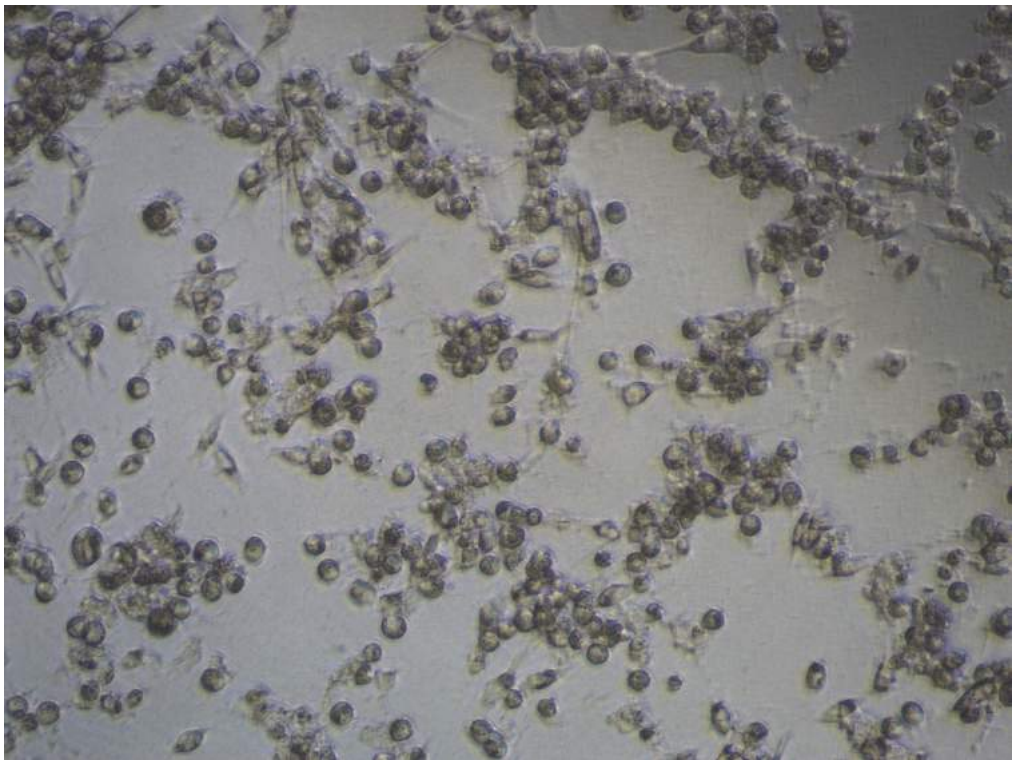


Cells treated with Laccase concentrated to 10 µg/ml

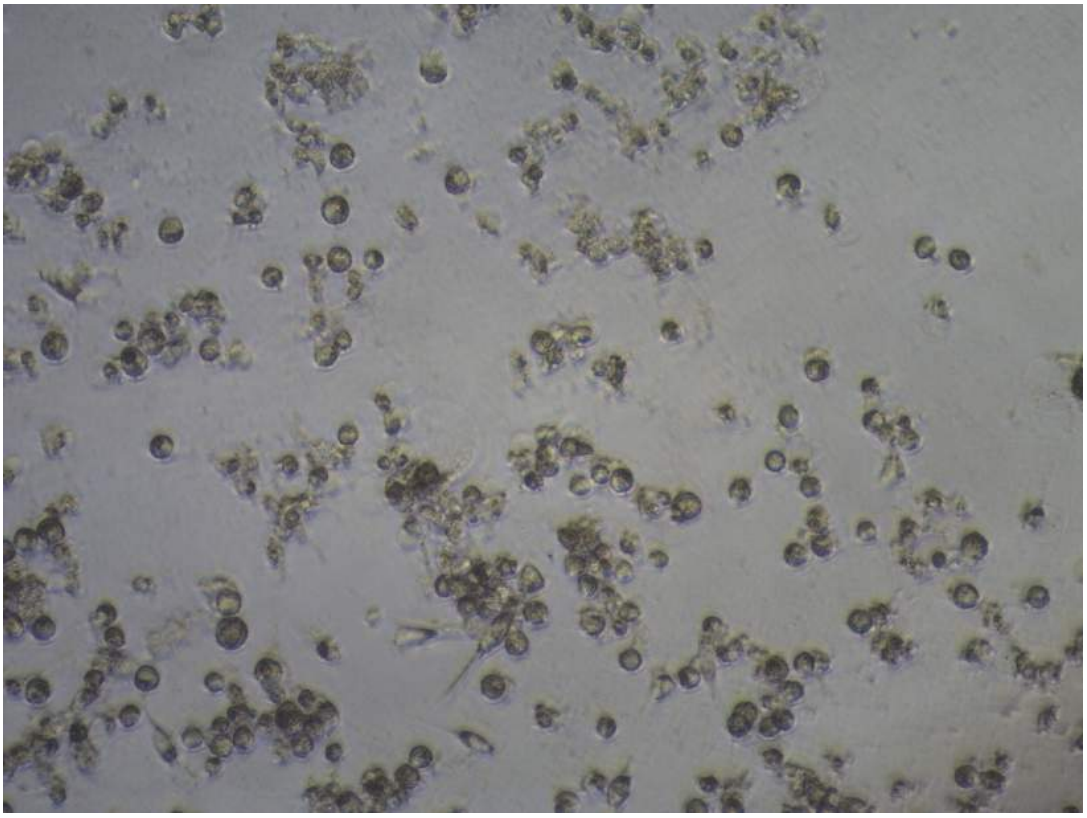
- **MDA-MB-231_DxR**



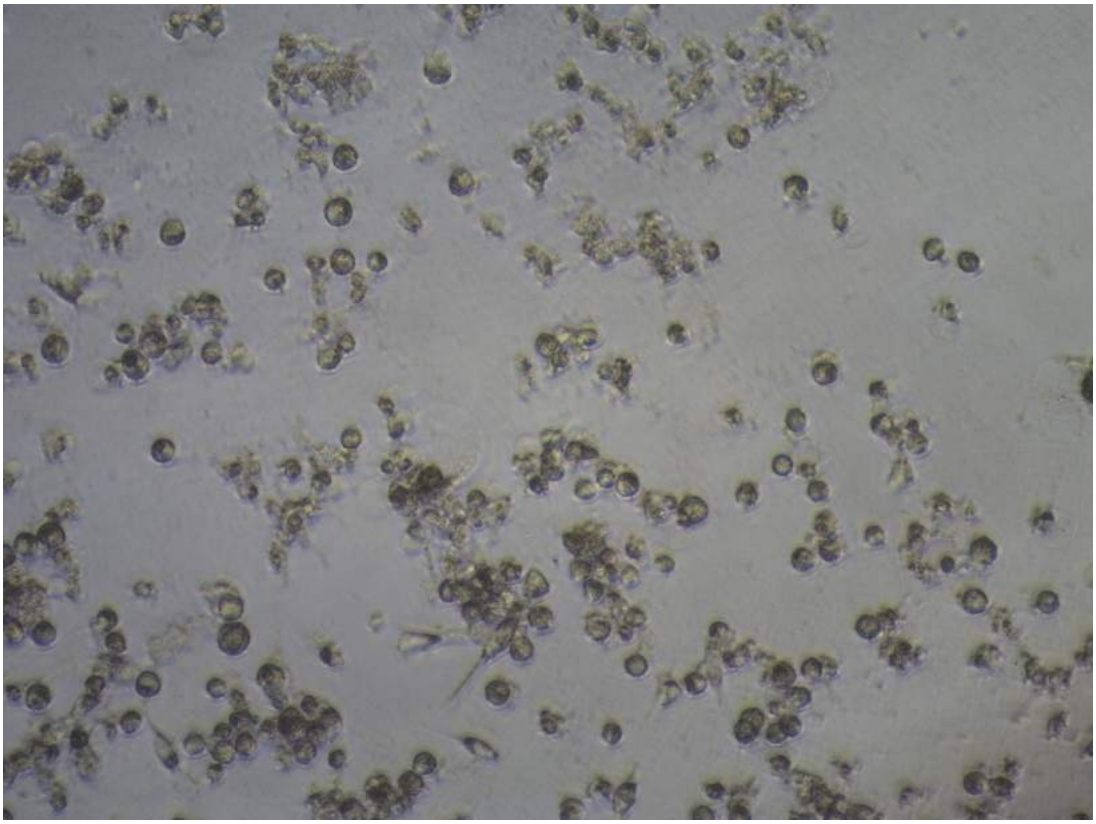
Cells treated with Laccase concentrated to 0.001 µg/ml



Cells treated with Laccase concentrated to 0.1 µg/ml

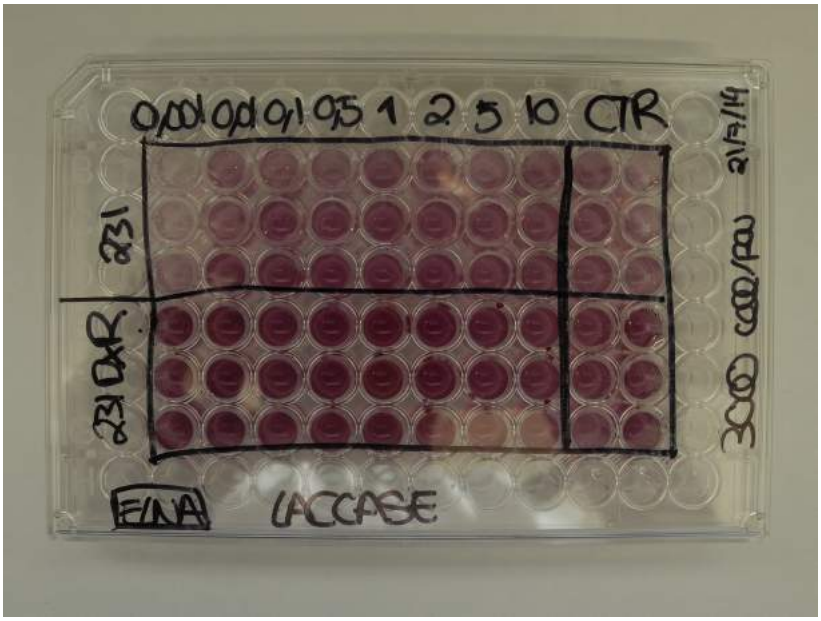


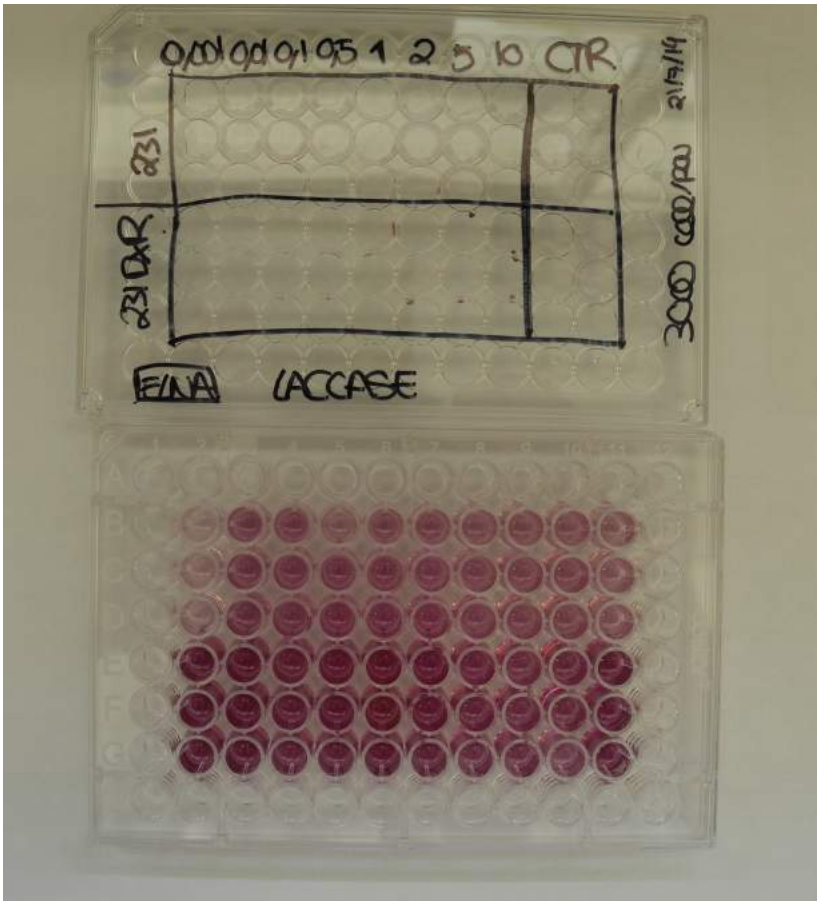
Cells treated with Laccase concentrated to 1 µg/ml



