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Biocompatibility of electrospun polymer nanofibers (EPNs) with NIH 3T3 cell line and interaction of quercetin with different flavonoids: Induction of phase II enzymes in vitro cancer cell lines

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BIOCOMPATIBILITY OF ELECTROSPUN POLYMER NANOFIBERS (EPNs) WITH NIH 3T3 CELL LINE AND INTERACTION OF QUERCETIN WITH DIFERENT FLAVONOIDS: INDUCTION OF PHASE II ENZYMES *IN VITRO* CANCER CELL LINES

A Thesis

by

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ABSTRACT

Benavides, Brenda S., Biocompatibility of Electrospun Polymer Nanofibers (EPNs) with NIH 3T3 Cell Line and Interaction of Quercetin with Different Flavonoids: Induction of Phase II Enzymes *in vitro* Cancer Cell Lines. Master of Science (MS), May, 2011, 110 pp., 24 tables, 32 figures, references, 37 titles.

Part I: In this study Electrospun nanofibers, Elasthane™ 55D, Elasthane™ 75D, Biospan®, Hydrothane™ and Lycra® were evaluated for their biocompatibility to be artificial tissue substitutes using NIH-3T3 fibroblast cells. A colorimetric based MTT dye reduction assay demonstrated that EPNs did not stop cell growth *in vitro*. Additionally, fluorescence confocal microscope images verified that cell adhesion occurred in the nanofibers, and cell growth on the EPNs over two weeks period was confirmed by DNA quantification.

Part II: This section of the thesis is focused on evaluation of chemopreventive properties of various phytochemicals by induction of Phase II enzymes. The interaction of Quercetin at an optimum concentration in combination with different flavonoids (Hesperedin, Ginger, Resveratrol and β-Carotene), were used to determine the induction of Phase II enzymes *in vitro* cancer cell lines; caffeine was incorporated in the experimental groups to assess its protective properties on the induction of phase-II enzymes.

DEDICATION

The conclusion of my master degree studies would not have been possible without the love and support of my husband Carlos R. Benavides; he is the light of my life. Carlos is that supportive friend who helped me when I was stressed due to a final test, presentation or the construction of this paper; he was a mentor who helped me throughout the writing of my thesis. He gave his love, patience, everything and I'm thankful to God to have Carlos as my life partner. Thanks to my parents, Ruben Ochoa Martinez and Modesta Ramos Delgado for their unconditional love; for giving me the tools, guidance and for being role models in my life. Thanks to my sisters, Any Ochoa de Guerrero and Ale Ochoa de Navarro, they have been always there for me, as I've always been there for them; I love you Girls. My love to my nephew and godson Ian; I want to let him know that in this life we need to cultivate our minds in order to be better persons. Finally, I would like to express my appreciation to my in-laws, Carlos R. Benavides H. and Magda Herrera for their words of encouragement.

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Thanks to my dissertation committee members: Dr.Aijie Han, Dr. Joanna Rampersad and Dr. Shivanni Maffi for their comments on my dissertation that helped me ensure the quality of my intellectual work. My gratitude to Dr. Maffi, for sharing her time to help me with the confocal microscope, without her knowledge, I could not have achieved my results.

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PART I

BIOCOMPATIBILITY OF ELECTROSPUN POLYMER NANOFIBERS (EPNs) WITH NIH 3T3 CELL LINE

CHAPTER I

INTRODUCTION

It is estimated that over two million people visit a physician for musculoskeletal injuries each year. Over 43% of these visits are for soft tissue injuries that occur to ligaments (connective tissues that link bone to bone at a joint) and tendons (connective tissues that link muscle to bone, or joint capsules). Some of the most commonly injured ligaments and tendons are the anterior cruciate (ACL), medial collateral ligaments (MCL), the Achilles tendon, and rotator cuff tendons (Guilak, et al. 2004, Kutz, 2003).

More than 200,000 ACL reconstruction procedures are performed in the United States annually (J. Y. Lim, et al. 2004), but injuries to tendons and ligaments are prevalent and result in a significant decrease in the quality of a patient's life. Tissue-engineering strategies hold promise as an alternative to current treatments for these injuries, which often fail to fully restore proper joint biomechanics and produce significant donor site morbidity (Guilak, et al. 2004).

Sometimes soft tissue structures must be replaced rather than repaired to avoid any rupture after the reconstruction; for example, the ACL heals incompletely when surgically repaired, producing a risk of ligament and bone fracture, and surgical intervention may be needed (Kutz 2003). The approaches of ACL replacement or reconstruction include the use of autografts, allografts and prostheses. Autografts allow the patient's own tendons to be used, the gold standard is the bone-patellar tendon-bone (BPTB), but there is a risk of patellar ruptures,

patellar tendonitis, and it is associated with an increased incidence of anterior knee pain (Frenot, et al. 2003). There are other options, for example the use of hamstring autograft, which is increasing in popularity, and the quadriceps tendon autograft which is less popular, but has shown excellent clinical results with low morbidity. No graft has been able to provide a faster recovery, however, patellar tendon autografts are preferable for high-performance athletes, while hamstring autografts and allografts have some relative advantages for lower-demand individuals (West and Harner, 2005). The specific concerns related to allograft are tissue availability, sterilization, graft cost, delayed graft incorporation, disease transmission, and long term graft strength, but improved sterilization techniques have led to an increase in safety and availability of allograft. Although ligament prostheses gained popularity in the 1980's, the materials that formed these prostheses were stiff with low ultimate tensile strengths and poor abrasion properties. Further, these prostheses showed cartilage destruction, and limited the popularity of synthetic ligaments in the late 1980's and 1990's, and after that, no current indications exist for synthetic ligaments (Holroyd and Fern 2009). The ideal graft for the anterior cruciate ligament reconstruction should have structural and biomechanical properties similar to those of the native ligament, allowing secure fixation, rapid biologic incorporation, and limiting donor site morbidity. Many options have been clinically successful as the use autografts, but the ideal graft remains controversial (West and Harner, 2005).

A potential alternative material for ACL replacement could be an artificially engineered ligament that may be formed by electrospun nanofibers. These nanofibers can be formed under the appropriate combination of biochemical and mechanical conditions that can induce synthesis of a ligament-like extracellular matrix (ECM). Central to this strategy is the achievement of an oriented extracellular matrix (ECM), since poor mechanical properties of healed ligament tissue

are associated with a disorganized ECM (Bashur et al. 2006). Therefore, the nanofiber should not only provide extensive surface area for cell attachment and matrix deposition, but it should induce cell orientation, which will guide the formation of an oriented ECM. One approach for designing exogenous ECMs for artificial ligament engineering is to mimic the functions of the ECM molecules naturally found in tissues.

One strategy to produce nanofibers is the electrospun method, which involves a polymer solution that is ejected to a target using an electric field, providing features around 3nm to 5µm in diameter. Features such as diameter and orientation on the electrospun method depend in electrical potential, distance, needle diameter, and solution concentration. (Li, et al W. 2002).

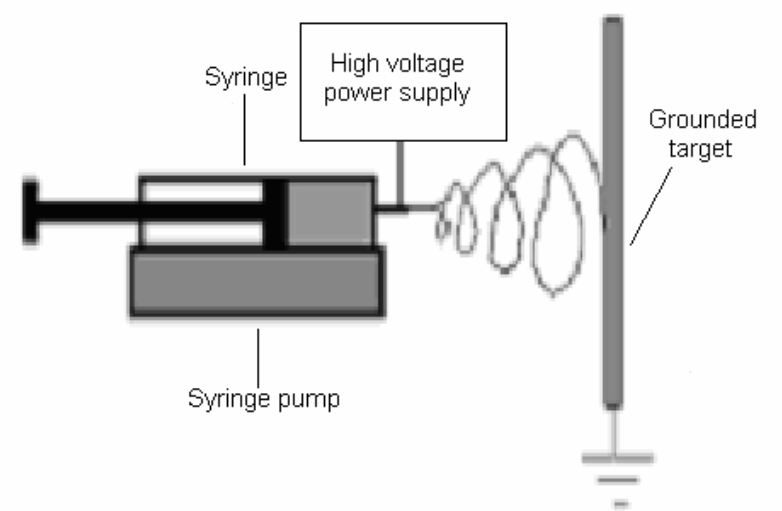


Figure 1.1: Electrospinning setup

One of the nanofibers features that plays an important role on cell adhesion are the fiber's diameters, because as the literature suggests, diameters lower than 100 nm are believed to allow adhesion of proteins such as fibronectin, laminin, and/or vitronectin to the surface of the nanofiber-textured layer, and to provide conformation for these proteins that better exposes

amino acid sequences such as RGD and YGSIR which enhance endothelial cell binding (Kutz, 2003).

As the literature suggests, polyurethanes are one of the most biocompatible and blood-compatible materials recognized today because of their properties, such as durability, elasticity, fatigue resistance and acceptance or tolerance in the body during the healing process. In this study five different nanofibers formed from Elasthane™ 55D, Elasthane™ 75D, Biospan®, Hydrothane™ and Lycra® were obtained. From the polymers chosen to form the EPNs, Elasthane™ and Biospan® are currently used in many medical devices ranging from catheters to total artificial heart. The proposed structures of the chosen polymers are showed in Figure 1.2 and 1.3. Hydrothane™ proposed structure is not shown because there is no information about the structure of this polymer.

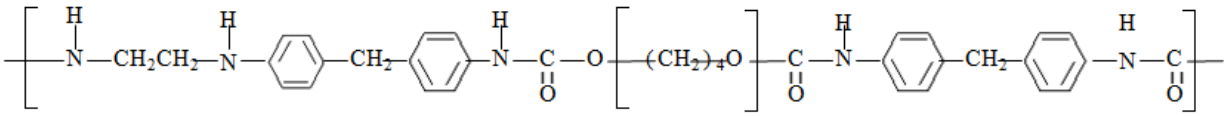


Figure 1.2: Proposed Structure of Elasthane™

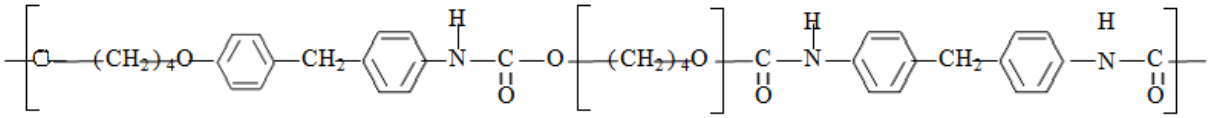


Figure 1.3: Proposed Structures of Biospan® and Lycra®

The use of nanofibers in tissue restoration is expected to result in an efficient ligament that will provide a large surface area, offered by nanofibers made from different polymers that

could be used as biocompatible prostheses, cosmetics, face masks, bone substitutes, artificial blood vessels, and valves, and drug delivery applications (Frenot, et al. 2003).

Artificial tissue-engineering strategies hold promise as an alternative to current treatments for musculoskeletal injuries, which often fail to fully restore proper joint biomechanics and produce significant donor site morbidity (Guilak, et al. 2004). This is one of the reasons to study these EPNs that could be biocompatible and be a promising candidate for the use of the reconstruction of ligament and tendons injuries, as in ACL reconstruction.

One of the objectives of this research was to work with micro- and nano-electrospun polymer nanofibers (EPNs). Because EPNs are not approved by the FDA, EPNs were seeded in NIH 3T3 cell line to evaluate biocompatibility using these fiber structures as potential artificial ligaments and tendons.