Cells treated with Laccase concentrated to 10 µg/ml

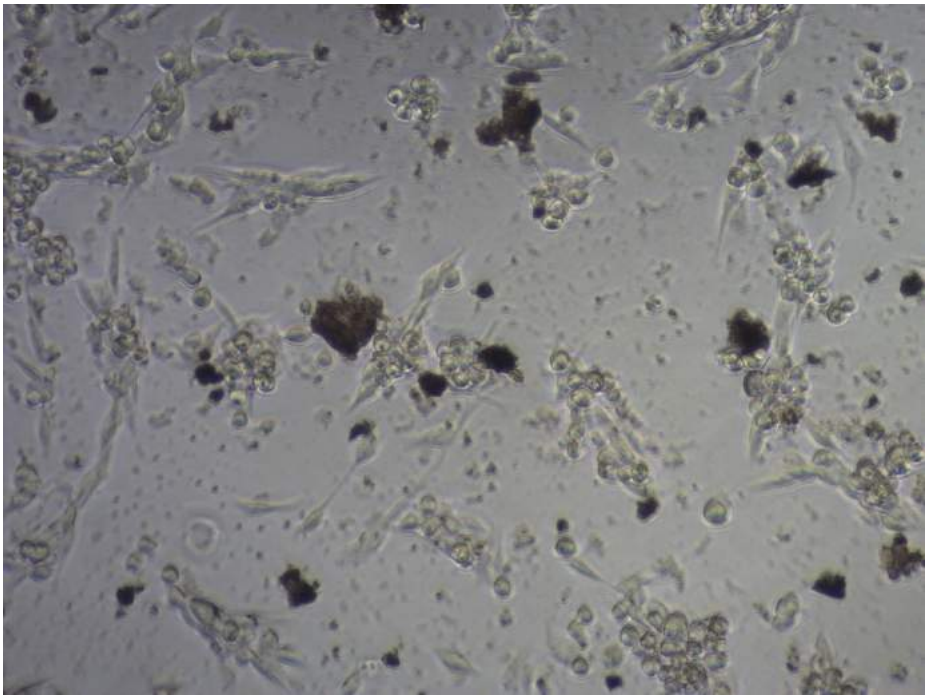
- **MICROWELL PLATE**



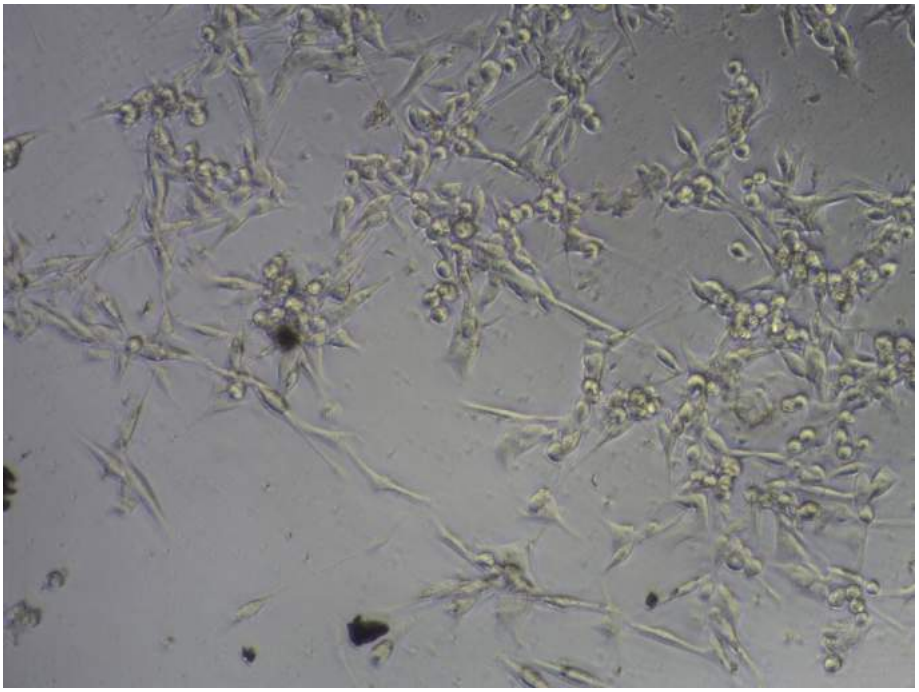


ANNEX 5. Images from Trial 4

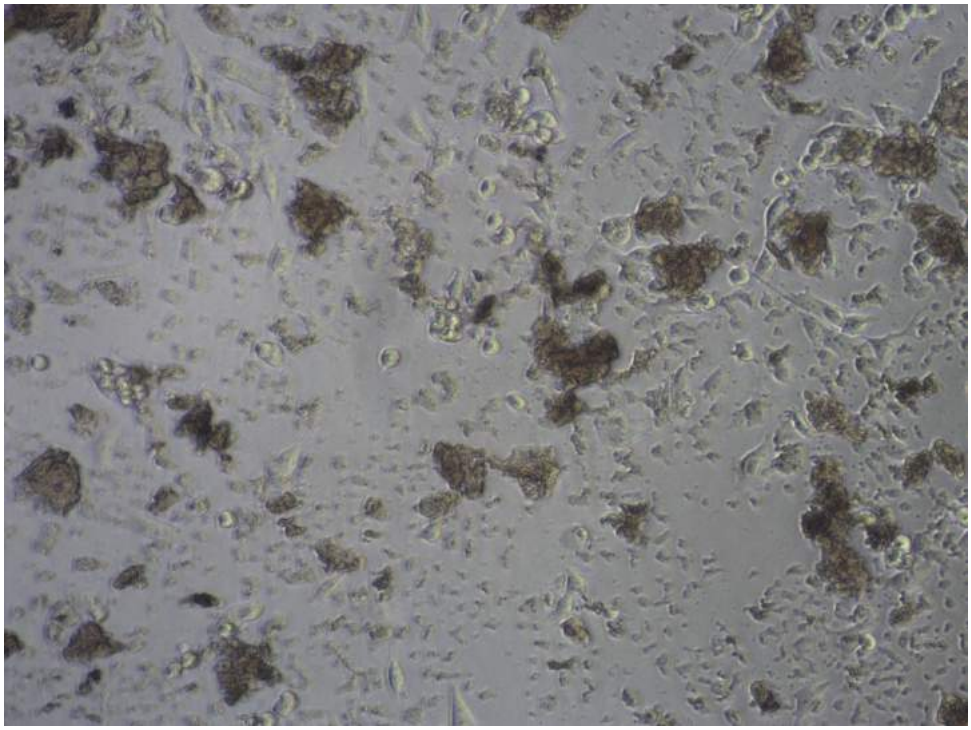
- **MDA-MB-231_WT cells**



Cells treated with Laccase concentrated to 50 µg/ml

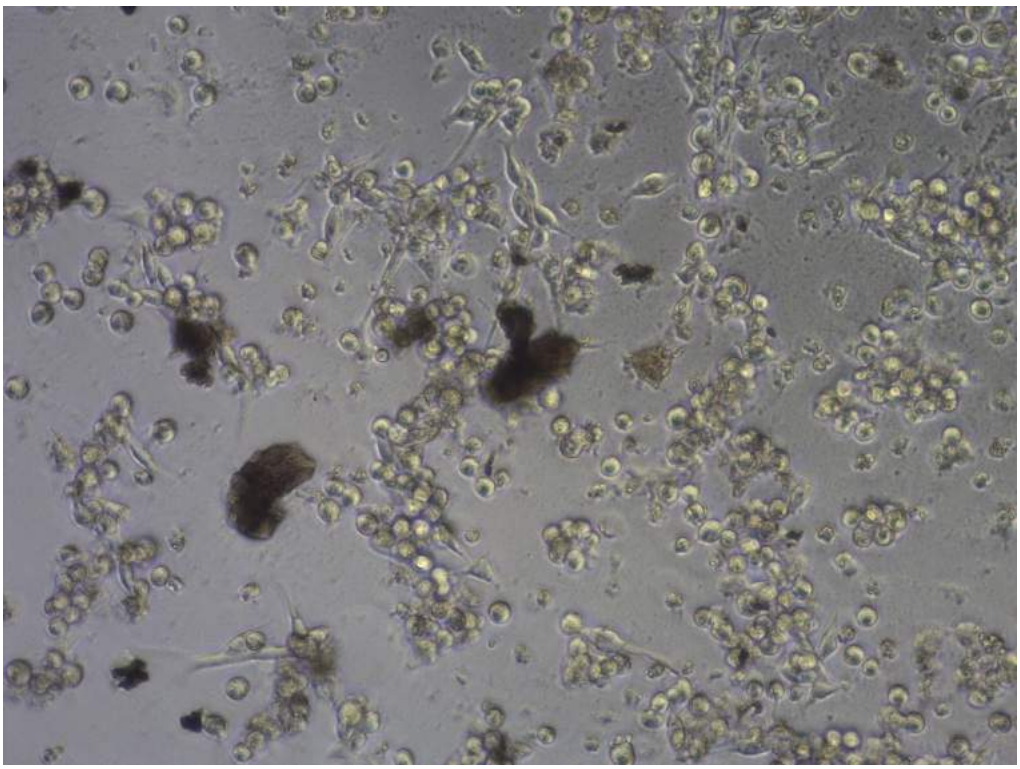


Cells treated with Laccase concentrated to 250 µg/ml

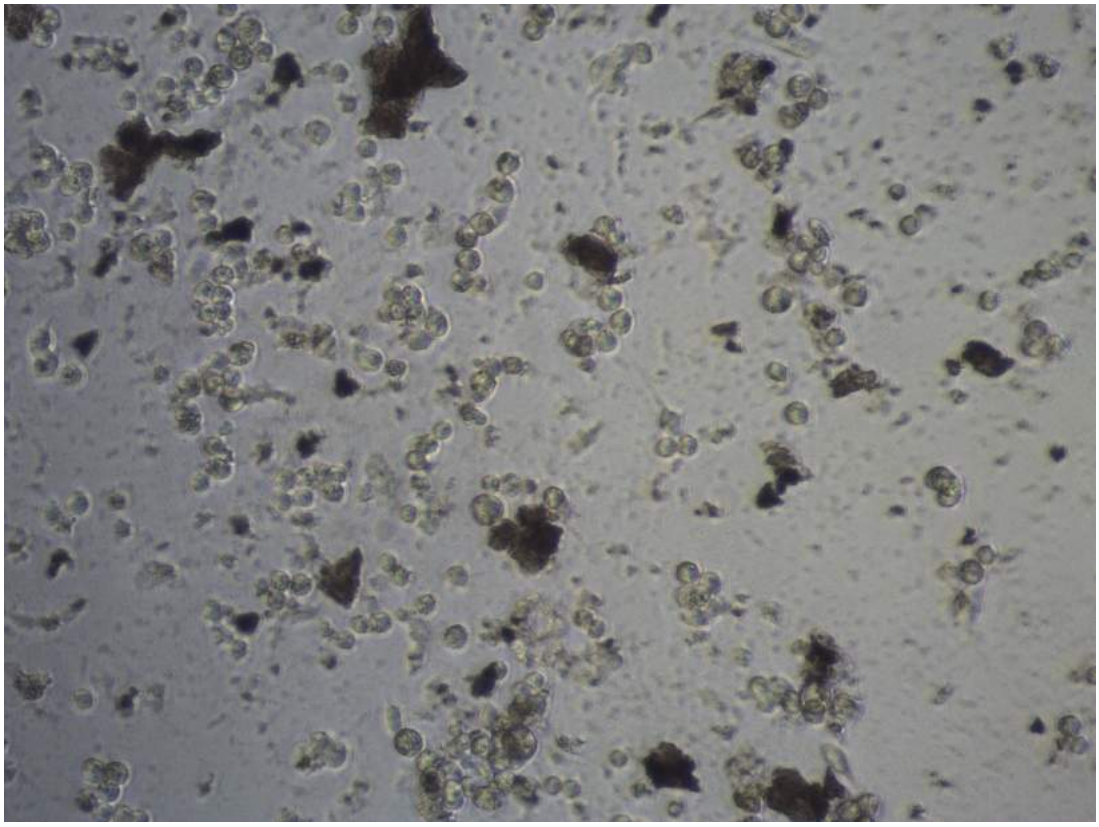


Cells treated with Laccase concentrated to 1.000 µg/ml

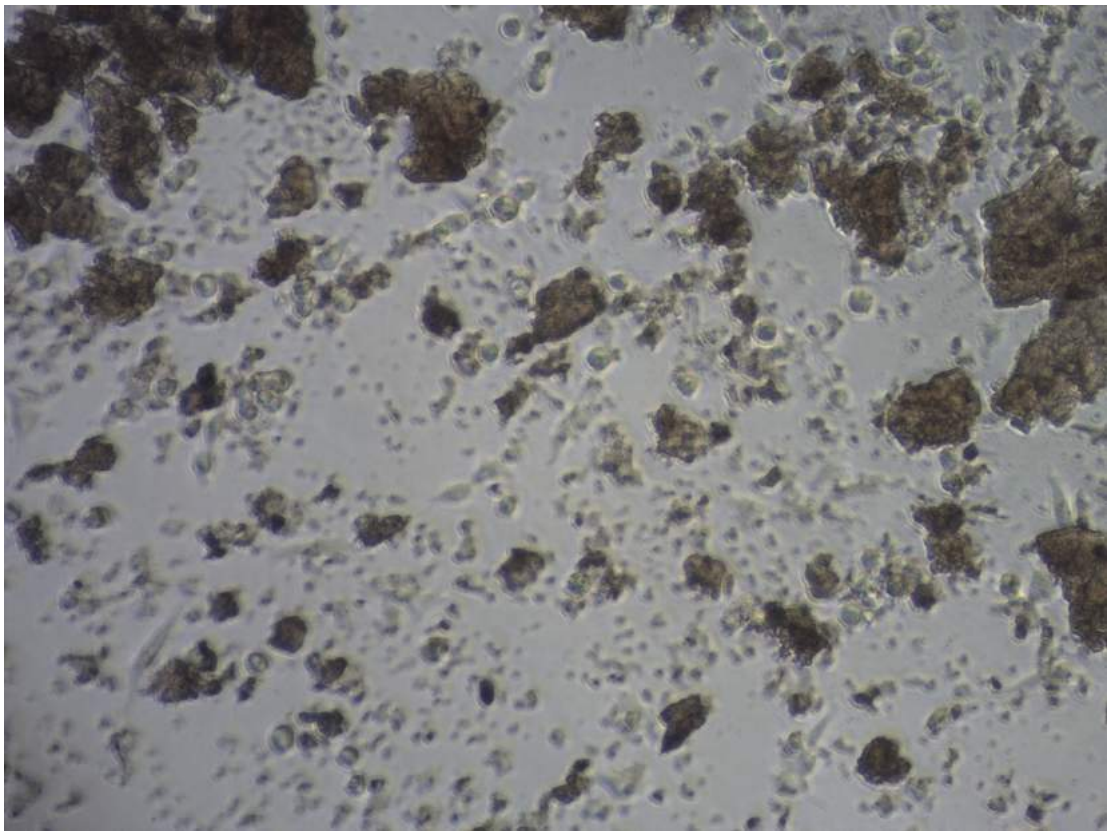
- **MDA-MB-231_DxR cells**



Cells treated with Laccase concentrated to 50 µg/ml

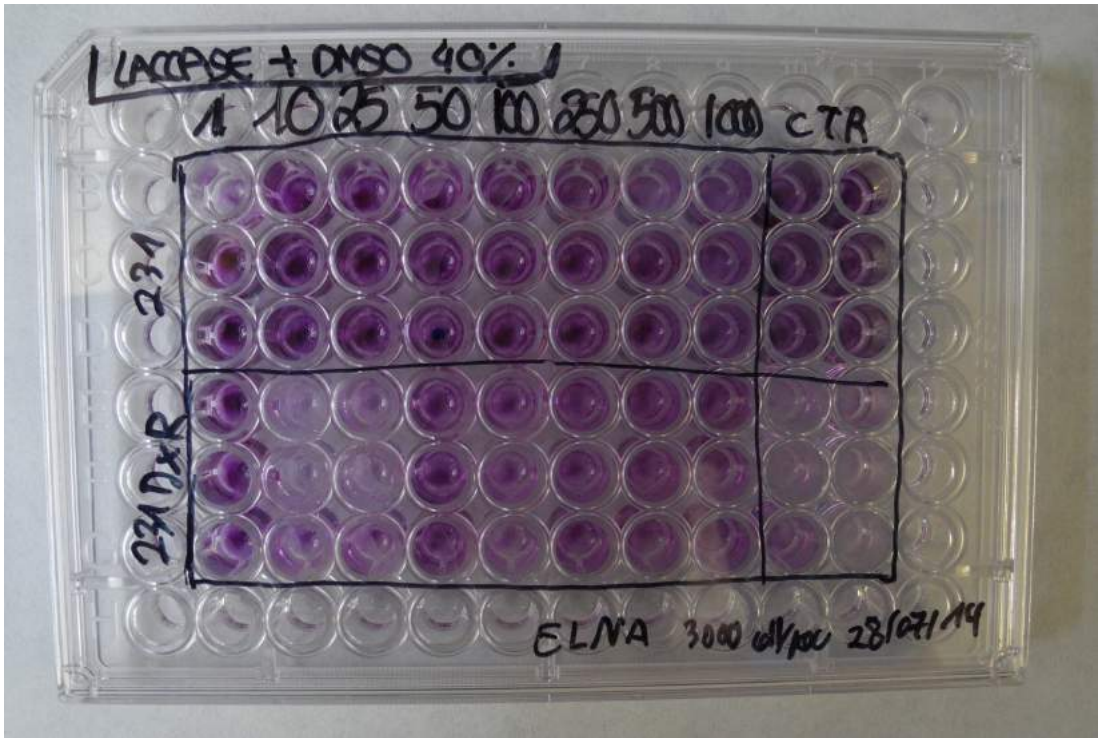


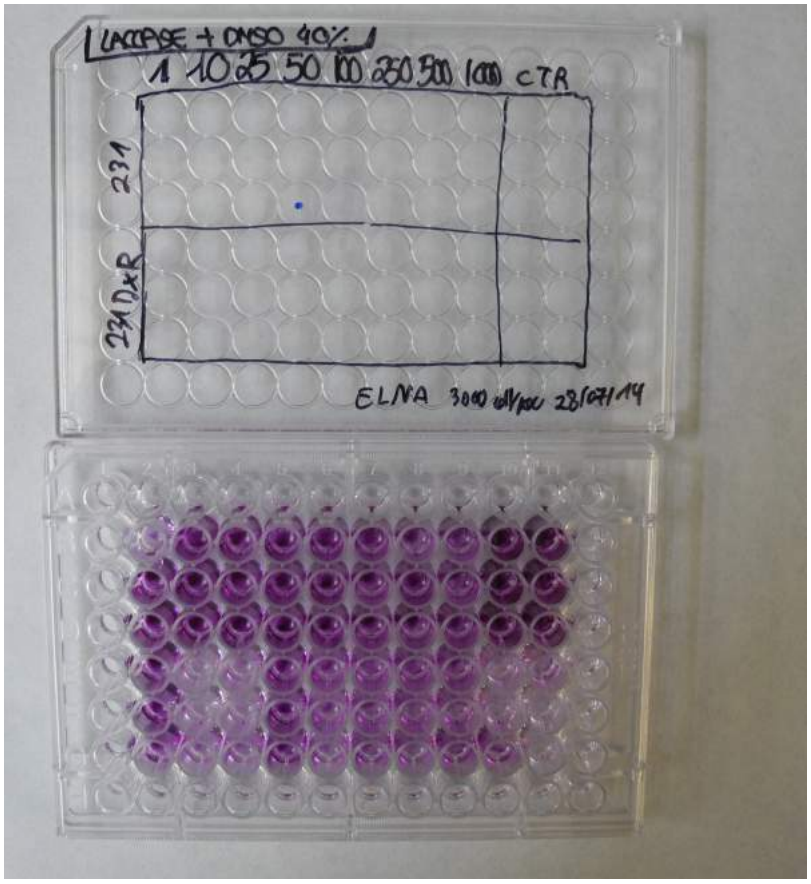
Cells treated with Laccase concentrated to 250 µg/ml



Cells treated with Laccase concentrated to 1.000 µg/ml

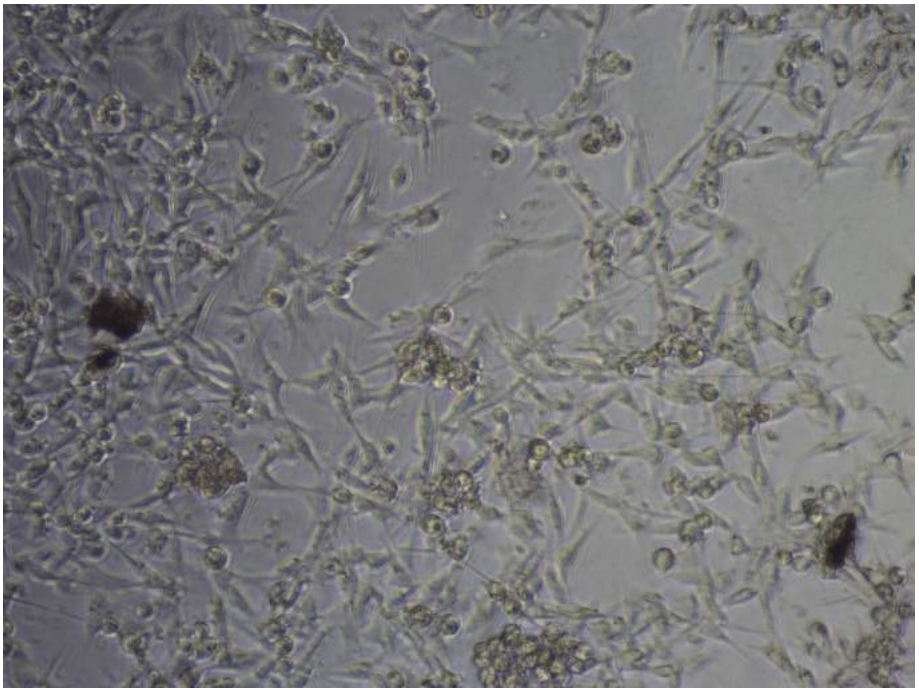
- **MICROWELL PLATE**



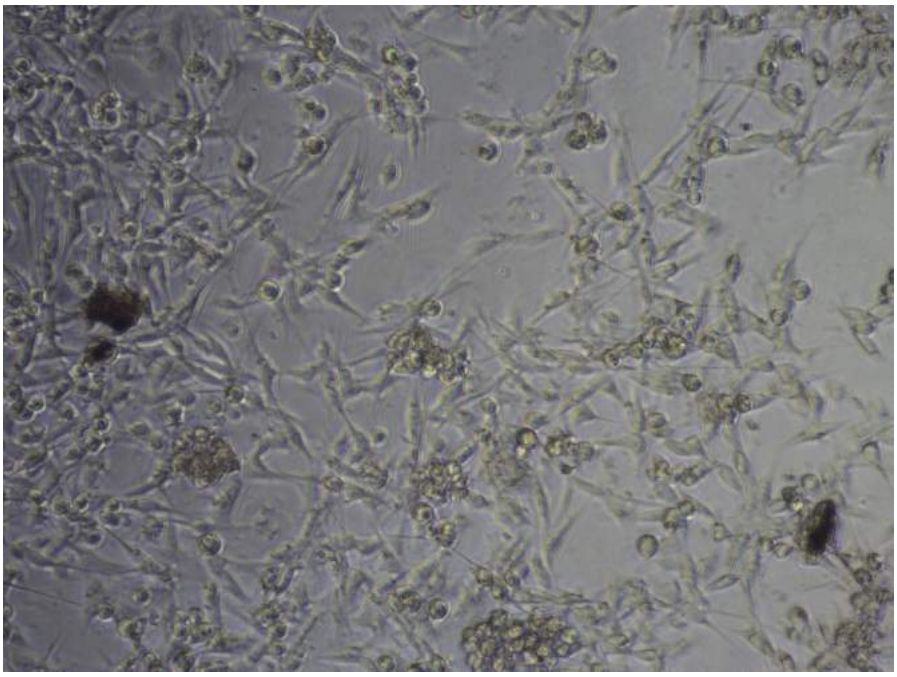


ANNEX 6. Images from Trials 5 and 6

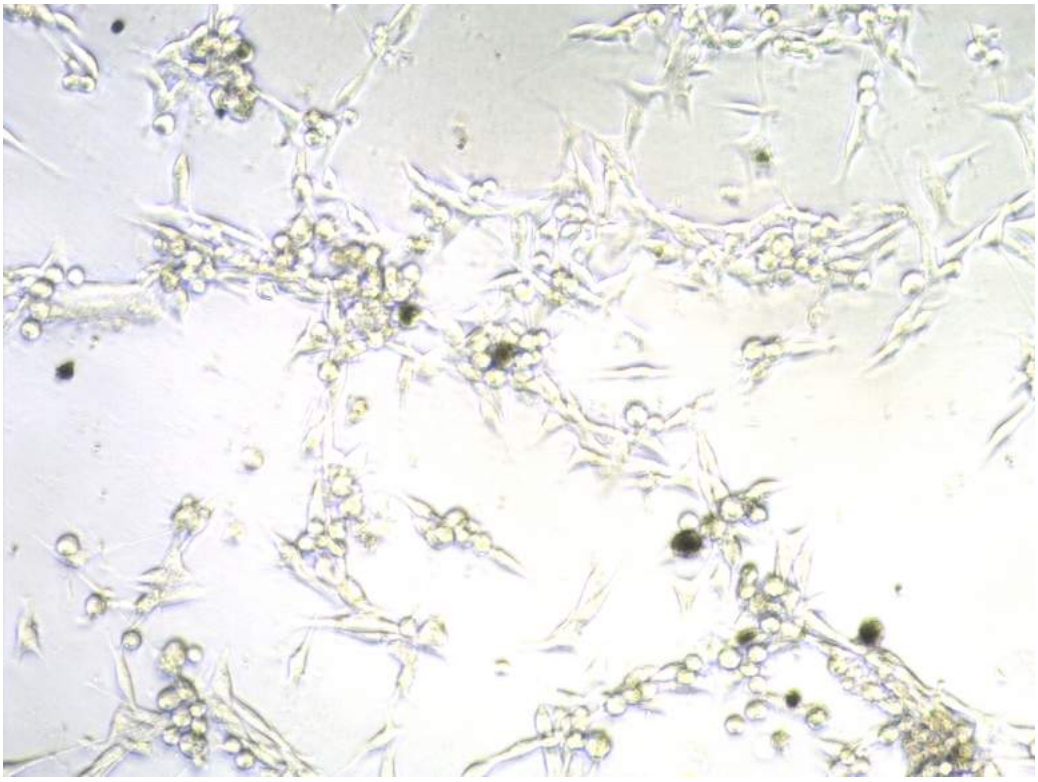
- **MDA-MB-231_WT cells**



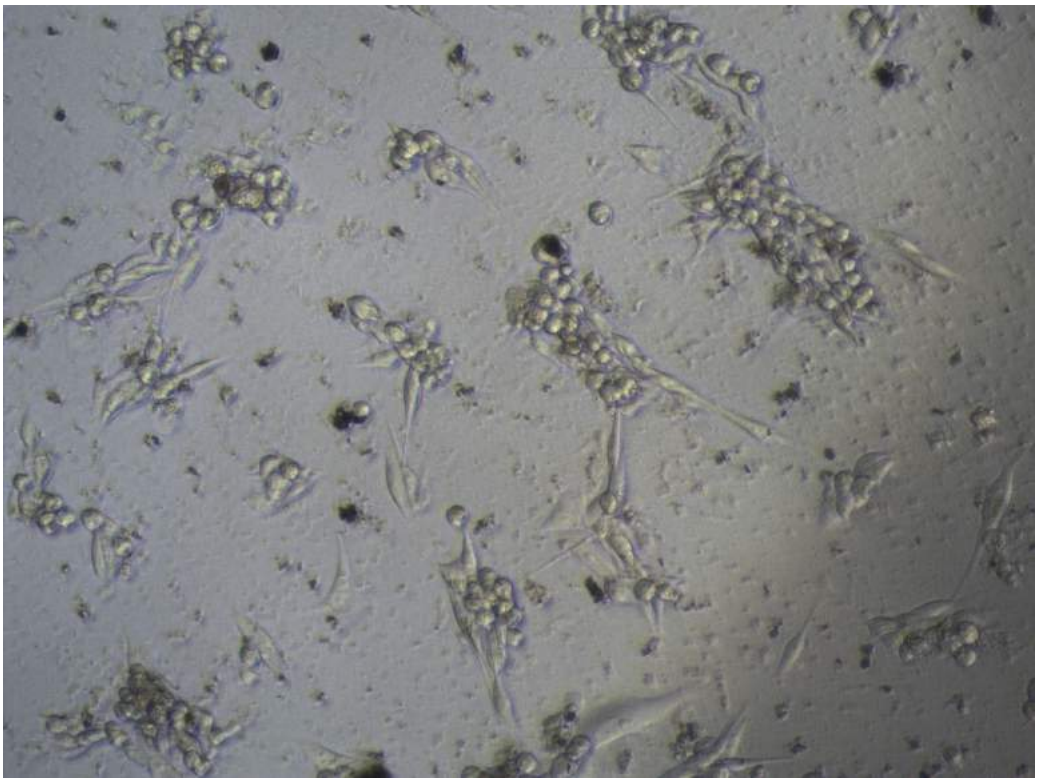
Cells treated with Laccase concentrated to 1 µg/ml



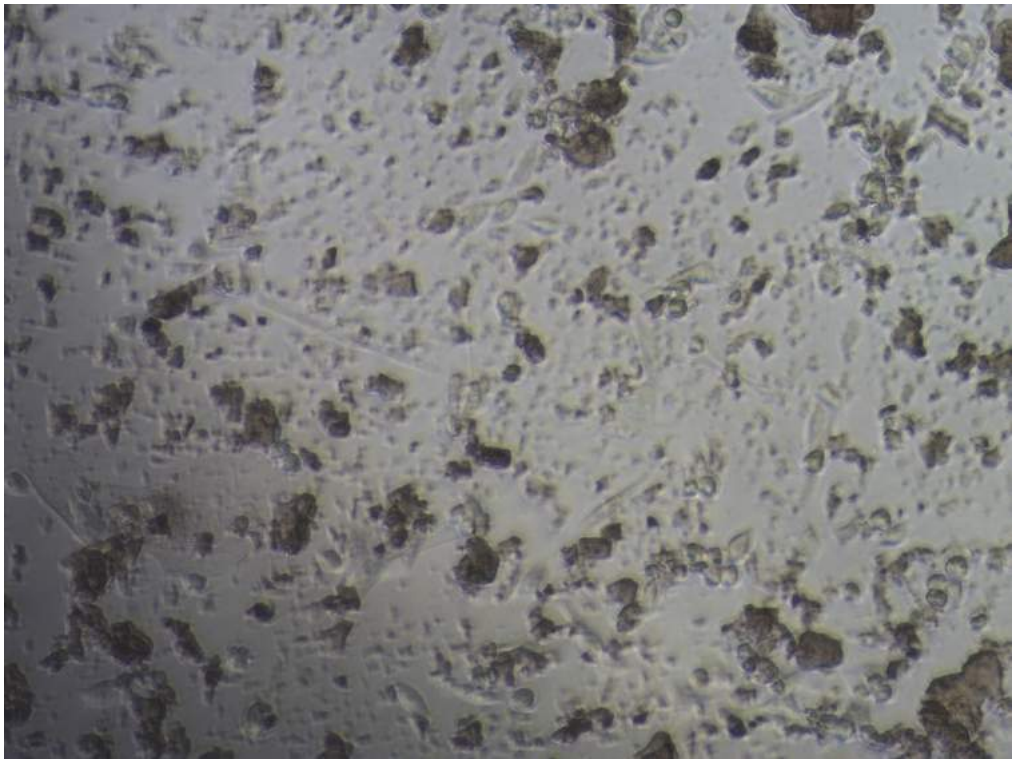
Cells treated with Laccase concentrated to 10 µg/ml