The major objectives of this study were to assess the biocompatibility of the micro- and nano-electrospun polymer nanofibers (EPNs). The biocompatibility evaluations of five different EPNs were carried out, since the EPNs with defined properties are not commercially available, the nanofibers were synthesized from Elasthane™ 55D, Elasthane™ 75D, Biospan®, Hydrothane™ and Lycra® by other individuals which I have acquired for this work. The EPNs were incubated with NIH 3T3 cell line (fibroblasts cells) to evaluate their biocompatibility for possible artificial ligaments and tendons substitutes. A colorimetric assay was performed to demonstrate the cytotoxicity of EPNs to the fibroblast cells. After establishing that the EPNs did not stop the cell growth, DNA quantification, cell morphology and proliferation analysis were performed to confirm biocompatibility in the NIH 3T3 cell line.

CHAPTER II

MATERIALS AND METHODS

2.1 Propagation of NIH 3T3 Cell Line

The cell culture was maintained according to the vendor's (ATCC, Manassas, VA) protocols as well as growth media.

Dulbecco's Modified Eagle's Medium (DMEM, ATCC) supplemented with a 10% calf serum (Invitrogen, Grand Island, NY), 7.5% sodium bicarbonate (Gibco™; Grand Island, NY) and a mixture of 1% penicillin/streptomycin (Gibco™) were used to propagate the NIH 3T3 cell line.

The cells from the original vial were transferred into a cell culture flask with a 75cm^2 growth area. The flask was placed in an incubator at 37°C with 5.2% CO_2. As ATCC protocols suggest, the flask was left in the incubator until 80% of confluency was obtained, the media was decanted and 7mL of 0.25% trypsin (Gibco™) was added to detach the cells from the flask. After approximately 7 minutes, the detached cells were removed from the flask, and transferred into a 15 mL conical tube and centrifuged at 200xg for 5 minutes in order to obtain a cell pellet. Trypsin was decanted and 7mL of DMEM was added to the centrifuge tube in order to break the cell pellet which was achieved by the repeated action of drawing up and dispensing liquid using the pipettor. An aliquot of 250µL of the suspended cells was transferred into each of the 2

culture flasks with 75cm^2 growth area, each previously filled with 35mL of DMEM. All flasks were placed in the incubator set at 37°C, 5.2% CO_2, and allowed to proliferate until the cells reached 80% confluency.

2.2 Preparation and Sterilization of the EPNs

EPNs were cut with a 6mm disposable biopsy puncher (Sklar Instruments; West Chester, PA) in order to have a constant diameter sample. All puncher cut EPN samples were sterilized by placing them in Petri dishes, and irradiating with UV light (Sanki Denki, UVC G30T8) for 12 hours (Bashur et al. 2006).

2.3 Cytotoxicity determination based on MTT dye reduction

Cytotoxicity is the cell-killing property of a chemical compound (such as a food, cosmetic, or pharmaceutical), or a mediator cell (such as a cytotoxic T cell) independent from the mechanisms of death (Roche, 3rd Edition).

The MTT assay is widely used to evaluate the cytotoxicity, particularly in the course of new drug development. In this study, an MTT assay was performed on the EPNs to evaluate two things:

1) If the EPNs kill the cells when incubated with fibroblast cells after 1 day incubation time and to select an appropriate cell density to future work.

2) To measure the effect on the cell growth when fibroblast cells were exposed to the EPNs in 1, 3 and 7 days.

In both determinations, cells were exposed to the nanofibers, and surviving cells were determined indirectly by MTT dye reduction. MTT is a yellow water soluble tetrazolium dye that is reduced by metabolically active cells (not by dead cells) to a purple formazan product that is insoluble in aqueous solutions. The amount of MTT-formazan produced can be determined spectrophotometrically (Brown & Boger-Brown, 1999).

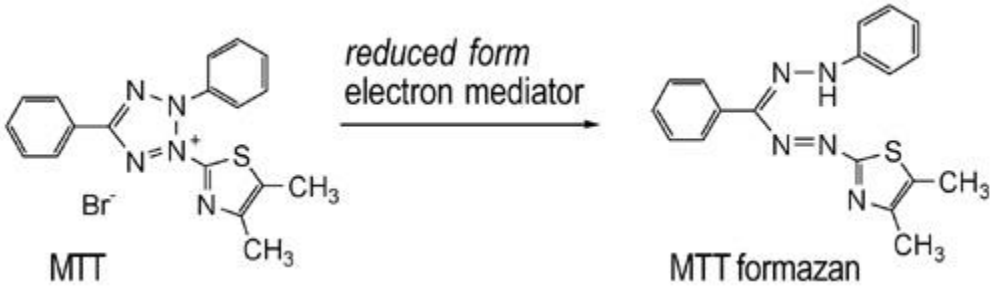


Fig 2.1: MTT reaction

2.3.1 One Day-EPN's cytotoxicity and cell density selection

The MTT assay was performed to evaluate the EPNs cytotoxicity on fibroblast cells after 24 hours of the incubation with EPNs. This evaluation process was started when cells reaches 80% of confluency; the cells were harvested by decanting the media and adding 7mL of 0.25% trypsin. The culture flasks with trypsin were incubated for 7 minutes at 37^0C with 5.2% CO_2. The cells were centrifuged to form a pellet of cells followed by decantation of trypsin. The pelleted cells were suspended in 7ml of DMEM. An aliquot of 10 µL (suspended solution) was combined with 10 µL of 0.4% Trypan Blue dye (Invitrogen), and cell count was performed using an automated cell counter (Countess™ automated cell counter; Invitrogen). The cell-

resuspension solution was diluted to achieve 1 $X10^4$-1 $X10^5$cells/ml density using DMEM. The diluted cells were transferred to the 96 wells microplate (Corning, NY), along with a blank (only DMEM). Ninety six wells microplates were placed in an incubator at 37^0C with and 5.2% CO_2. After 24 hours, the EPNs were added to each well in the microplate and incubated at 37^0C with 5.2% CO_2. Following 24 hours incubation, the cytotoxicity assay was performed. The MTT reagent (Sigma, St. Louis, MO) was prepared at 5mg/mL in phosphate buffer saline (PBS). Before adding the MTT reagent, the DMEM on the microplate was replaced with DMEM without phenol red to avoid interference on the spectrophotometric reading, 40µL of MTT reagent were added to each well in the microplate and incubated at 37^0C and 5.2% CO_2. After 4 hours, the microplate was centrifuged at 200xg for 10 min in order to pellet cells in each well. After centrifugation, the media was aspirated without disturbing the cell pellet. 160 µL of DMSO (≥99.9%, Sigma-Aldrich) and 20 µL of glycine buffer (0.1M glycine from Bio-Rad, plus 0.1M NaCl (Sigma-Aldrich) equilibrated to pH 10.5 with 0.1N NaOH) were added to each well. Microplates were mixed for 10 minutes, and read spectrophotometrically at 595nm in a Bio-Rad microplate reader (Brown & Boger-Brown, 1999).

2.3.2 MTT Assay to evaluate cell proliferation in 1, 3 and 7 days

The MTT assay was performed to evaluate the cell proliferation of NIH 3T3 cells after 1, 3 and 7 days of incubation with the EPNs. This assay was performed when 80% of the cell confluency was obtained. The cell layer was harvested, and cells were collected in a 15mL conical centrifuge tube. The cell pellet was formed after 7 minutes of centrifugation at 200xg. Trypsin was decanted, and 7mL of DMEM was added to re-suspend the cell pellet. Cell count

was carried out by mixing 10µL of the homogenized solution and 10 µL of 0.4% Trypan Blue. After the cell count, the re-suspended cells were diluted to 25,000 cells/mL. 160µL of the 25,000 cell/mL solution were added to each experimental microplate. Microplates were incubated at 37°C with a humidified atmosphere of 5.2% CO_2 for 24 hours. After 24 hours, the media was taken out and 80µL of fresh media was added on the wells. Sterilized nanofibers were added to the 96 well microplates in triplicates and 80 µL of fresh media was added to each experimental well in order to achieve a final volume of 160µL. In each determination, a cell control in triplicate was added to evaluate cell growth by direct comparison. The microplates were incubated at 37°C with 5.2% of CO_2 for 1, 3, and 7 days. Following these incubation periods, media from the microplates was replaced with 160µL of fresh media (DMEM) without phenol red and 40µL of the MTT reagent at 5mg/mL in PBS was added to each well, and the microplates were incubated. After 4 hours of incubation, the microplates were centrifuged to pellet cells and removed all media. One hundred sixty µL of DMSO and 20µL of glycine buffer (0.1M glycine from, plus 0.1M NaCl and equilibrated to pH 10.5 with 0.1N NaOH) was added. The microplates were placed in a shaker for five minutes to let the formazan crystals dissolve followed by removal of EPNs from each well. Subsequently, the microplates were read at 595nm. Analysis of variance (ANOVA) was done to MTT assay raw data in order to evaluate cell growth after 1, 3 and 7 days of EPNs exposure with the fibroblast cells.

2.4 Cell seeding to assess biological compatibility

Cell culture methods have been used to evaluate the biological compatibility of materials for more than two decades (North, 1986). By definition, biocompatibility is "The ability of a

material to perform with an appropriate host response in a specific application"(Williams, 1999), this definition is also referred in the European Society for Biomaterials, another definition for biocompatibility is defined by Dorland Medical as "The quality of not having toxic or injurious effects on biological systems".

2.5 Cell morphology/ Cell proliferation

Cell morphology was determined by image analysis of cells stained by Calcein-AM. Calcein-AM (Invitrogen) is transported into the cytoplasm of live cells; where intracellular esterases cleave the acetoxymethyl (AM) ester group. This transformation leaves the fluorescently active calcein, which emits a green color when the sample has live cells. It is important to note that dying cells leak Calcein-AM out, thus cells lose Calcein-AM overtime and surroundings become brighter. Calcein-AM analysis was performed to observe the morphology of the fibroblast cells and to demostrate that cell density increased after 1, 2, 3, 7 and 14 days of incubation the EPNs with NIH 3T3 cells.

2.5.1 Determination of Cell morphology/ Cell proliferation for up to 14 days

For the cell morphology and cell density analysis, the nanofibers were sterilized (as described in section 2.2) and incubated for 1 hour at room temperature with 2mL of a 10 µg/mL fibronectin (Invitrogen) in phosphate buffered saline (PBS; 10 mM Phosphate buffer from Sigma, 150 mM NaCl from Sigma, pH 7.0).

A culture flask with 80% cell confluency was harvested and cells were collected in 15mL conical centrifuge tubes. After centrifugation, trypsin was decanted, and a solution was formed with 7mL of DMEM. Cell count was performed to form a 25,000 cell/mL solution. The treated nanofibers were added in 12 wells microplates (Corning), and glass cylinders (Pyrex-cloning cylinders; Corning Inc, NY) were put in each experimental well to hold the nanofiber in the bottom of the well. A 25,000 cell/mL solution was added to each experimental well and microplates were put in the incubator for 4 hours at 37°C and 5.2% CO_2. After 4 hours, the media was taken out, and the glass cylinders were removed. Fresh DMEM media was added to each experimental well, and microplates were put in the incubator at 37°C and 5.2% CO_2 for 1, 2, 3, 7 and 14 days for cell morphology, and cell proliferation analysis. For long periods the media was completely changed every two days for fresh media.

Cell morphology and cell density determinations were done through staining the nanofibers with 1.5μg/mL calcein-AM. Before staining, the nanofibers were washed twice with PBS in order to remove all cells that were not attached to the nanofiber. The washed nanofibers were placed in a new 12 wells microplate followed by the addition of 2 mL of 1.5 μg/mL calcein-AM in DMEM without phenol red, and allowed to incubate at 37°C for 30 min. Scanned images were obtained using a confocal microscope (Olympus FluoView® 1000 Ver. 2.01) with a 20x objective were continuous images across the x, y and z planes of the nanofiber were captured and analyzed with FluoView® Software in order to observe cell morphology and cell density in the EPNs.