Cells treated with Laccase concentrated to 50 µg/ml

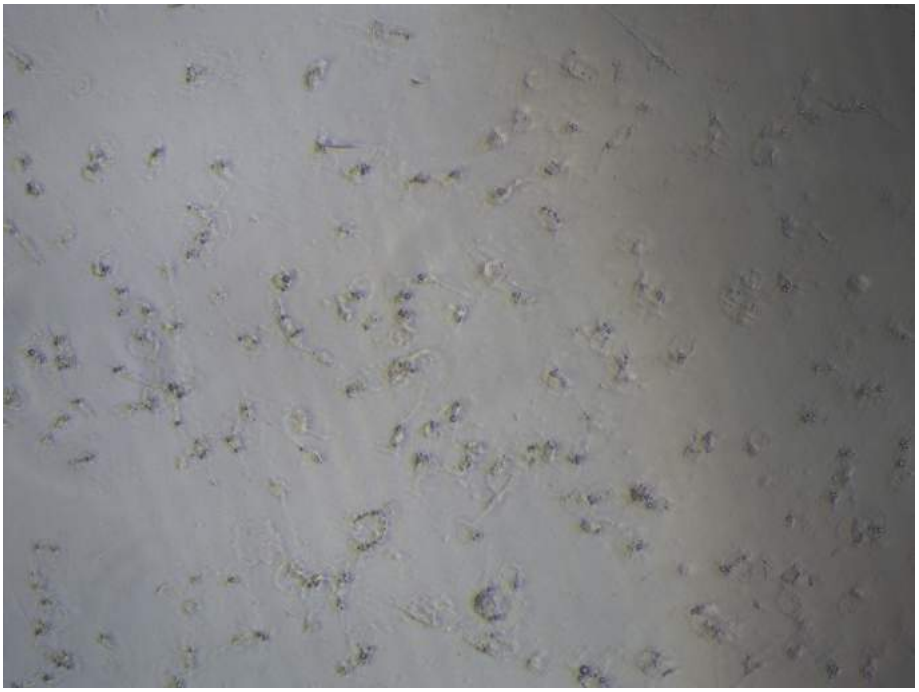


Cells treated with Laccase concentrated to 250 µg/ml

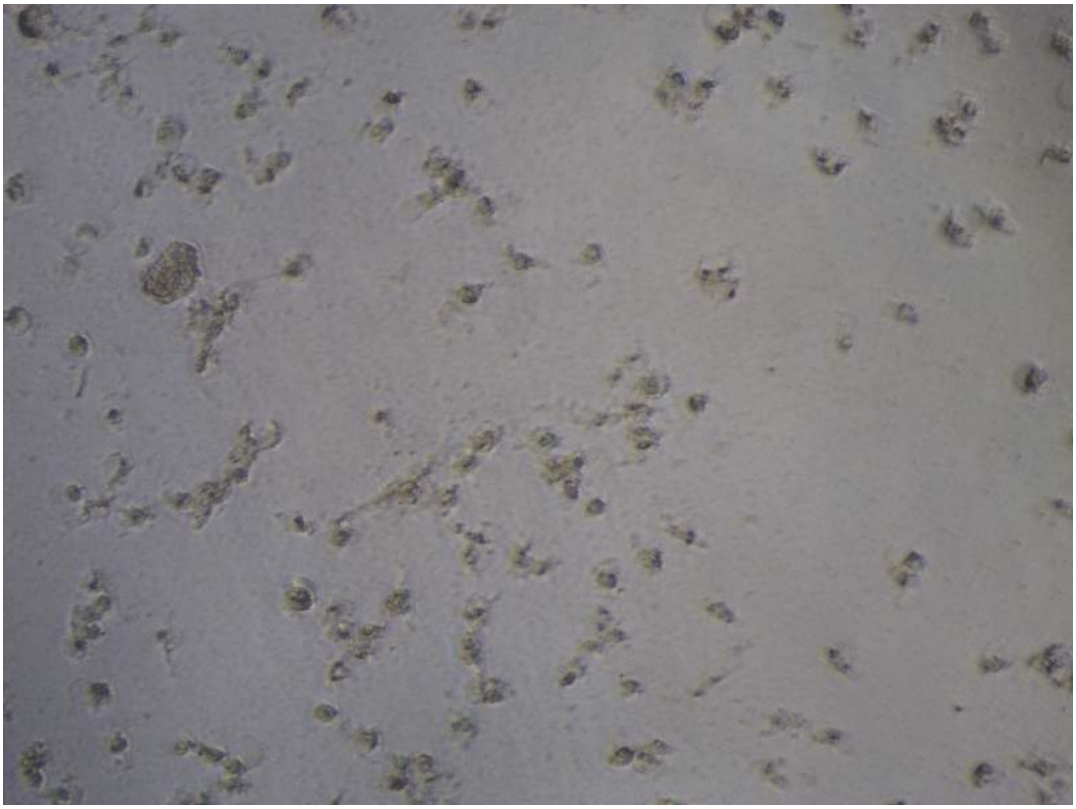


Cells treated with Laccase concentrated to 1.000 µg/ml

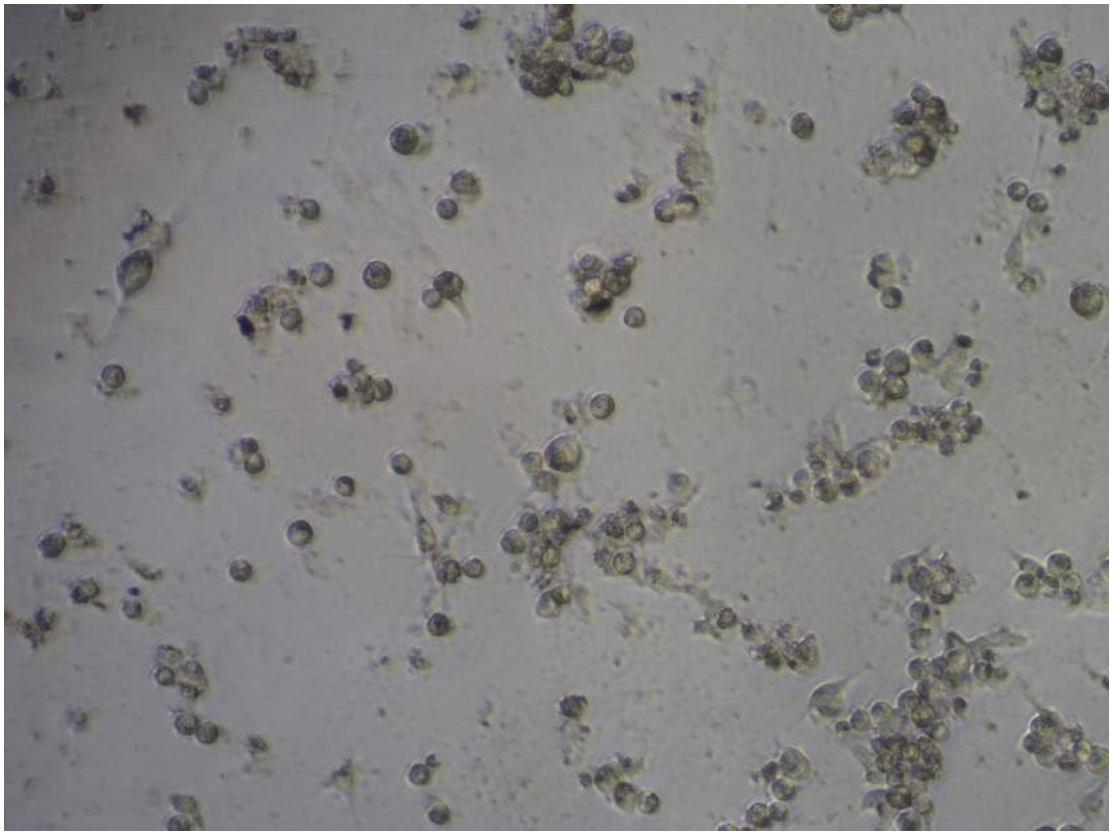
- **MDA-MB-231_DxR cells**



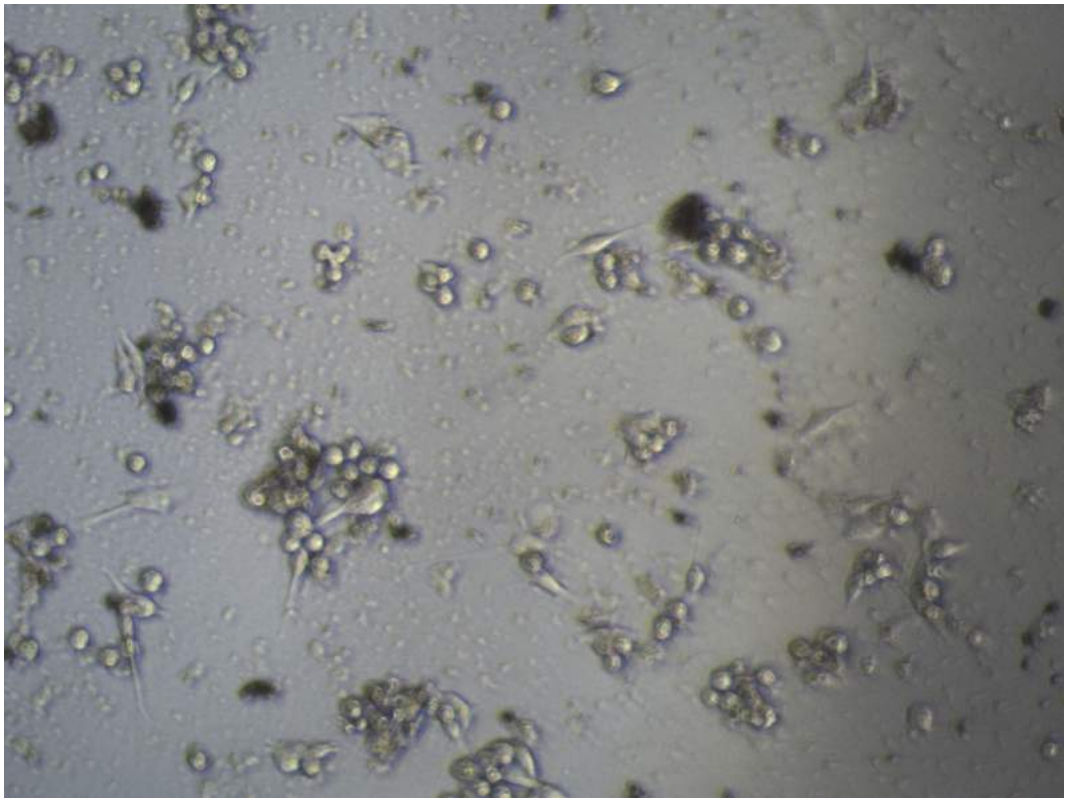
Cells treated with Laccase concentrated to 1 µg/ml



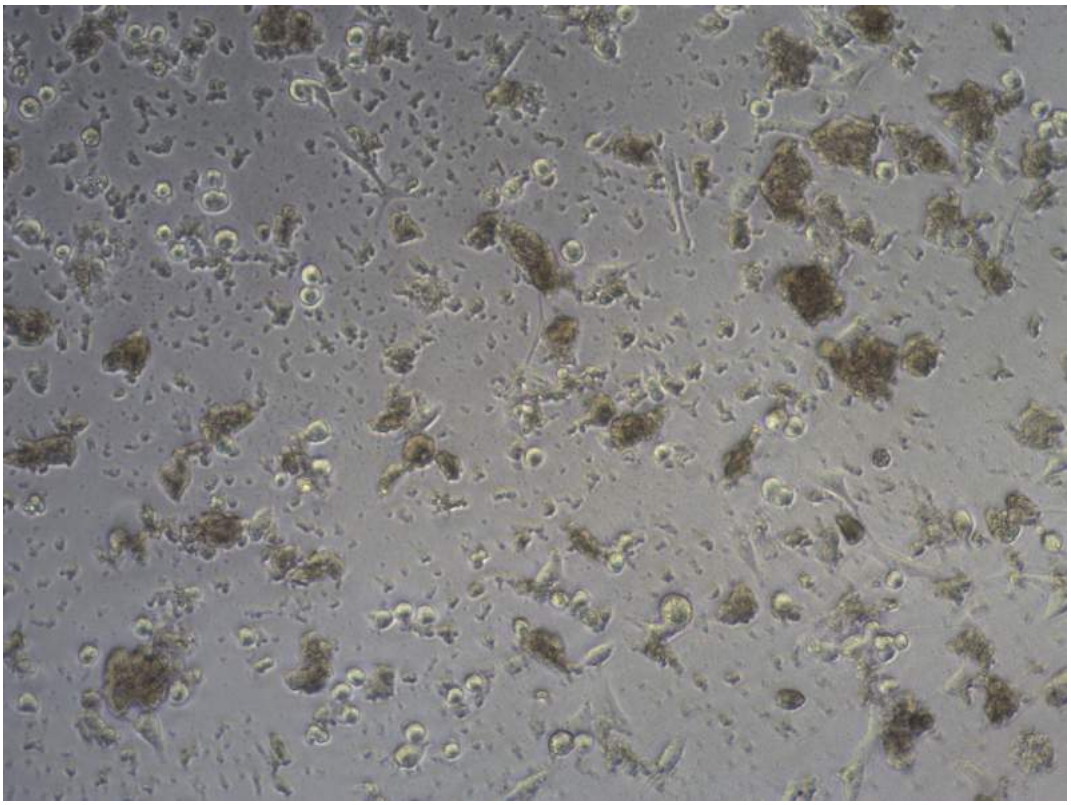
Cells treated with Laccase concentrated to 10 µg/ml



Cells treated with Laccase concentrated to 50 µg/ml

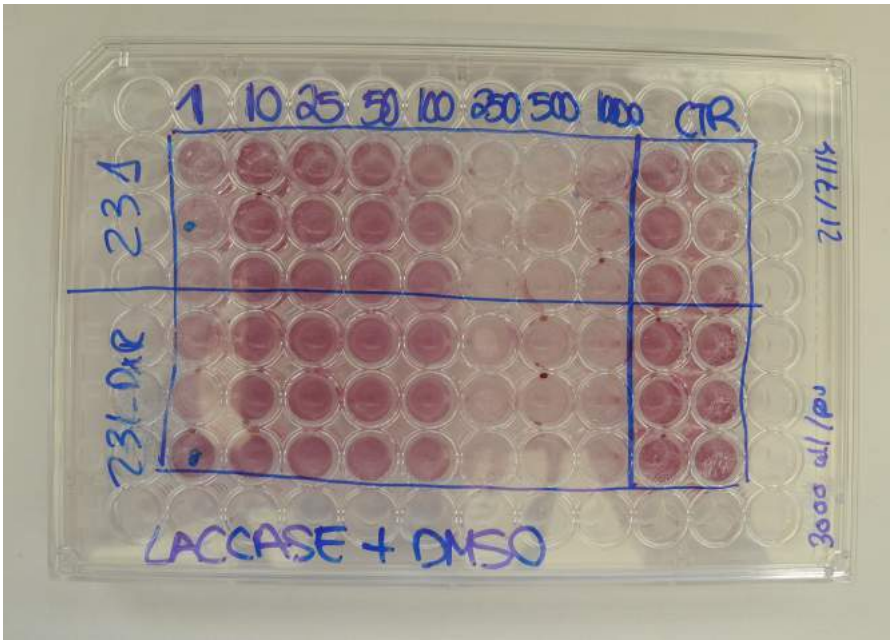


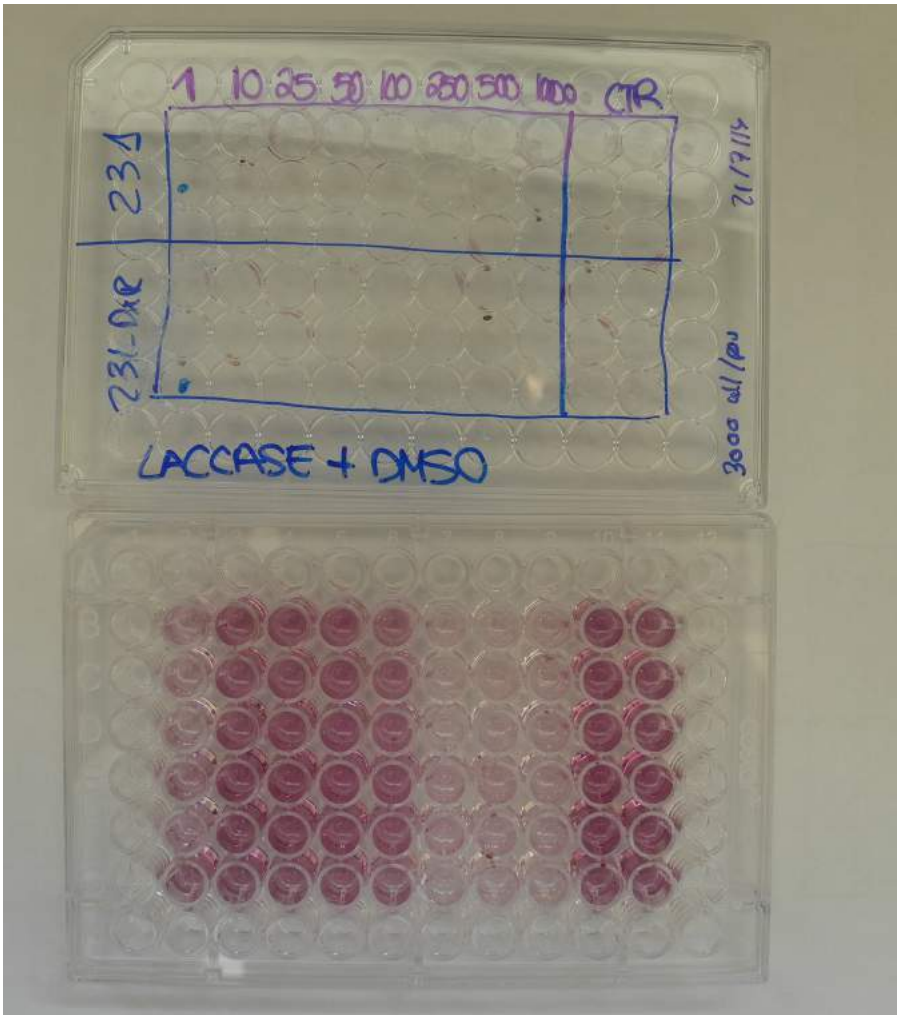
Cells treated with Laccase concentrated to 250 µg/ml