2.6 DNA quantification to demonstrate cell proliferation

DNA quantification was done to demonstrate cell proliferation by incubating the nanofibers (triplicate) the nanofibers with a solution of 25,000 cell/mL solution. Glass cylinders were used to hold the nanofibers on the bottom of the well. After 4 hours of incubation, the 25,000 cell/mL solution was changed for fresh DMEM media and the microplates were incubated for 3, 7, and 14 days at 37°C and 5.2% of CO_2.

Cell proliferation was confirmed determining the DNA of cells attached to the EPNs that were incubated for 3, 7, and 14 days. Before the fluorometric analysis, cell layers were rinsed twice with PBS, and collected in glass tubes, then 1mL of 10mM EDTA (Bio-Rad; Richmond, CA) at pH = 12.3 was added to each tube. The cells were sonicated on ice to avoid the denaturation of DNA. A 0.2mL aliquot of 1M KH_2PO_4 was added to all tubes after sonication to neutralize pH. Each sample was sonicated three times at 100 amps for 3 seconds.

DNA standards were prepared by adding known volumes of DNA solution (0-375 µL) of a 200 µg/mL to varying volumes of EDTA (pH=12.3), followed by the addition of 1M KH_2PO_4 to neutralize pH. Aliquots of 35µL DNA standard and sonicated EPN were added to 140 µL of a 100 ng/mL solution of Hoechst 33258 dye (Sigma) in 100mM NaCl, and 10mM Tris buffer. Fluorescence measurements were made in triplicate in a LS 55 Luminescence Spectrometer (Excitation/emission 346nm/460nm). The standards curve was prepared using the fluorescence obtained at different concentrations of DNA. The concentration of DNA in the cells incubated with the nanofiber was determinate with the standard curve. Analysis of variance (ANOVA) was performed to evaluate the raw data in order to obtain the statistical results.

CHAPTER III

RESULTS AND DISCUSSION

Different assays were performed to determine cytotoxicity, cell morphology and cell proliferation, and DNA quantification of cells growing on the EPNs. First, a colorimetric assay based on MTT reduction was done on NIH 3T3 cells exposed to the EPNs for one day to establish that the electrospun polymer nanofibers did not extinguish or stop NIH 3T3 cell growth. The cells could die as a result of potential residual solvent or inappropriate sterilization. After establishing the viability of the NIH 3T3 cells in presence of EPNs, the cytotoxicity assay was carried out for 1, 3, and 7 days to evaluate of the long term exposure of EPNs on cell viability. The results were compared to NIH 3T3 cells without EPN's exposure (cell control); NIH 3T3 cells with and without EPNs exposure were carried out in triplicate and the average of each triplicate was compared with the average of the cell control in order to obtain data with statistical significance. In addition, EPNs were seeded without NIH 3T3 cells in order to obtain the absorbance of the nanofiber, followed by their subtraction from the corresponding EPN seeded with NIH 3T3.

Confocal microscope were use to evaluate NIH 3T3 morphology and cell proliferation in presence of the EPNs in 1, 2, 3, 7, and 14 days. From these images the attachment of the

fibroblast cells was visible in all EPNs. However, variations in cell number between the EPNs were observed throughout days (1, 2, 3, 7 and 14). In most cases, cell morphology of the cells attached to the EPNs showed unstressed cells, but some EPNs showed stressed cells. Nevertheless, images from cell proliferation determination on different days confirmed that all EPNs showed an increase in cell number up to 14 days of incubation. There was a higher cell growth in Elasthane™ 55D, Elasthane™ 75D and Biospan® nanofibers compared to Hydrothane™ and Lycra® nanofibers.

DNA quantification was performing using Hoechst 33258 on cell growth on the EPNs for 3, 7 and 14 days to further confirm cell attachment. Results from each EPN were extrapolated to the standard curve in order to obtain the DNA content in each sample. Results by DNA quantitation did not completely correlated with the results of proliferation obtained by the confocal images suggesting the possibility of imperfect sonication process which might have resulted in uncompleted DNA extraction from cells attached to the EPNs.

3.1 Day One- EPNs cytotoxicity

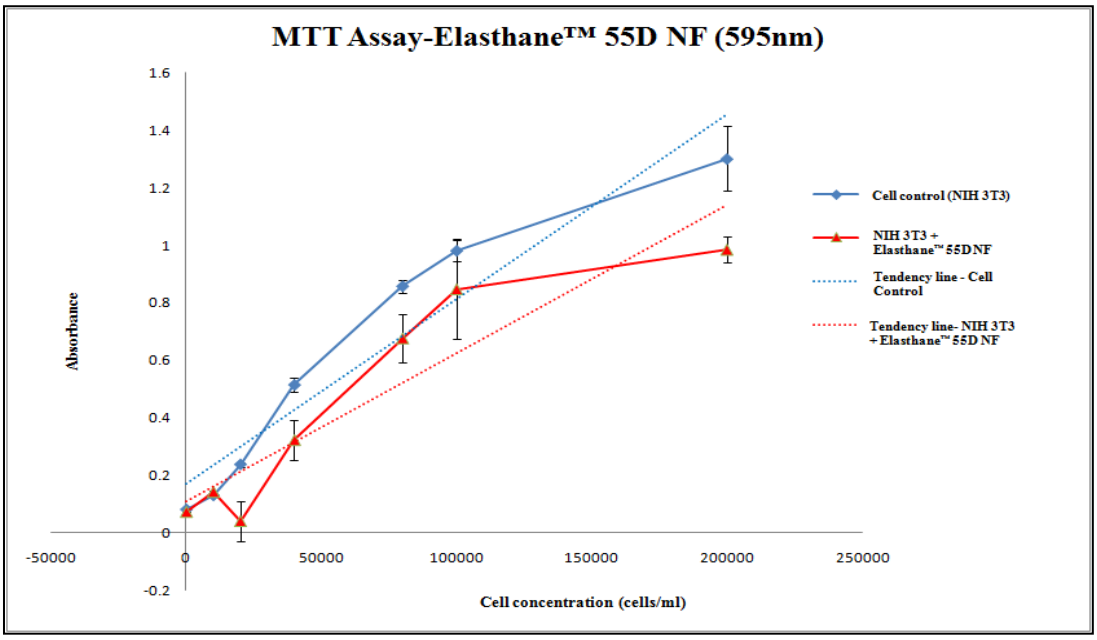


Figure 3.1.1: MTT Assay- 1 Day Elasthane™ 55D NF* (595nm)

*NF=Nanofiber

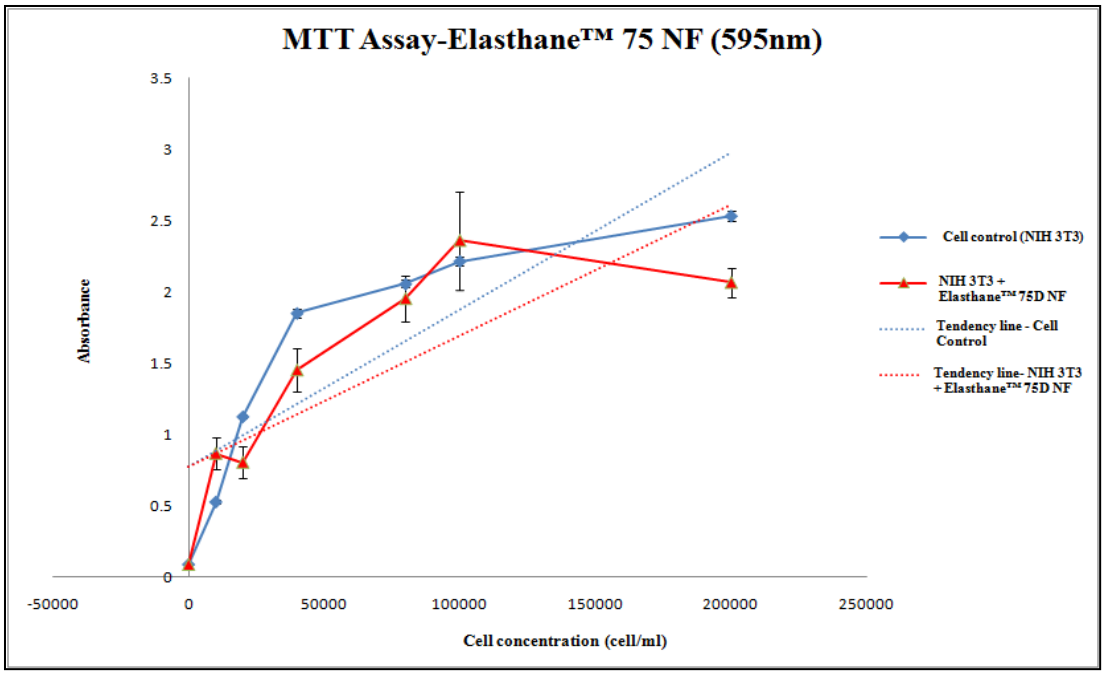


Figure 3.1.2: MTT Assay- 1 Day Elasthane™ 75D NF (595nm)

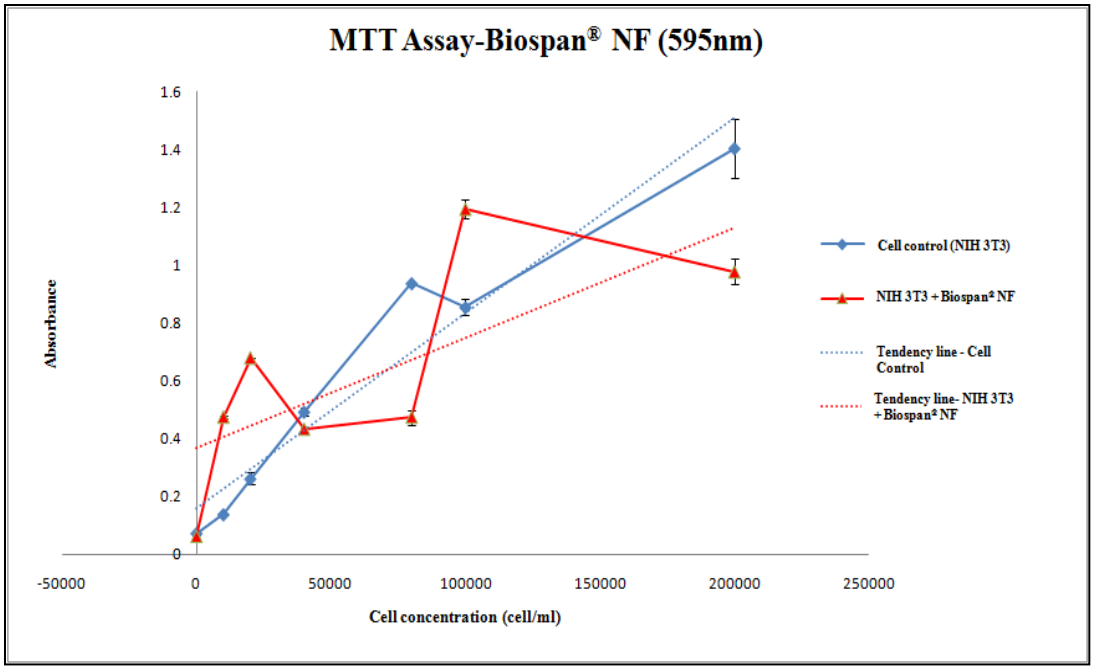


Figure 3.1.3: MTT Assay- 1 Day Biospan® NF (595nm)

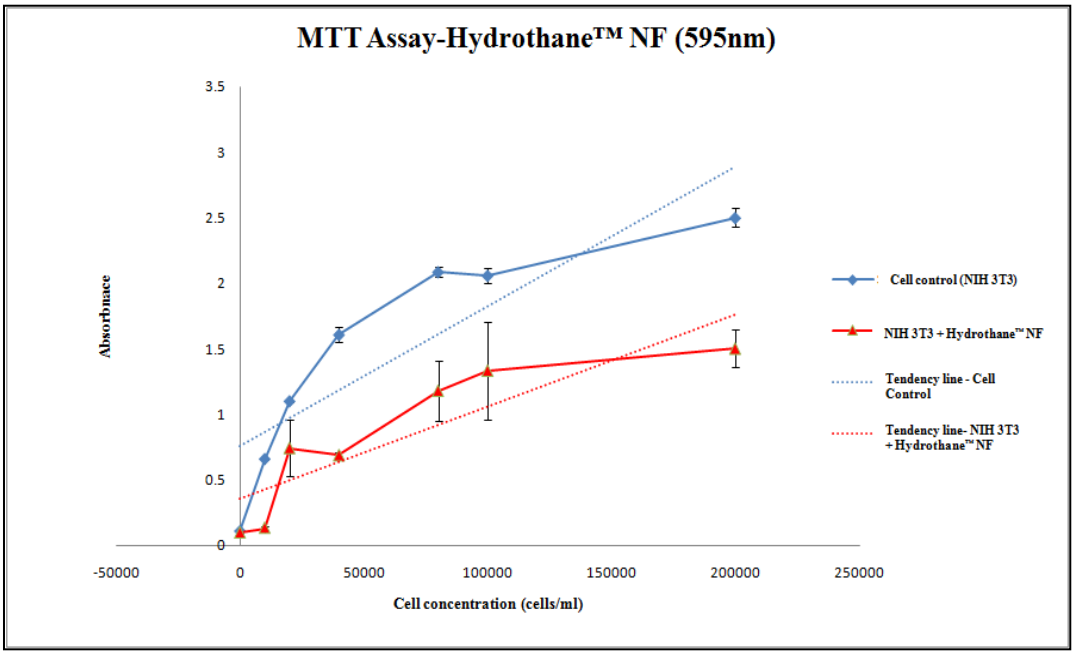


Figure 3.1.4: MTT Assay- 1 Day Hydrothane™ NF (595nm)

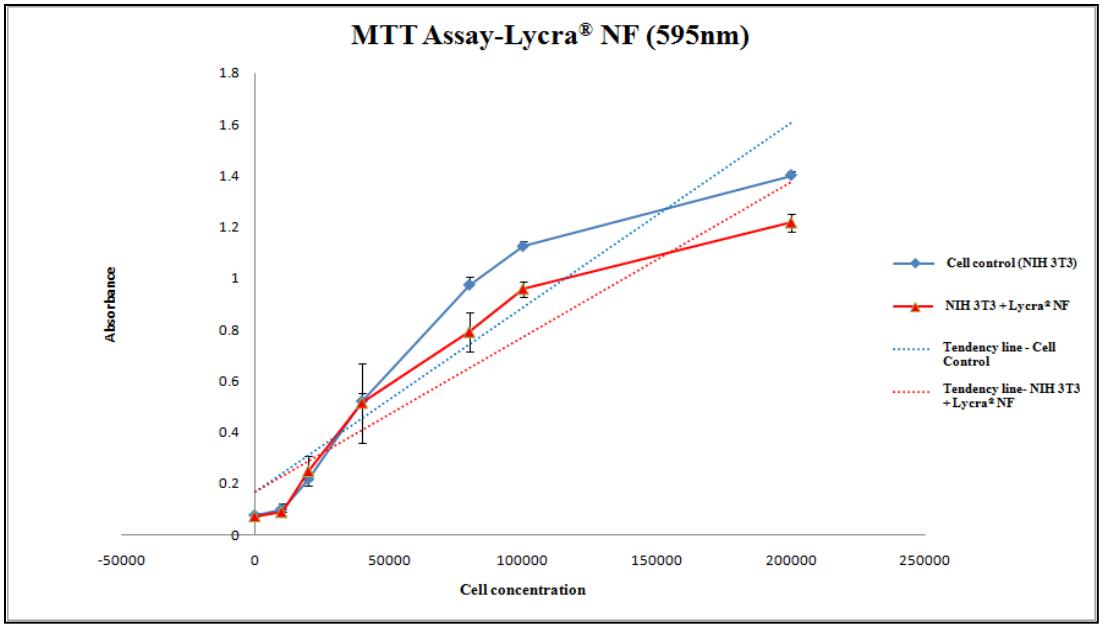


Figure 3.1.5: MTT Assay- 1Day Lycra® NF (595nm)

Figures 3.1.1 to 3.1.5 illustrate the results from MTT assay 1 day at 595nm. This MTT assay was done to demonstrate that NIH 3T3 cells did not die when exposed to the EPNs. All graphs show a data series that represent the cell control at different cell densities, and the results for NIH 3T3 cells exposed to EPNs for 1 day. These series were corrected subtracting the average absorbance that each polymer nanofiber produced. From the cell control and NIH 3T3 exposed to the EPNs, tendency lines were created, these tendency lines in all EPNs' graphs resulted in a higher absorption, when the cell density increased; thus demonstrating that EPNs did not stop the cell growth of the fibroblasts (NIH 3T3). It was observed that EPNs promoted different behaviors on the cells in the *in vitro* model, by analyzing the behavior of the cells in Figures 3.1.1 to 3.1.5; it is possible to suggest that each polymer affects in a specific way the fibroblasts without stopping cell growth. From these results, it was also confirmed that EPNs are

potential candidates for ligaments and tendons prostheses. As the literature suggests, results confirmed that 25,000 cell/mL starting solution fits in the experiments using NIH 3T3 cells.

3.2 MTT Assay- EPNs' cytotoxicity 1, 3 and 7 days

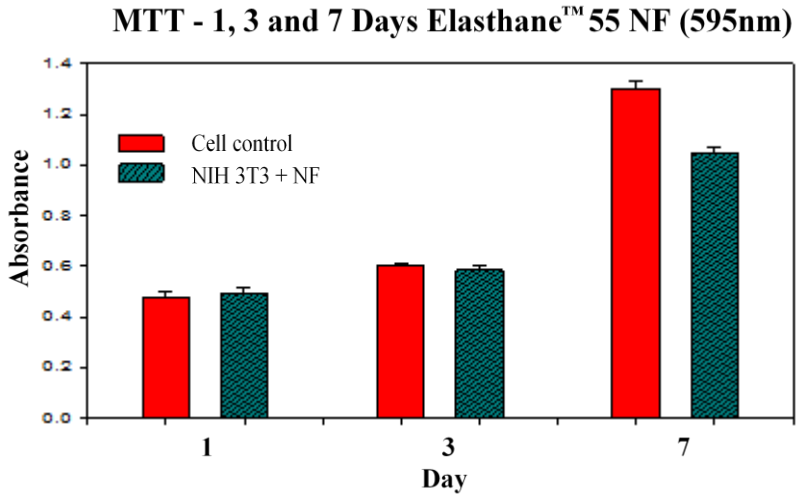


Figure 3.2.1: MTT Assay- 1, 3 and 7 Days Elasthane™ 55D NF (595nm)

Figure 3.2.1 illustrates the cell growth of NIH 3T3 (cell control) in 1, 3 and 7 days, versus the cell growth of NIH 3T3 exposed to Elasthane™ 55D nanofiber in 1, 3 and 7 days. The cells exposed to the nanofiber showed an increase in cell growth rate from day 1 to day 7, but when cells exposed to the nanofibers were compared to their corresponding cell control it was shown that on the first and third day, NIH 3T3 cells exposed to Elasthane™ 55D nanofiber did not have a statistical difference compared to the cell control $p>0.05$ (Appendix A, Table 6), which means that both the cell control and the cells exposed to the nanofiber had cell growth at almost the same rate. By day 7, cells exposed to the nanofibers revealed a decrease in the growth

rate when compared to the cell control, and the t-Test confirmed that the difference was statistically significant, since $p<0.05$.

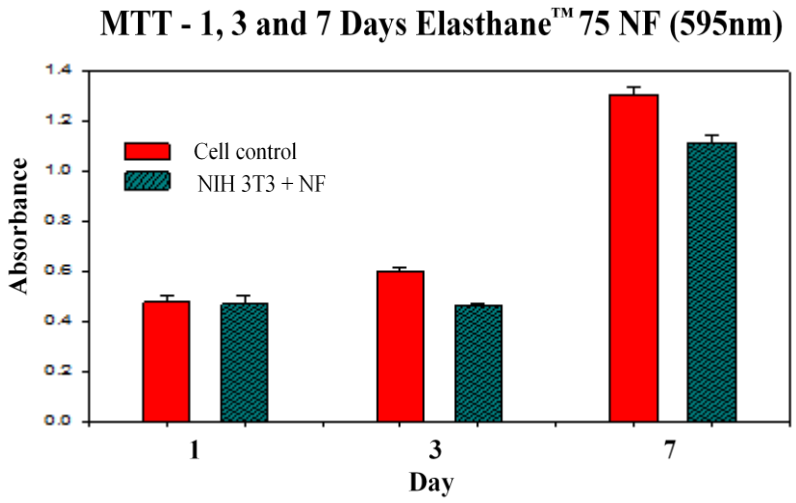


Figure 3.2.2: MTT Assay- 1, 3, 7 Days Elasthane™ 75D NF (595nm)

Figure 3.2.2 illustrates cell growth of NIH 3T3 (cell control) in 1, 3 and 7 days versus, cell growth of NIH 3T3 exposed to Elasthane™ 75D nanofiber in 1, 3 and 7 days. The cells exposed to the Elasthane™ 75D nanofiber showed an increase in the cell growth rate from day 1 to day 7. However, when the cell growth of 1, 3, and 7 days are compared to their corresponding cell control, it was observed that on the first day NIH 3T3 cells exposed to the nanofiber had no statistical difference compared with the cell control since $p>0.05$ (Appendix A, Table 7); suggesting that both the cell control and the cells exposed to the nanofiber in first day had approximately the same cell growth rate. By the third day, cells exposed to the nanofibers demonstrated practically the same cell growth as the first day (referred to the cells exposed to the nanofiber), but showed a statistical difference ($p<0.05$) when compared to the control on day 3;

by day 7, cells exposed to the nanofibers revealed an increase in cell growth, but were statistically different to the cell control of day 7 since $p<0.05$.

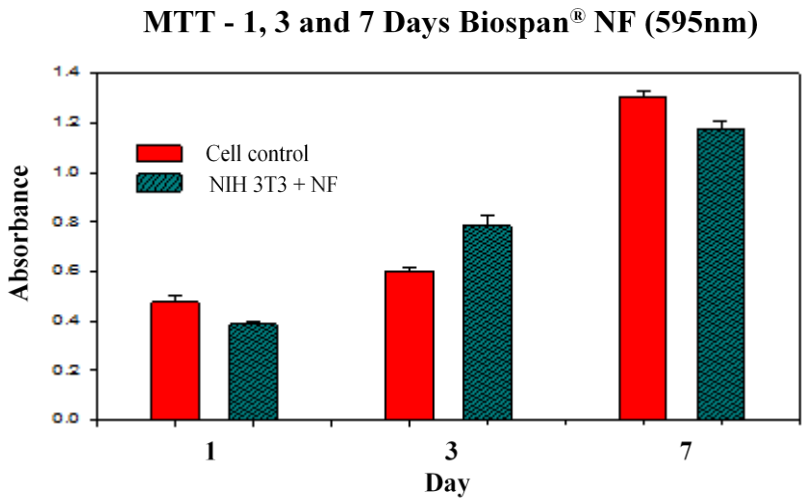


Figure 3.2.3: MTT Assay- 1, 3 and 7 Days Biospan® NF (595nm)

Figure 3.2.3 illustrates cell growth of NIH 3T3 (cell control) in 1, 3 and 7 days, versus cell growth of NIH 3T3 exposed to Biospan® nanofiber in 1, 3 and 7 days. The cells exposed to the Biospan® nanofiber showed an increase behavior in the cell growth rate throughout 1, 3 and 7 days. But, when the cell growth of 1, 3, and 7 days are compared to their corresponding cell controls, it was observed that on the first day NIH 3T3 cells exposed to the nanofiber did not have a statistical difference compared with their respective cell control, since $p>0.05$ (Appendix A, Table 8). By the third day, cells exposed to the nanofibers demonstrated a higher cell growth than the cell control of day 7 resulting in a statistically difference between them, since $p<0.05$;

by day 7 cell growth rate was less compared to the cell control of day 7, and the difference was statistically significant since $p<0.05$.