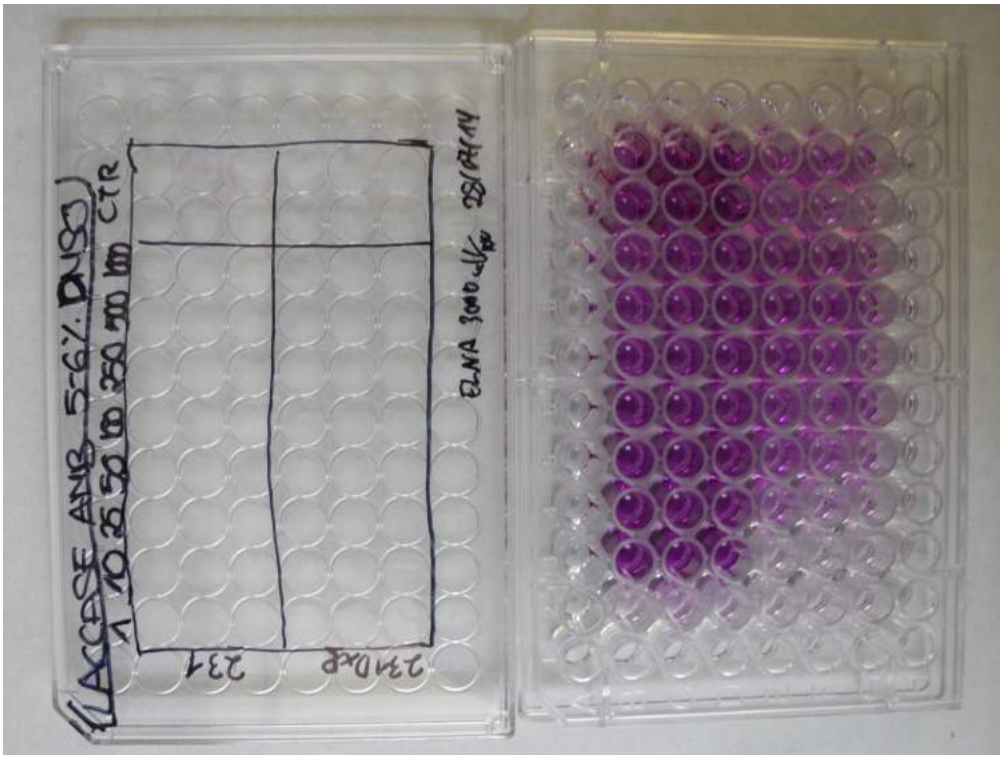
Cells treated with Laccase concentrated to 1.000 µg/ml

- **MICROWELL PLATES**






GLUCOSE ANB 5-6% DNS
1 10 25 50 100 250 500 1000 CTR
231
231 DxR
ELNA 3000 28/04/14


GLUCOSE ANB 5-6% DNS
1 10 25 50 100 250 500 1000 CTR
231
231DxR
ELNA 3000 28/04/14

ANNEX 7. Alternative Experiments with Different Solvents

These qualitative alternative experiments where carried out to check whether Laccase dissolved in other substances would precipitate less when mixed to other compounds than medium. The drug was not applied to the cells in those trials given that the goal was to observe the crystals and compare them with each other.