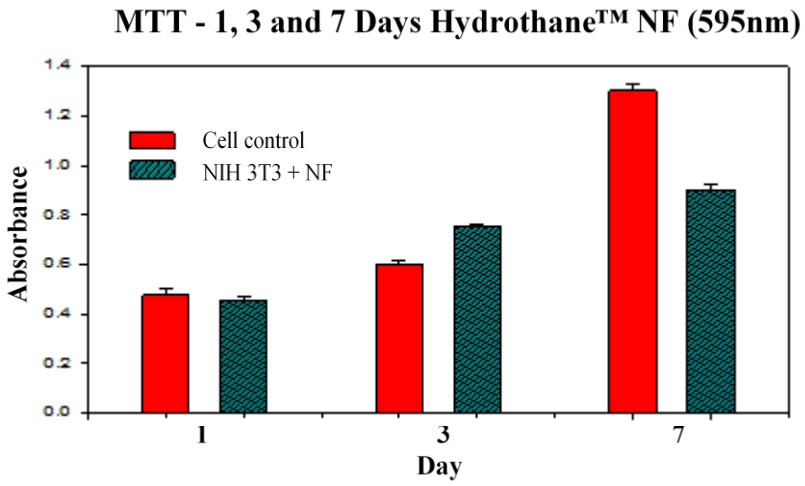


Figure 3.2.4: MTT Assay- 1, 3 and 7 Days Hydrothane™ NF (595nm)

Figure 3.2.4 illustrates cell growth of NIH 3T3 (cell control) in 1, 3 and 7 days, versus cell growth of NIH 3T3 exposed to Hydrothane™ nanofiber in 1, 3 and 7 days. The cells exposed to the Hydrothane™ nanofiber had an increase cell growth behavior throughout days 1, 3 and 7. When results from cells exposed in 1, 3, and 7 days were compared to their corresponding cell control it was observed that day 1 had almost the same rate as its cell control since $p< 0.05$ (Appendix A, Table 9), on day 3 cells exposed to the nanofiber had higher growth rate than cell control obtaining a statistical relevance, since $p<0.05$; by day 7, cells exposed to the nanofiber revealed a decrease in the cell growth rate resulting in a statistically difference when compared to the cell control of day 7 since $p<0.05$.

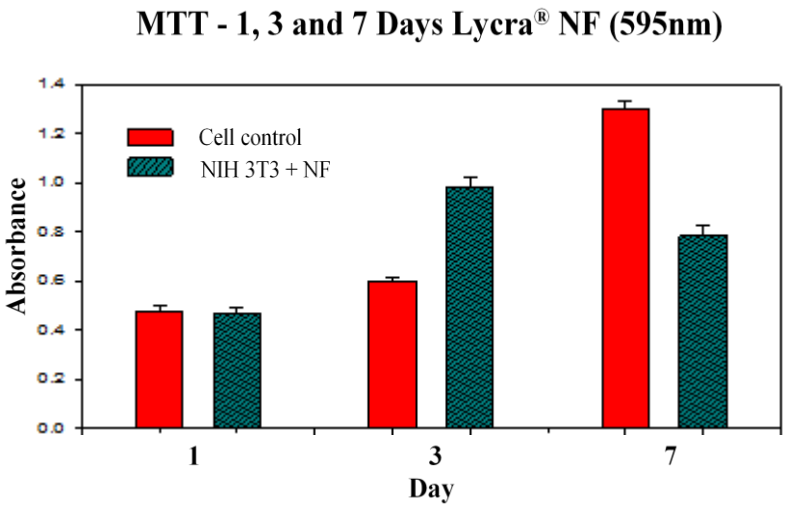


Figure 3.2.5 MTT Assay- 1, 3 and 7 Days Lycra® NF (595nm)

Figure 3.2.5 illustrates cell growth of NIH 3T3 (cell control) in 1, 3 and 7 days, versus cell growth of NIH 3T3 exposed to the Lycra® nanofiber. The cells exposed to the Lycra® nanofiber had an increase in cell growth from day 1 to day 3, but on day 7 these cell growth decreased. When these cells exposed to the Lycra® nanofiber in 1, 3 and 7 days were compared to their corresponding cell control, on day 1 cells exposed to the nanofibers did not have statistical difference compared to the cell control, on day 3 cell growth was higher than cell control resulting in a statistical difference since $p<0.05$ (Appendix A, Table 9); by day 7, cells exposed to the nanofiber revealed a decrease on the cell growth rate resulting to a statistically difference when compared to the cell control of day 7 since $p<0.05$.

3.3 Cell Morphology by Calcein-AM

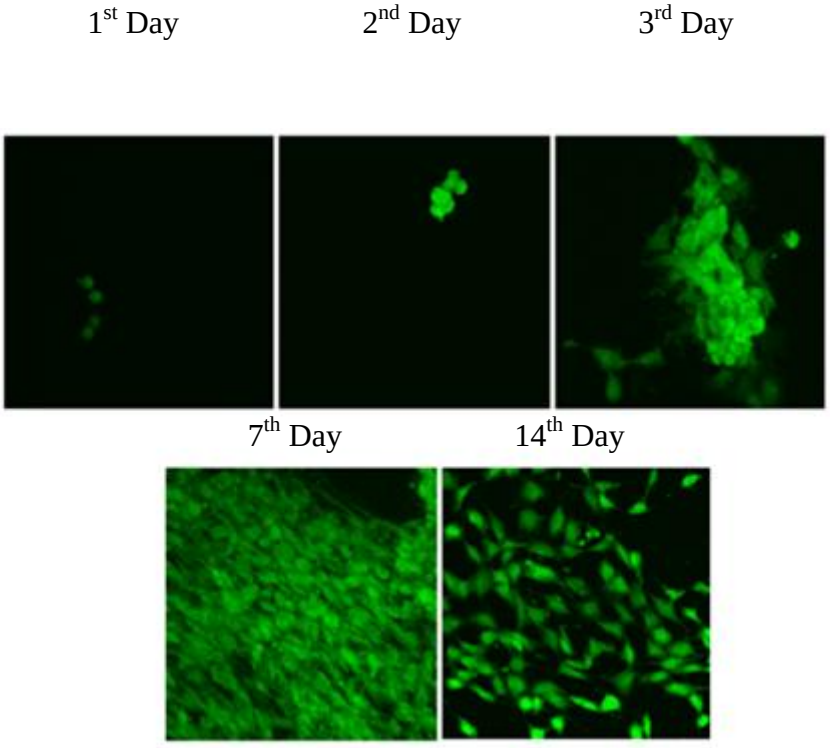


Figure 3.3.1 Confocal section showing NIH 3T3 cells (fibroblasts) seeded in Elasthane™ 55D nanofiber: EPNs were treated with 10µg/ml fibronectin, and incubated for 1, 2, 3, 7 and 14 days. The images show X, Y and Z axis level, 30 minutes after 1.5µM- Calcein addition. Magnification 20X, NA: 0.75.

Figure 3.3.1 illustrates NIH 3T3 cells attached to Elasthane™ 55D electrospun nanofibers in day 1, 2, 3, 7 and 14. An increase in cell proliferation was observed from day 1 to day 7, while cell proliferation decreased on day 14. Stressed cells or cells starting to divide were observed in all images (rounded shape cells); from day 3 to day 14 elongated cells were visible.

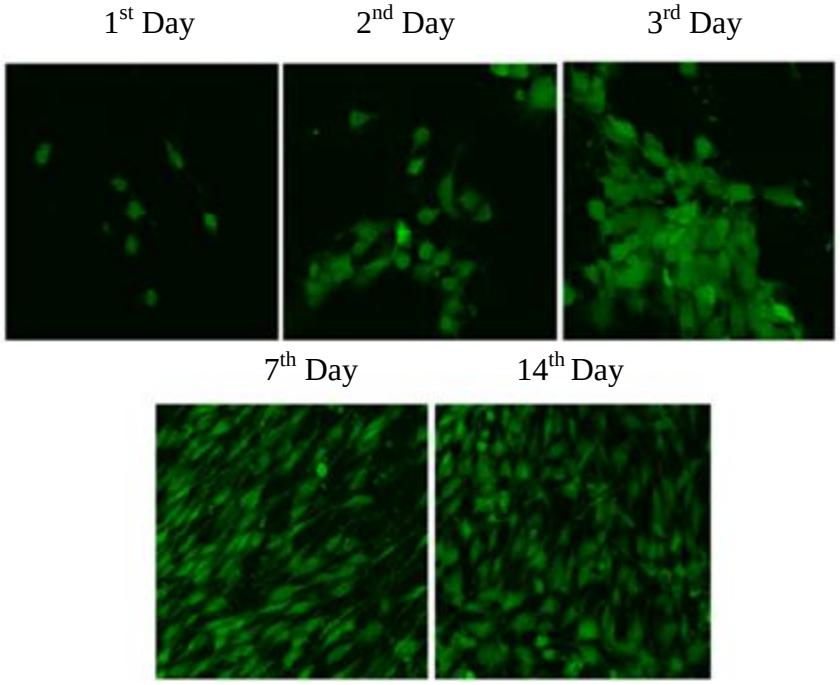


Figure 3.3.2 Confocal section showing NIH 3T3 cells (fibroblasts) seeded in Elasthane™ 75D nanofibers: EPNs were treated with 10µg/ml fibronectin, and incubated for 1, 2, 3, 7 and 14 days. The images show X, Y and Z axis level, 30 minutes after 1.5µM- Calcein addition. Magnification 20X, NA: 0.75.

Figure 3.3.2 illustrates fibroblast cells seeded on Elasthane™ 75D electrospun nanofibers in 1, 2, 3, 7 and 14 days. It was observed that from day 1 to day 7 there was an increase in cell density, and on day 14the cell number seemed to be approximately the same cell number as on day 7. When cell proliferation throughout 1, 2, 3, 7 and 14 days was described per day, on day 1 stressed cells or cells starting to divide, and cells starting to elongate were visible; on day 2 elongate cells were more noticeable; from day 7 to day 14 a homogenous spread of cells on the EPN was observed.