For the first trial of this set of experiments, Laccase was diluted in 10% DMSO. When observed in the microscope, it was seen that large brown crystals covered the well. They were spread all around and only a few blank spaces were present. Laccase in this condition would not work as a treatment.

For another trial, the oxidase was mixed in 40% DMSO. Even though in the well more spaces were observed, larger agglomerations of crystal Laccase coated the surface. The ratio crystal-surface was too great for Laccase in this form to be an anti-cancer cure.

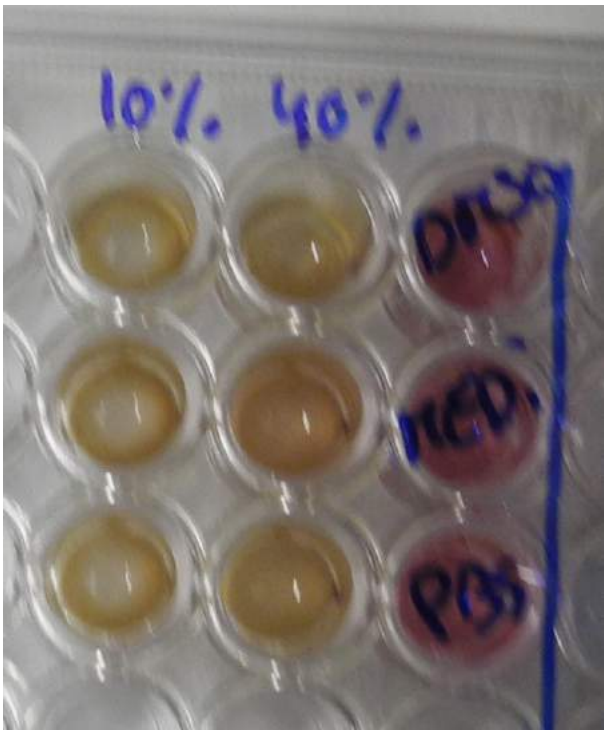
In another well, Laccase was diluted in 10% of medium. Some crystals were present but they only covered about 20% of the surface. However, the crystals were large. This condition would be a fine way to administer Laccase.

For the fourth trial, Laccase was diluted in 40% of medium. Medium-sized crystals were present, and they capped about half of the surface. There were too many crystals for this form to be used as an anti-cancer remedy.

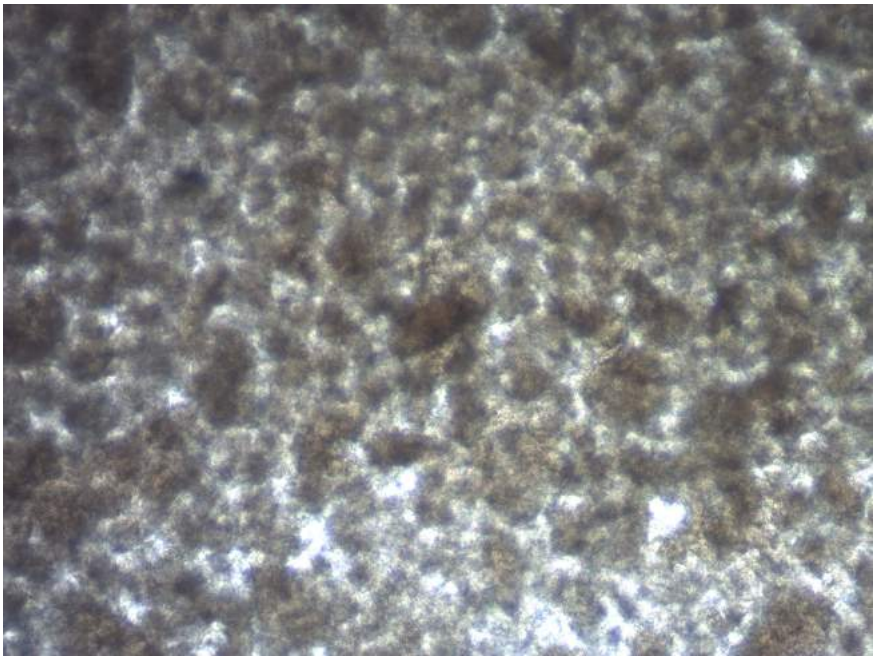
Furthermore, Laccase was diluted in 10% of PBS. Small crystals appeared covering most of the space, which shows that Laccase diluted this way would not work as an anticancer drug.

In contradiction with the previous trials, Laccase diluted in 40% of PBS created fewer crystals that coated about 40-50% of the well's surface. The crystals were larger and, for their clearer color, appeared to be less dense. Still, the concentration of crystals was too high for this condition to be used as a treatment.

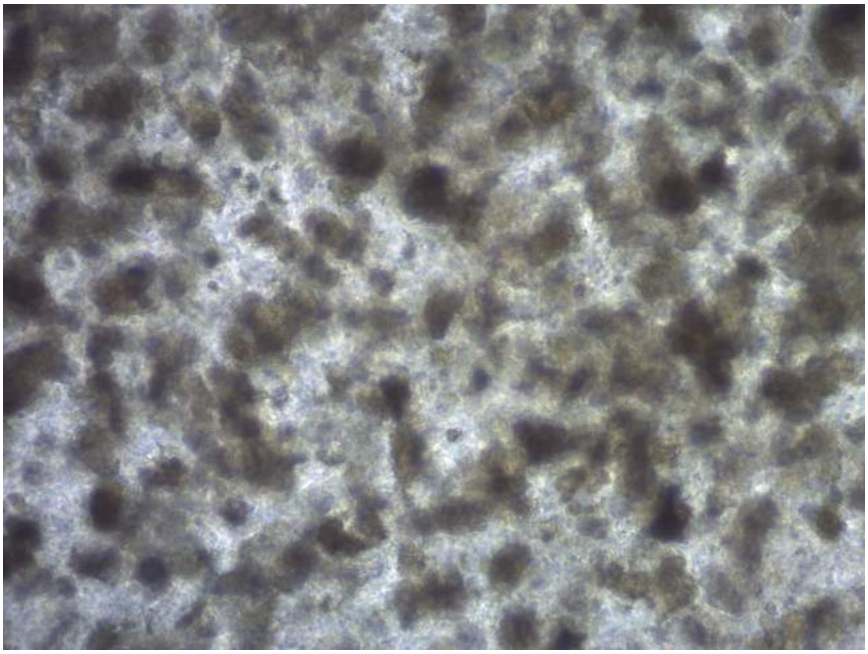
- **THE MICROWELL PLATE**



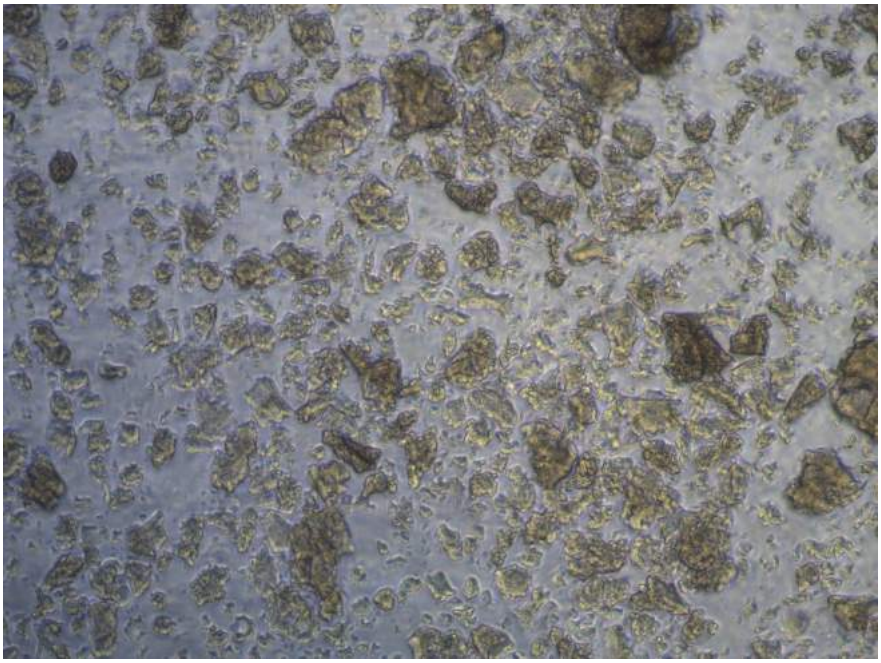
- **Laccase diluted in DMSO**



Laccase crystals when diluted in 10% of DMSO

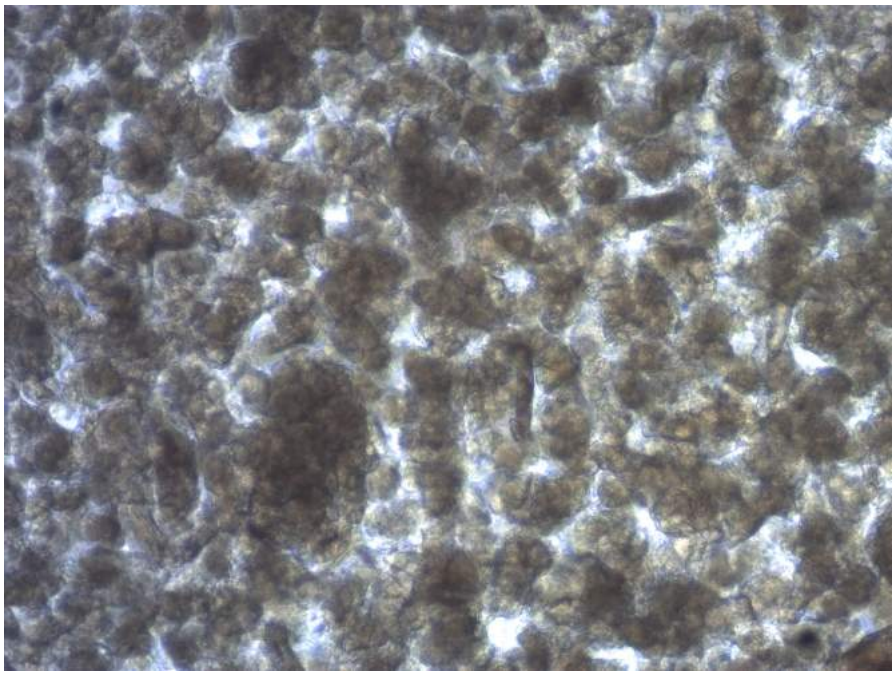


Laccase crystals when diluted in 40% of DMSO

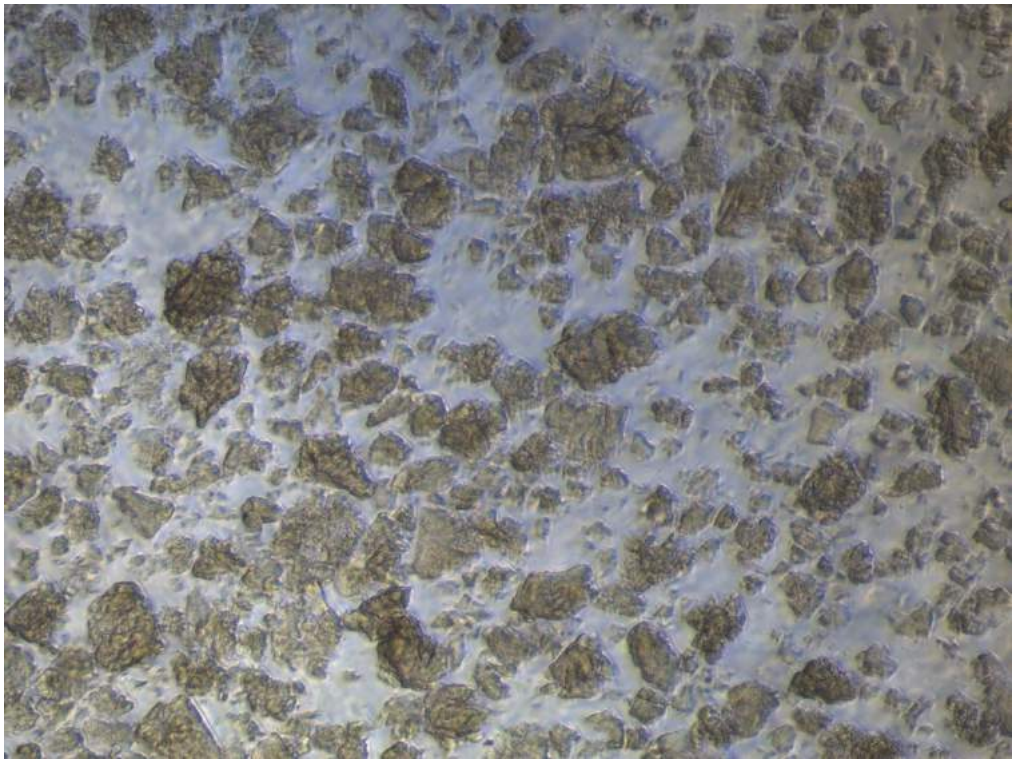


Laccase crystals when diluted with medium and DMSO (control replica)

- **Laccase diluted in Medium**

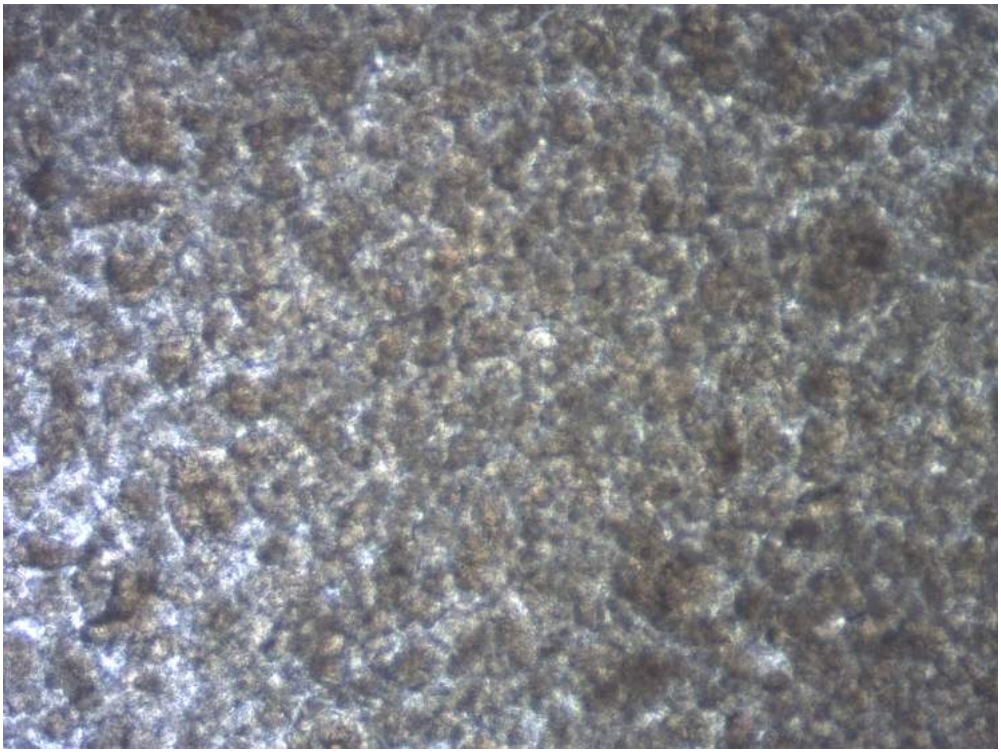


Laccase crystals when diluted in 40% of medium

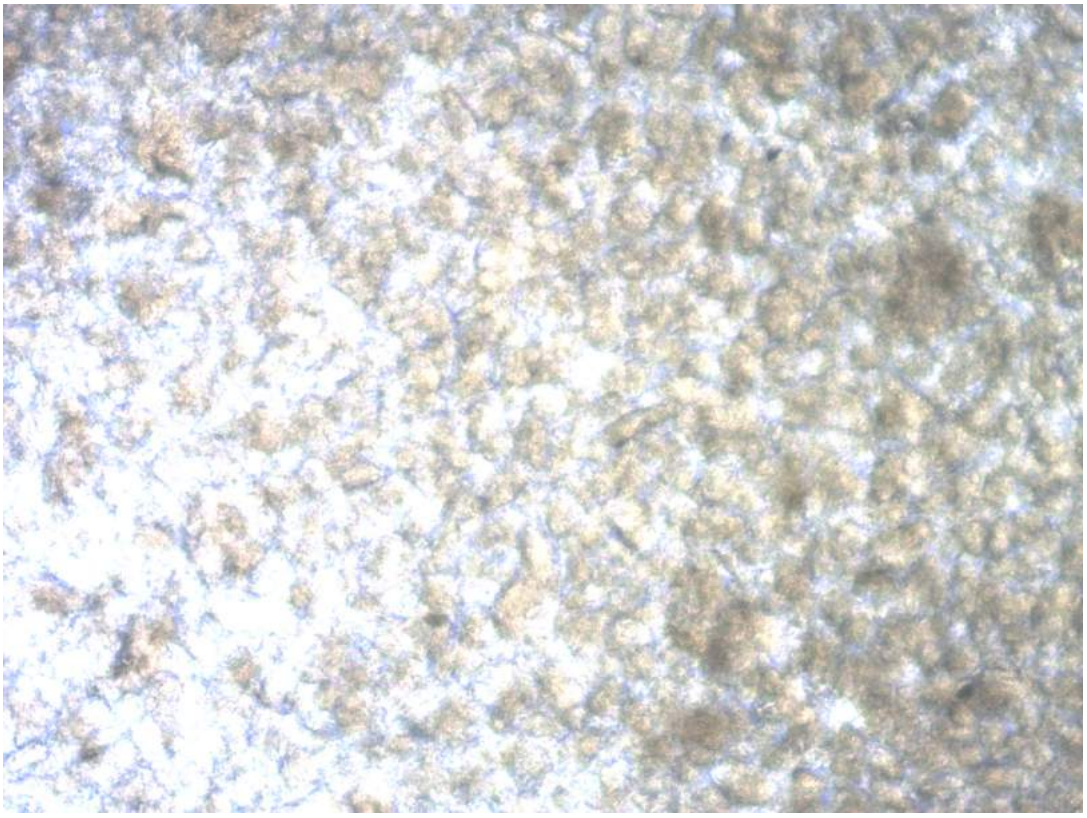


Laccase crystals when diluted with medium and medium (control replica)

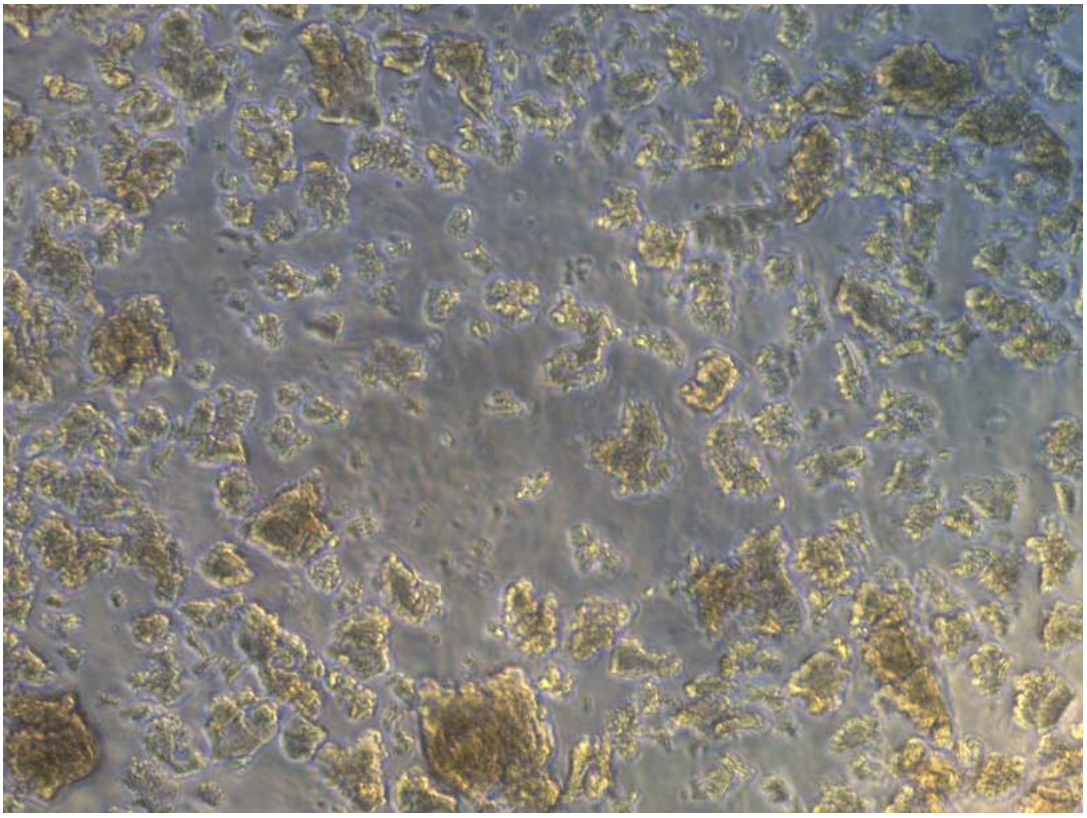
- **Laccase diluted in PBS**



Laccase crystals when diluted in 10% of PBS

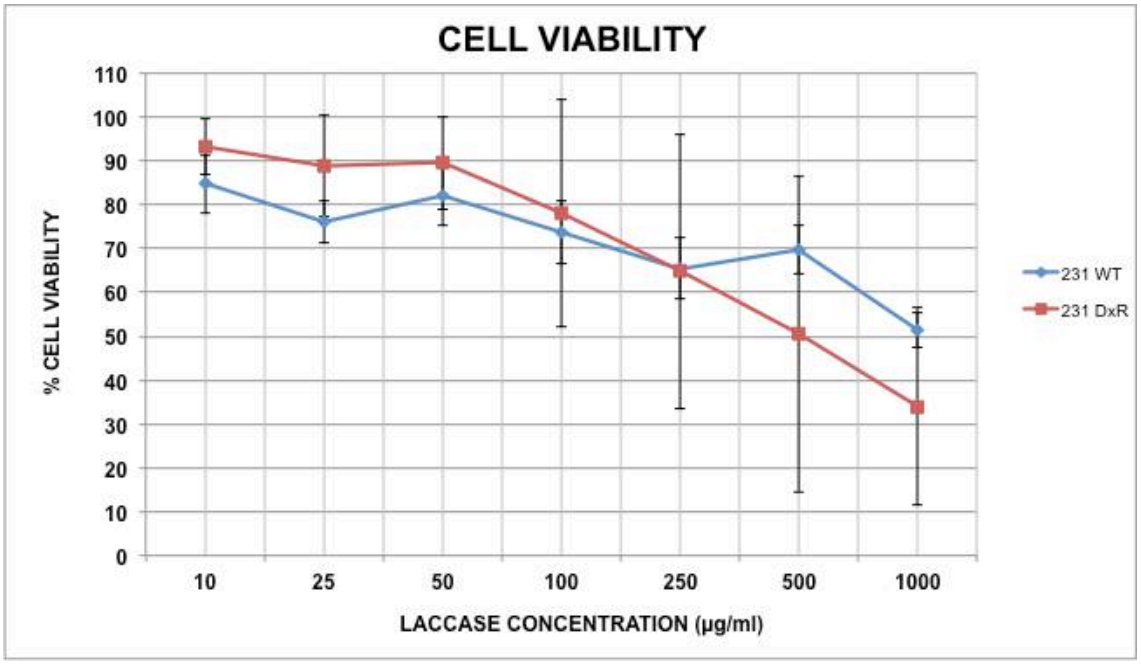


Laccase crystals when diluted in 40% of PBS

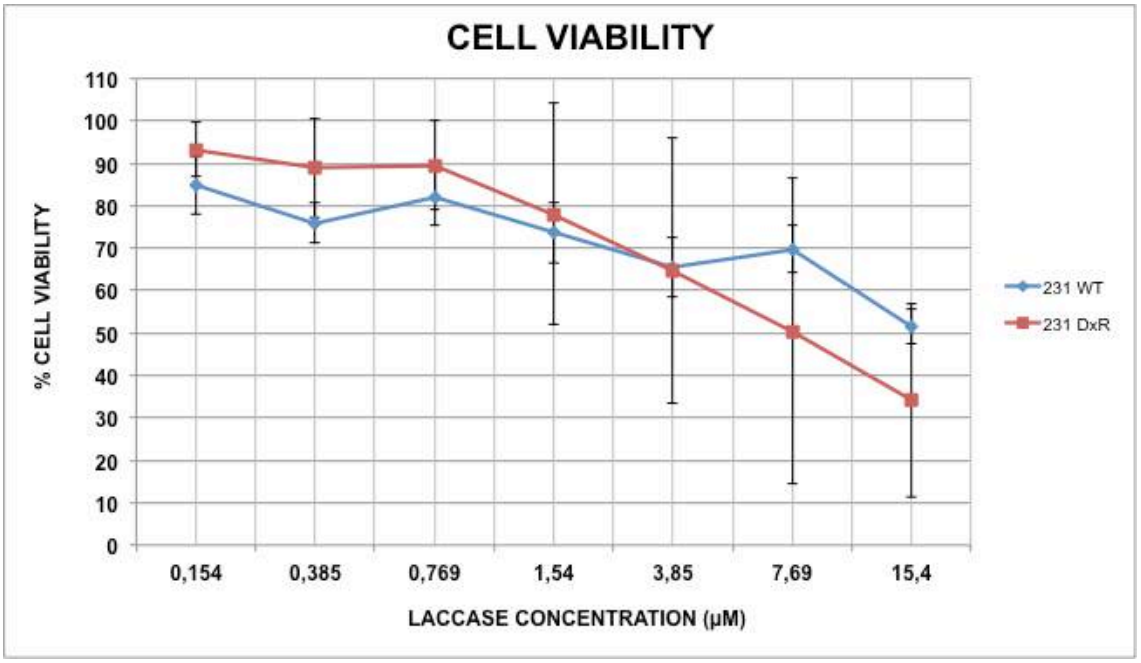


Laccase crystals when diluted with medium and PBS (control replica)