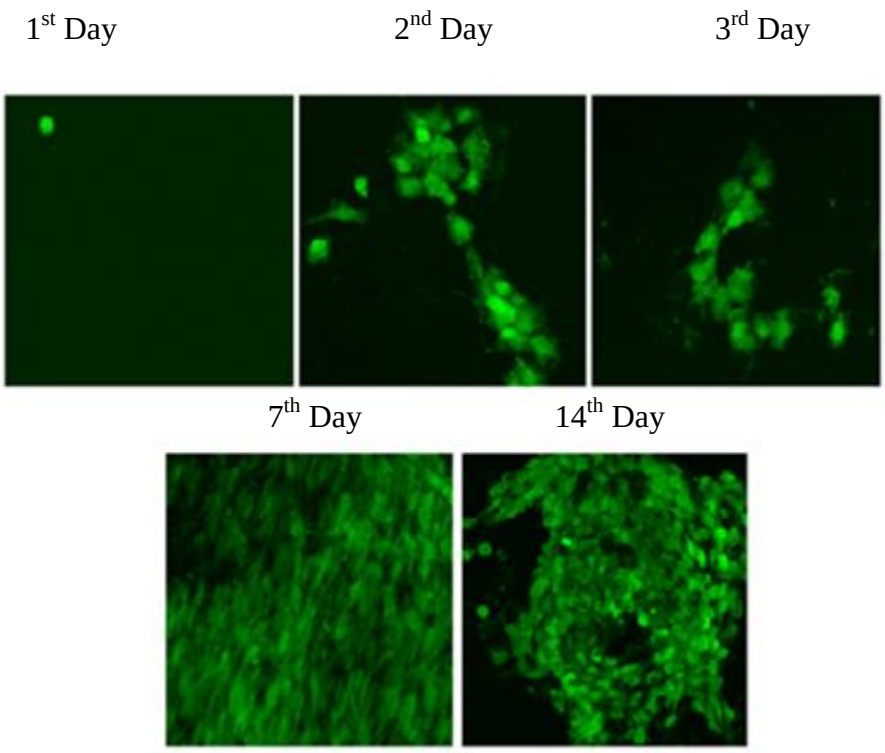


Figure 3.3.3 Confocal section showing NIH 3T3 cells (fibroblasts) seeded in Biospan® nanofibers: EPNs were treated with 10µg/ml fibronectin, and incubated for 1, 2, 3, 7 and 14 days. The images show X, Y and Z axis level, 30 minutes after 1.5µM- Calcein addition. Magnification 20X, NA: 0.75.

Figure 3.3.3 illustrates NIH 3T3 cells attached to Biospan® nanofiber in 1, 2, 3, 7 and 14 days. Cell growth from day 1 to day 7 was observed, and a drop of cell growth in day 14. When the images were analyzed individually, it was observed that day 1 had stressed cells or cell starting to divide; in day 2, 3, 7 and 14, stress cells or starting to divide and elongated cells were visible.

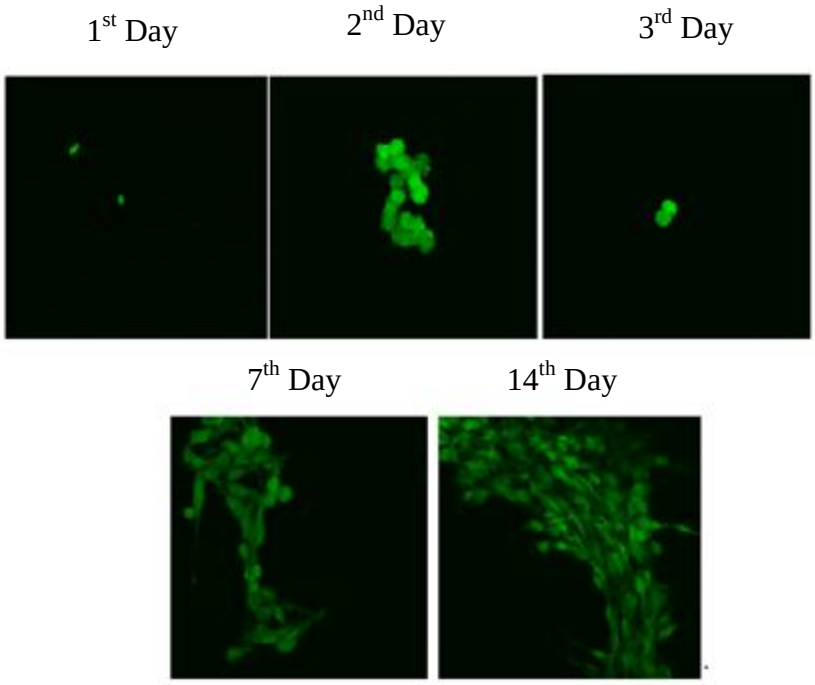


Figure 3.3.4 Confocal section showing NIH 3T3 cells (fibroblasts) seeded in Hydrothane™ nanofibers: EPNs were treated with 10µg/ml fibronectin, and incubated for 1, 2, 3, 7 and 14 days. The images show X, Y and Z axis level, 30 minutes after 1.5µM- Calcein addition. Magnification 20X, NA: 0.75

Figure 3.3.4 illustrates fibroblast cells seeded on Hydrothane™ electrospun nanofibers in 1, 2, 3, 7 and 14 days. An increased in cell density was observed from day 1 to day 14. From day 1 to day 3 stressed cells or cells starting to divide were observed; in day 7 and day 14 stressed cells or cells starting to divide and elongated cells were visible.

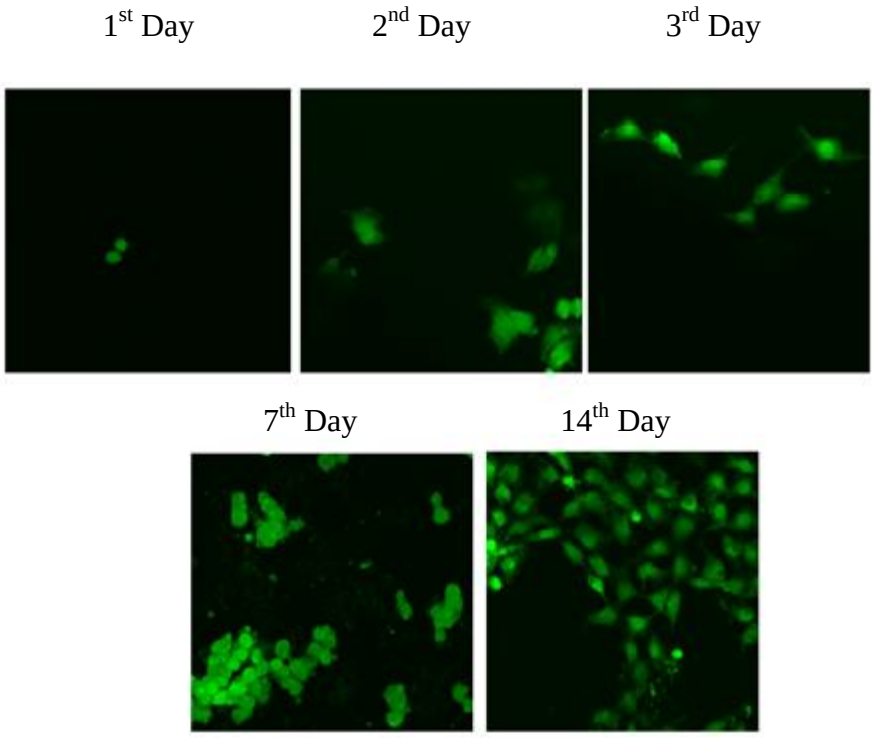
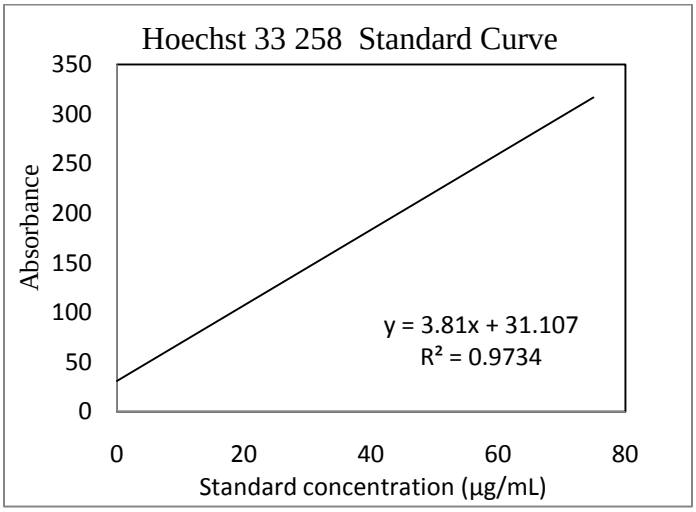


Figure 3.3.5 Confocal section showing NIH 3T3 cells (fibroblasts) seeded in Lycra® nanofibers: EPNs were treated with 10µg/ml fibronectin, and incubated for 1, 2, 3, 7 and 14 days. The images show X, Y and Z axis level, 30 minutes after 1.5µM- Calcein addition. Magnification 20X, NA: 0.75.

Figure 3.3.5 illustrates fibroblast cells seeded on Lycra® electrospun nanofibers in 1, 2, 3, 7 and 14 days. An increase in cell density was observed from day 1 to day 14, but this increase in the cell number was in a low rate when compared to the first cells seeded on the first nanofibers (Elasthane™ 55D, Elasthane™ 75 D and Biospan®). On day 1 stressed cells or cells starting to divide were observed; from day 2 to day 3 stressed cells or cells starting to divide and elongated cells were visible; by day 7 stressed cell were abundant and elongated cells were hardly visible, by day 14 the cells number was increased, but this increase on the cell number was not in all the EPN, there were some gaps (Regions without cells) visible on the nanofiber.

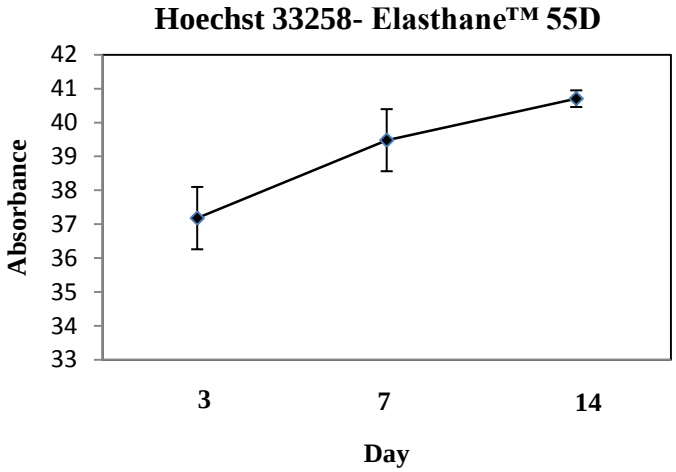
3.4 DNA quantification to demonstrate cell proliferation



	1	2	3	4
1	31.606	149.42	233.73	292.54
2	31.054	152.38	237.94	306.62
3	30.66	155.52	227.16	302.65
average	31.1067	152.4400	232.9433	300.6033
std. deviation	0.4752	3.0504	5.4329	7.2597
(std. dev.^2)/ n	4.516E-02	1.861E+00	5.903E+00	1.054E+01
std. error	0.2376	1.5252	3.1367	4.1914

Figure 3.4.1: Hoechst 33258- DNA Standard Curve

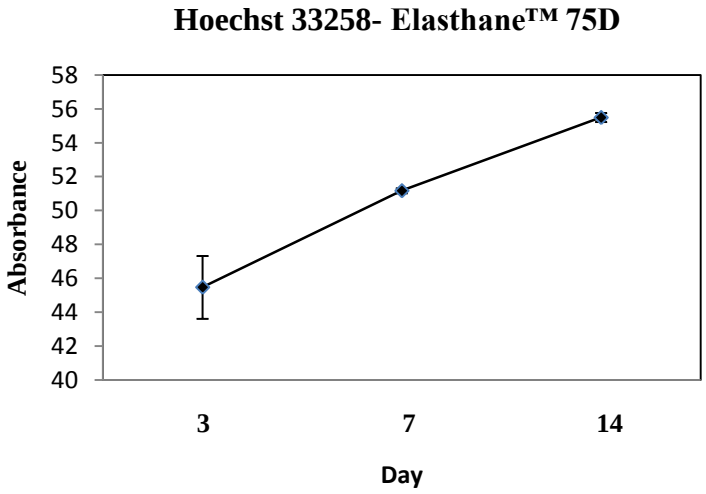
Figure 3.4.1 illustrates the Standard Curve that was formed by 25, 50 and 75µg/mL standards of DNA. This standard curve was used to extrapolate the results from the DNA extracted from the EPNs seeded with NIH 3T3 cells in order to get a quantitative analysis from cell proliferation in 3, 7 and 14 days in all EPNs.



Elasthane™ 55D NF		
Day	Abs	Conc. (µg/mL)
3rd Day	37.181	1.594
7th Day	39.479	2.197
14th Day	40.703	2.519

Figure 3.4.2: Hoechst 33258- Elasthane™ 55D NF

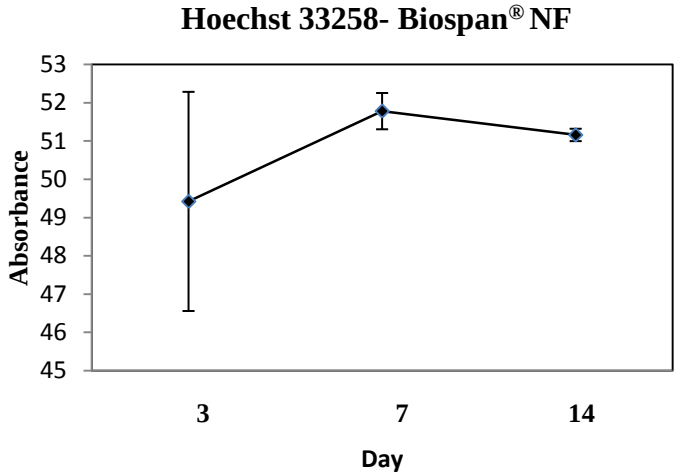
Figure 3.4.2 illustrates the DNA quantification of cells that were attached to Elasthane™ 55D nanofiber in 3, 7, and 14 days after seeded with fibroblasts cells. As illustrated, cell proliferation increased throughout these days.



Elasthane™ 75D NF		
Day	Abs	Conc. (µg/mL)
3rd Day	45.470	3.770
7th Day	51.170	5.266
14th Day	55.495	6.401

Figure 3.4.3: Hoechst 33258- Elasthane™ 75D NF

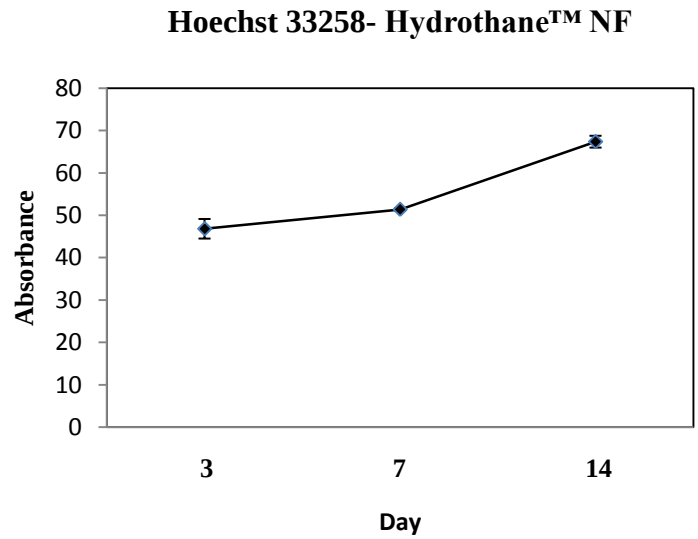
Figure 3.4.3 illustrates the DNA quantification of cells that were attached to Elasthane™ 75D nanofiber in 3, 7, and 14 days after seeded with fibroblasts cells. As illustrated, cell proliferation increased throughout these days.



Biospan® NF		
Day	Abs	Conc. (µg/mL)
3rd Day	49.422	4.807
7th Day	51.781	5.426
14th Day	51.159	5.263

Figure: 3.4.5 Hoechst 33258- Biospan® NF

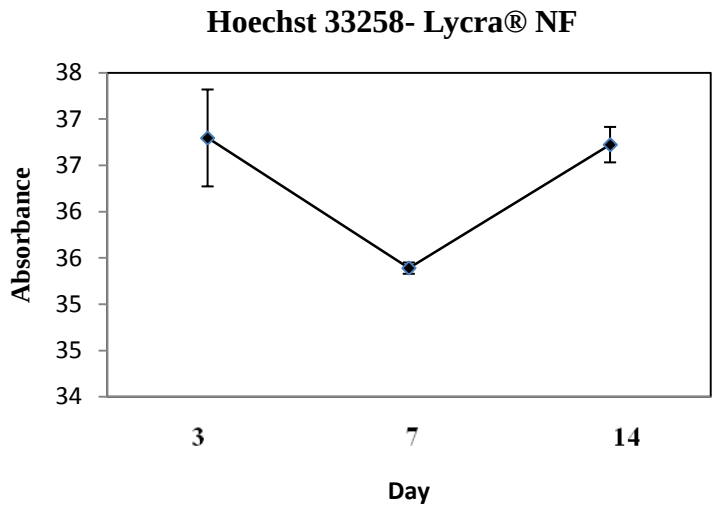
Figure 3.4.4 illustrates the DNA quantification of cells attached to Biospan® NF nanofiber in 3, 7, and 14 days after seeded with fibroblasts cells. As illustrated, cell proliferation increased from day 3 to day 7, but by day 14 the DNA amount decreased.



Hydrothane™ NF		
Day	Abs	Conc. (µg/mL)
3rd Day	46.822	4.125
7th Day	51.396	5.325
14th Day	67.376	9.519

Figure 3.4.5: Hoechst 33258- Hydrothane™ NF

Figure 3.4.5 illustrates the DNA quantification of cells attached to Hydrothane™ nanofiber in 3, 7, and 14 days after seeded with fibroblasts cells. As illustrated, cell proliferation increased from the day 3 to day 14.



Lycra® NF		
Day	Abs	Conc. (µg/mL)
3rd Day	36.796	1.493
7th Day	35.390	1.124
14th Day	36.724	1.474

Figure 3.4.6: Hoechst 33258- Lycra® NF

Figure 3.4.6 illustrates the DNA quantification of cells attached to Lycra® NF nanofiber in 3, 7, and 14 days after seeded with fibroblasts cells. As illustrated, cell proliferation decreased from day 3 to day 7 and by day 14 DNA amount increased.

Results of this study strongly suggest that the sterilization process used for the EPNs was sufficient to carry out all the experiments, without causing contamination of the NIH 3T3 cells seeded on the EPNs. In this study, the cellular response and cellular proliferation of NIH 3T3 cells seeded on Elasthane™ 55D, Elasthane™ 75D, Biospan®, Hydrothane™ and Lycra® electrospun nanofibers was assessed. The NIH 3T3 cells showed continuous growth when exposed to EPNs, as indicated by the high values on the MTT assay by the fibroblast cells for up to 7 days. The growth of the fibroblast in Elasthane™ 55D, Elasthane™ 75D, Biospan® and Hydrothane™ nanofibers was optimal on day 7 but for Lycra® nanofiber the highest value was on the day 3; suggesting that EPNs provide an environment for rapid proliferation of NIH 3T3 cells. The increase observed in the in cell number under confocal microscope images in presence of calcein-AM at day 14 culture indicate that NIH 3T3 cells proliferated on all polymer surfaces. The cell density was greater on nanofibers formed by Elasthane™ 55D, Elasthane™ 75D and Biospan® nanofibers than on and Hydrothane™ and Lycra® nanofibers. In the case of Elasthane™ 55D, Elasthane™ 75D and Biospan®, they had a continuous cell growth pattern from day 1 to day 7, and after day 7, the cell growth dropped or remained equal. This may be due the high confluency of the cells on the EPN might have caused the cells to die. DNA quantification of the EPN attached cells did not correlate with the cell increase that is evident on MTT assay and Calcein-AM based confocal images. This discrepancy in the results might be due to the inadequate sonication process which did not extract all DNA from the NIH 3T3 cells attached to the EPNs.

CHAPTER IV

CONCLUSION

The results of this study conclude that, fibroblast cells showed good biocompatibility with some of the EPNs as oppose to others. These variations were visible when the cells growth rate and the confocal microscope images were compared with various EPNs. For example, Elasthane nanofibers showed a remarkable cell growth response and cell proliferation. Results on the confocal microscopy images showed that NIH 3T3 cells remain viable and showed a notable increase in cellular proliferation on some EPNs.

These results conclude that Elasthane™ and Biospan® nanofibers showed better biocompatibility results on the fibroblast cell proliferation than Hydrothane™ and Lycra® nanofibers. This study also strongly suggests that Elasthanes™, Biospan®, Hydrothane™ and Lycra® showed significant response to cell growth and biocompatibility to the NIH 3T3 cells and thus can be consider as a promising material for tissue-engineering ligaments.

In the future, carbon nanotubes (CNTs) will be incorporated to electrospun nanofibers to enhance their mechanical properties. Biocompatibility assays will be run to determinate the effect of introducing the carbon nanotubes in the electrospun nanofibers. Furthermore, alignment in the electrospun nanofibers will be done to these EPNs to obtain a material with

more uniformed physical, structural and biomechanical/physical properties similar to those of the native ligaments.

PART II

INTERACTION OF QUERCETIN WITH DIFERENT FLAVONOIDS: INDUCTION OF PHASE II ENZYMES *IN VITRO* CANCER CELL LINES

CHAPTER V

INTRODUCTION

Cancer is defined as the malfunction of genes that control cell growth and cell division. Only 5% of cancer is hereditary, so most do not result from inherited genes (American Cancer Society, 2009). Mutation in genes can produce cancer; this mutation can be generated by internal and external factors. Some examples of internal factors are hormones and digestions of nutrients within cells, and external factors such as tobacco chemicals, sunlight, among others (American Cancer Society, 2009).

Around the world, cancer is a predominant factor in the global burden of diseases, with estimations of cases that will increase from 10 million in 2000 to 15 million by 2020 (World Health Organization, 2002). The fact that cancer treatment facilities are not universally available and the rapidly increasing rate of cancer cases around the world may produce a real crisis for public health and health systems worldwide. Currently, priorities for global cancer control will focus on risk factors and prevention research of cancer (Boyle and Levin, 2008).

In the United States it is reported that more than half a million people died from cancer in 2009 (Fig 5.1). The American Cancer Society estimates that about one-third of the half million cancer deaths will be related to overweight, obesity, physical inactivity and poor nutrition (ACS, 2009).

US Mortality, 2009

Rank	Cause of Death	No. of deaths	% of all deaths
1.	Heart Diseases	631,636	26.0
2.	**Cancer**	**559,888**	**23.1**
3.	Cerebrovascular diseases	137,119	5.7
4.	Chronic lower respiratory diseases	124,583	5.1
5.	Accidents (unintentional injuries)	121,599	5.0
6.	Diabetes mellitus	72,449	3.0
7.	Alzheimer disease	72,432	3.0
8.	Influenza & pneumonia	56,326	2.3
9.	Nephritis*	45,344	1.9
10.	Septicemia	34,234	1.4

*Includes nephrotic syndrome and nephrosis.
Source: US Mortality Data 2006, National Center for Health Statistics, Centers for Disease Control and Prevention, 2009.

Fig 5.1: US Mortality, 2009

Cancer is the second most common cause of death in the US, surpassed only by heart disease (Fig 5.2). At least one-third of annual cancer deaths in the U.S. are due to dietary factors. The American Cancer Society estimates that up to 80 percent of cancers of the large bowel, breast, and prostate are due to dietary factors.