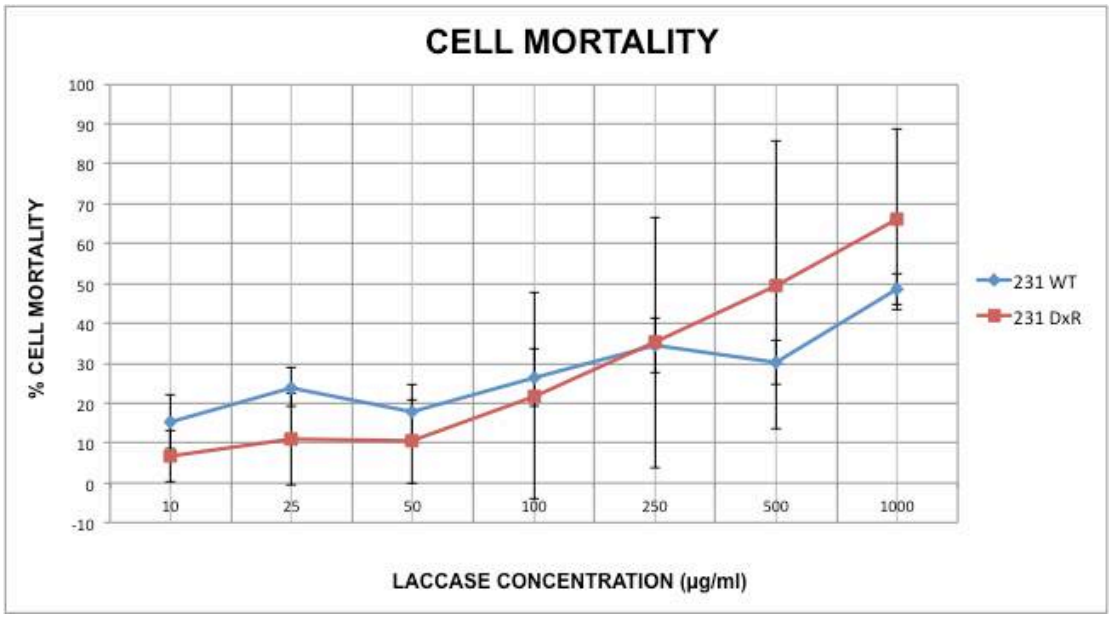
ANNEX 8. Overall Data Graphs



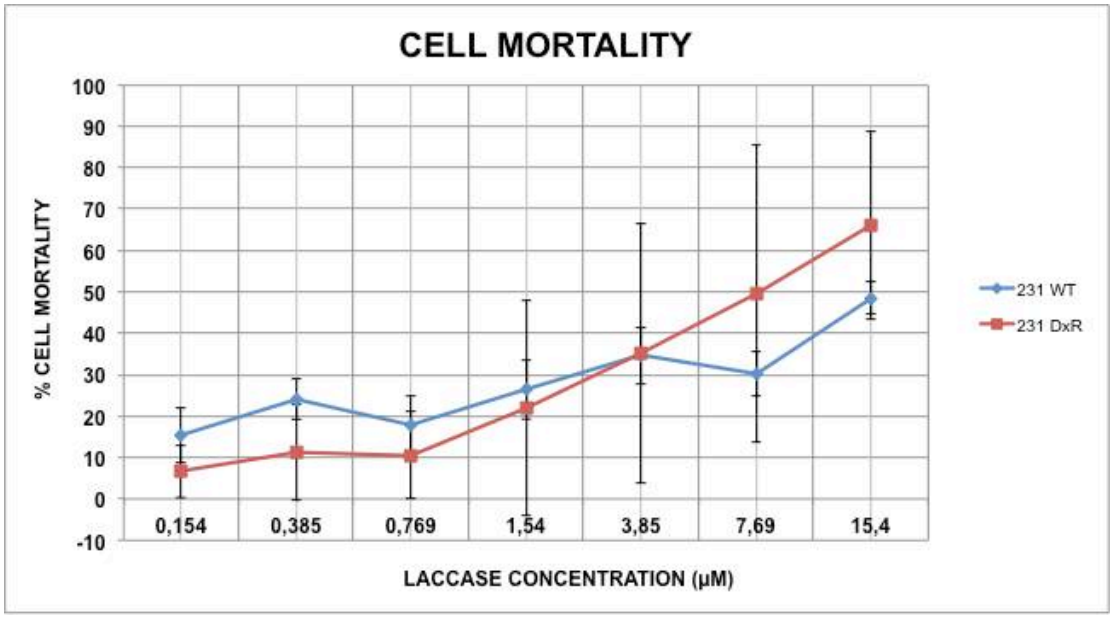
Cell viability based on Laccase concentrations in µg/ml



Cell viability based on Laccase concentrations in µM



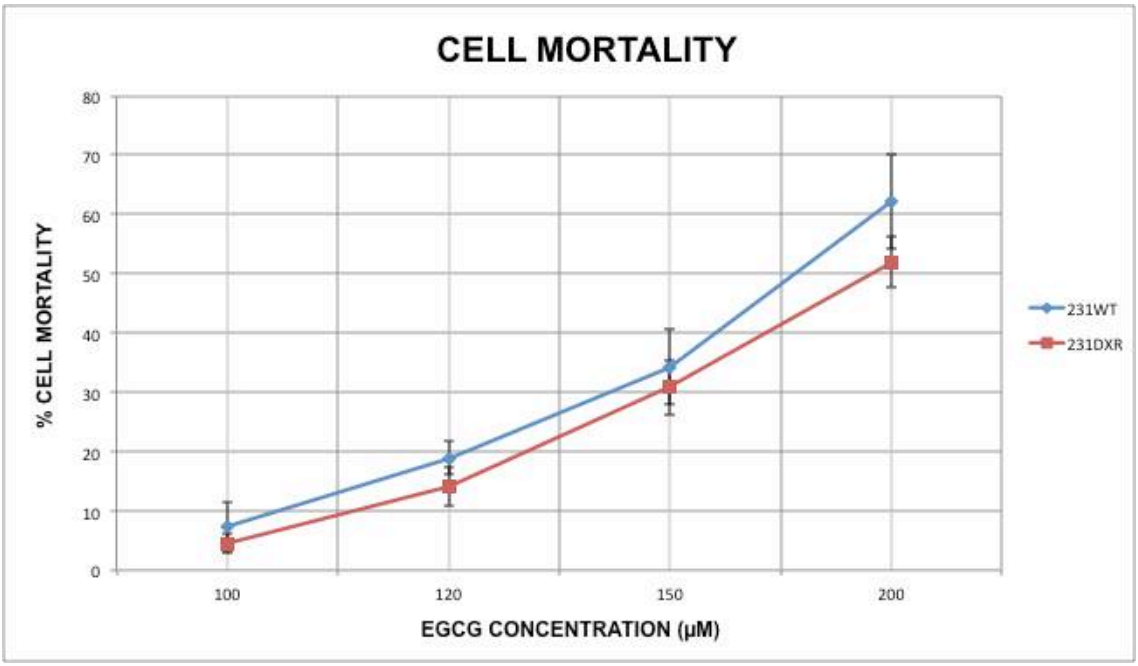
Cell mortality based on Laccase concentrations in µg/ml



Cell mortality based on Laccase concentrations in µM

	LACCASE (µg/ml)	LACCASE (µM)
IC50	> 500	15
IC30	175	2,7
IC50	500	7,7
IC30	185	1,5

Laccase Inhibitory Concentrations



Cell mortality based on EGCG concentrations in µM

ECGC (µM)				
	IC50	180	**IC50**	195
	IC30	145	**IC30**	150

EGCG Inhibitory Concentrations

ANNEX 9. Biological Therapies

There are seven types of biological therapies drugs: *monoclonal antibodies*, which recognize and attach to specific proteins produced by the cell; *cancer vaccines* that can be either used for preventing cancer or getting the immune system to recognize and attack carcinoma cells; *blood cell growth factors*, which stimulate the production of white blood cells that help the immune system; *angiogenics*, which inhibit the invasion of blood vessels by cancer (angiogenesis); *immunotherapy* that uses interferon and interleukin (substances made by cells a.k.a. cytokines) to boost the immune system and interfere with the way carcinoma cells multiply; *gene therapy*, which is still experimental but would use genes to manipulate the carcinoma cells; and *cancer growth blockers*, which include a variety of substances capable of inhibiting cells' growth factors.

ANNEX 10. Green-tea polyphenols and EGCG

• Polyphenols

Polyphenols are a class of micronutrients that contain phenol units and that are present in fruits, vegetables and other plant−derived products, in their cell vacuoles. They are known for their health-promoting effects in prevention and treatment of different illnesses such as cancers, cardiovascular and neurodegenerative diseases, which appear to be linked to their strong antioxidant activity. Polyphenols range from simple molecules, such as phenolic acids, to highly polymerized compounds. They are divided in Phenolic acids, Flavonoids, Lignans and Stilbenes [7].

Polyphenols have many distinct uses. For instance, pomegranate peel, which contains tannin polyphenol, is used to dye fabrics, and cashew nut shell liquid (CNSL) is an important phenolic raw material used for paints and wood preservatives. Furthermore, they play an important role in ecology and plant tissues. They release and suppress growth hormones, they create UV screens to protect against ionizing radiation and to provide coloration (plant pigments), they give sensory properties, they prevent microbial infections, and they signal molecules in ripening and other growth processes. High levels of polyphenols in some woods can explain their natural preservation.

As antioxidants, polyphenols control the presence of free radicals. As it has been appointed, free radicals are threatening because they start attacking normal molecules so as to gain an electron, which can alter cells. They

commonly begin chain reactions that lead to oxidative stress, which can develop into illnesses like arthritis, premature aging, edemas and swelling, artery hardening, and cancer. Antioxidants are molecules that give electrons to free radicals so they will not try to get it from stable molecules. They inhibit the oxidation reactions that the free radicals try to carry out and they break the chain reactions. Antioxidants are of highly importance because they prevent or delay cell damage. Some examples are Beta-carotene, Lutein, Lycopene, Selenium, and Vitamins A, C, and E.

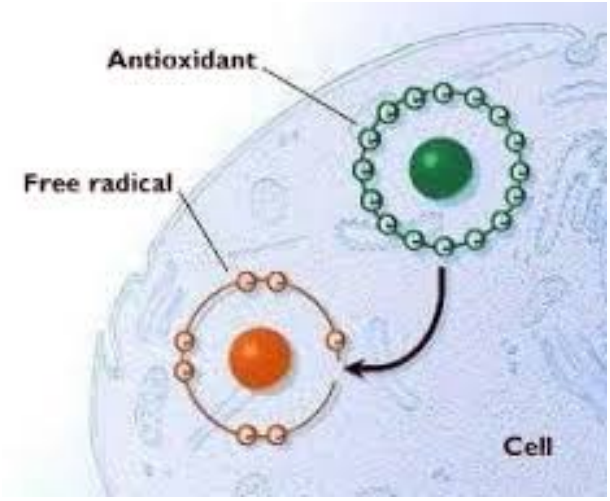


Figure 2.5. Antioxidants' mechanism of action.
Source: http://currentscienceperspectives.com/tag/antioxidants/

There exist many polyphenols such as *Phloretin* (apple), *Oleuropein* (olive), *Coraligin* (wine), *Lavanone* (citrus), *Isoflavone* (soya), *Quercetin* (plant products), and the one used in this study: *(−)−epigallocatechin−3−gallate* (EGCG). Some of the top foods and beverages that contain polyphenols are green teas, red wines, olives, spinaches, broccolis, apples, pomegranates, dark berries, basils, and oreganos [14].

- **EGCG**

(−)−epigallocatechin−3−gallate (EGCG) is a Green Tea Polyphenol. Its molecular formula is $C_{22}H_{18}O_{11}$ and its molecular weight 458.37 g/mol. EGCG can be found in many products such as tea (from *Camellia* plant), apple skin, plums, and onions. One cup of green tea contains approximately 300 to 400 mg of polyphenols, but only 8 to 12 percent of the entire cup is polyphenol substance [5].

This polyphenol has been a focus in the past years mainly because of its therapeutic potentials. For instance, EGCG can be beneficial in treating human immunodeficiency virus (HIV) infections, and it is regarded as a possible cure for AIDS. It is also used to improve mental alertness and thinking, to lose weight, to treat stomach disorders and osteoporosis (bone loss), to cure diseases of heart and blood vessels, to take care of skin damage, and to treat Parkinson's and Crohn's diseases. EGCG is able to cure the previous disorders because of its high levels of antioxidants and its 2-4% of caffeine [13].

Evidence from studies by Oliveras (2012), Colomer et al. (2009), and Braicu et al. (2013) captures this polyphenol is a cancer inhibitor and preventer. EGCG inhibits receptors and multiple downstream signaling pathways in human head and neck, breast, and colon cancer cell lines. It decreases the proteins in carcinoma cells and suppresses FASN (Fatty Acid Synthase, a trigger to cancer formation) activity [7]. Therefore, it is a great cancer preventer and inhibitor.