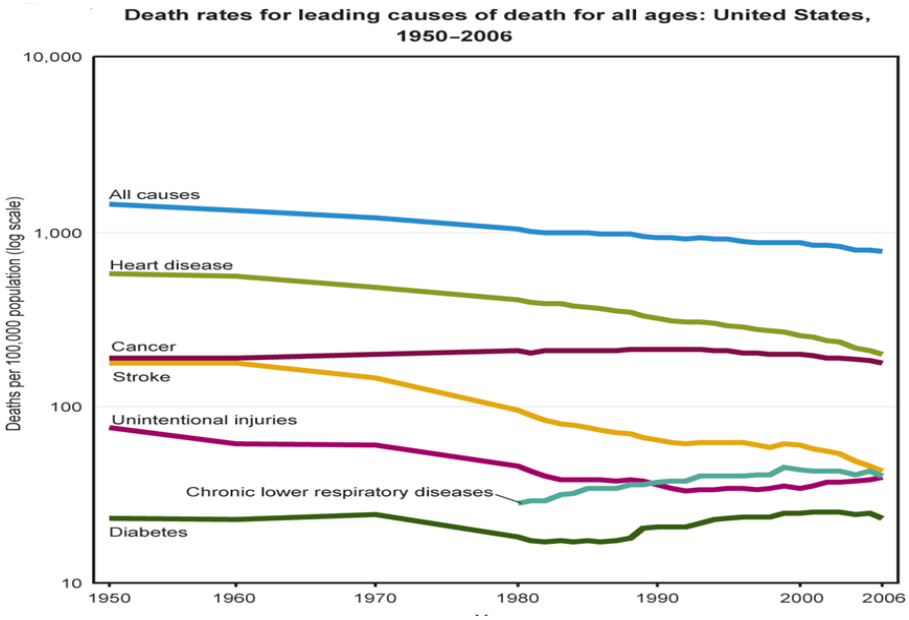


Fig 5.2: Death rates for leading causes of death for all ages: United States, 1950-2006

Studies suggest and provide evidence that certain foods contain specific components that function as chemoprotective agents against cancer (Watson and Mufti, 1996). These chemoprotective agents against cancer are among the vast group of compounds known as a phytochemicals.

Chemoprevention involves managing specific amounts of a particular natural or synthetic chemical in an attempt to identify agents that will prevent, halt, or reverse the process of carcinogenesis (Nixon, 1995). Using phytochemicals is currently accepted as one of the leading strategies of cancer chemopreventive drug discoveries and development (Maghes, 2007).

Induction of Phase II drug metabolizing enzymes such as Glutathione S-transferase (GST) and NADPH: Quinone Oxidoreductase (QR) is considered a major mechanism of protection against chemical stress and initiation of carcinogenesis (Talalay et al., 1995). Induction of Phase II enzymes seems to be sufficient to obtain chemoprevention, and can be achieved by administering any of the diverse arrays of naturally occurring and synthetic chemical agent (Kensler, 1997).

GSTs are ubiquitous enzymes that protect cells by detoxification of carcinogens by catalytic and non-catalytic binding mechanisms, thus yielding less toxic hydrophilic conjugates which are readily excreted (Zheng, 1993). QR catalyzes the two-electron reduction of quinone, leading to the formation of hydroquinone, and thereby shields their ability to generate oxidative stress and prevent carcinogenesis (Talalay, 1991).

The protection mechanism against cancer include the detoxification and enhanced excretion of carcinogens, the suppression of inflammatory processes, inhibition of mitosis and the induction of apoptosis (Johnson, 2007).

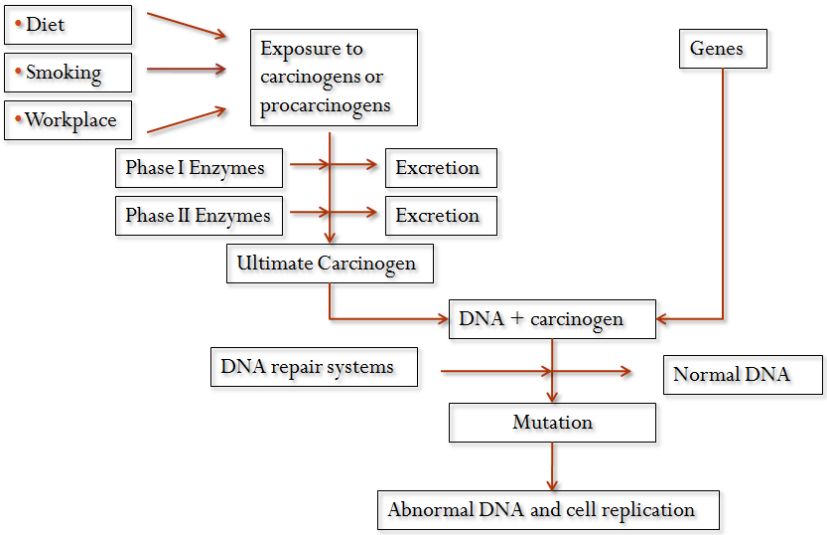


Fig 5.3: Model of Chemical Carcinogenesis

Primary cancer prevention includes educating individuals and policy makers about the relationship between weight control, diet, physical activity and cancer. It is important to emphasize to the public that cancer risks can be reduced with a healthy diet and daily exercise (American Cancer Society, 2009). In order to assist the public in making lifestyle changes to their diet and exercise, it is imperative that we identify chemopreventive agents.

In previous studies conducted in our laboratory, data obtained from the 4-nitroquinoline1-oxide (4NQO) biochemical assay clearly showed the induction of the Phase II enzymes GST and NADPH: Quinone Oxidoreductase by Quercetin with a second phytochemical. Nevertheless, in other mixes the induction was not promising, suggesting that is possible to modify the concentration of the secondary flavonoids and test in different concentrations, in order to increase the induction of the Phase II enzymes. The experimental groups will be formed with a constant concentration of Quercetin, a constant concentration of Caffeine and a secondary

flavonoid in two different concentrations. The phytochemicals that will be used as a second flavonoid in this study will be Hesperedin, Ginger, Resveratrol and β-Carotene.

The purpose of this study is to determinate whether greater induction of Phase II enzymes GST and QR can be achieved through the treatment of Hepa-1c1c7 (mouse hepatoma) and MCF7 (human cancer breast) cells with various phytochemicals in combination with Quercetin and Caffeine. The strategy used in this study is to investigate the induction of phase II enzymes with phytochemicals such as Hesperedin, Ginger extract, Resveratrol and β-Carotene in combination with Quercetin at a predetermined optimal inducing concentration. Caffeine was added to determine the protective effect of cells survival.

CHAPTER VI

LITERATURE REVIEW

6.1 Quercetin

Quercetin belongs to a family of naturally occurring, water-soluble plant compounds known as polyphenols, flavonoids, flavonols and bioflavonoids, and it appears to have both anti-inflammatory and antioxidant properties. In 2003, the U.S. Department of Agriculture developed a database of the quercetin contents and in common foods. Apples, onions and teas are the main sources of Quercetin in diets, but it is also present in red wine, berries, seeds, leafy green vegetables, hot peppers, parsley and red grapes, and it is also available as a dietary supplement (Manach, et al., 2004). Quercetin appears to be associated with small toxicity indexes when administered orally or intravenously. Studies *in vitro* and some preliminary animal and human data indicate that Quercetin inhibits tumor growth. More research is needed to clarify the absorption of oral doses and the magnitude of the anti-cancer effect (Lamson, et al., 2000)

6.2 Caffeine

Caffeine is a natural stimulatory compound that is present in many plants including cocoa beans, coffee beans, cola nuts, and tea leaves. Caffeine is also commonly used as a stimulant to

prevent sleepiness and is found in several over-the-counter medications, including pain remedies. Because of its frequent and common consumption in tea, coffee and soft drinks, caffeine may very likely be the most frequently ingested neuroactive drug in the world (Bode and Dong, 2007).

Caffeine has been reported to affect cell cycle function, induce programmed cell death or apoptosis and perturb key cell cycle regulatory proteins. Although the effects of caffeine have been heavily investigated, much of the research data regarding caffeine's effects on cell cycle and proliferation seem ambiguous (Bode and Dong, 2007).

Caffeine has generally been reported to induce G1/S arrest, and to reverse or abrogate the G1/S and G2/M checkpoint delay periods. Studies have shown that reversal of DNA-damage-induced checkpoint function or arrest can be produced with caffeine (Bode and Dong, 2007).

6.3 Hesperedin

Hesperidin (5, 7, 3′-trihydroxy-4′-methoxyflavanone7-rhamnoglucoside) belongs to the class of flavonoids called flavanones and is found mainly in citrus fruits. It has several biological functions such as antioxidant, anti-inflammatory, prostaglandin-synthesis inhibition, anti-mutagenic activity, modulation of drug-metabolizing enzyme etc. The effects of hesperidin in prevention and treatment of diseases have recently received considerable attention with particular interest in the use of flavonoids as anti-cancer compounds. This compound has been reported to have several beneficial health effects, including the inhibition of skin tumorigenesis, and carcinogenesis in the bladder. Additionally, hesperidin suppresses cell proliferation in azoxymethane-induced rat colon carcinogenesis (Kamaraj et al., 20

6.4 Ginger

Ginger has been cultivated for thousands of years as a spice and for medicinal purposes. Ginger contains phenolic substances which generally possess strong anti-inflammatory and anti-oxidative properties, anti-carcinogenic and anti-mutagenic activities. Ginger can be consumed as a fresh or dried root and is often prepared in teas, soft drinks, and breads. Ginger's root contains 6-paradol and a large amount of 6-gingerol; which is a pungent ingredient that has been found to exert various pharmacological effects such as anti-inflammatory, analgesic, antipyretic and antioxidant activity. It was discussed that these compounds suppress proliferation of human cancer cells through the induction of apoptosis (Kather D., 2010).

6.5 Resveratrol

Resveratrol is a flavonoid, largely found in skins of red grapes. Resveratrol has antioxidant activity and inhibits radical formation. Resveratrol exhibits cancer chemopreventive activity. Resveratrol was found to act as an antioxidant and antimutagen, to induce Phase II drug-metabolizing enzymes, mediated anti-inflammatory effects, inhibited cyclooxygenase and hydroperoxidase functions; and it induced human promyelocytic leukemia cell differentiation. In addition, it inhibited the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture and inhibited tumorigenesis in a mouse skin cancer model. These data suggest that resveratrol, a common constituent of the human diet, deserves investigation as a potential cancer chemopreventive agent in humans (Jang, 1997).

6.6 β-Carotene

β-Carotene is derived from the latin name for carrot, is a type of pigment found in plants, fruits and vegetables, its structure was described by Karrer et al. in 1930; in nature, β-Carotene is a precursor (inactive form) to vitamin A via the action of beta-carotene 15,15'-monooxygenase (Van Armun, 1998). β-Carotene is an antioxidant that protects the body from free radicals that can cause damage to cells through oxidation. Various natural carotenoids were proven to have anticarcinogenic activity; epidemiological investigations have shown that cancer risk is inversely related to the consumption of green and yellow vegetables and fruits. Since β-carotene is present in abundance in these vegetables and fruits, it has been investigated extensively as possible cancer preventive agent (Nishino, 1997).

CHAPTER VII

MATERIAL AND METHODS

7.1 Propagation of Hepa 1c1c7 Cell Line

Cell culture protocols that were carried out during the experiment, as well as the required cell-growth media, were obtained from American Type Culture Collection (ATCC) protocols.

The base medium for Hepa 1c1c7 (mouse hepatoma cells); ATCC, Manassas, VA) is α-Minimum Essential Media (Gibco™; Grand Island, NY) supplemented with a 10% fetal bovine serum (Atlanta Biologicals; Lawrenceville, GA), 7.5% sodium bicarbonate (Gibco™; Grand Island, NY) and a mixture penicillin/streptomycin (GibcoBRL™; Grand Island, NY) were used to propagate the cells.

The content from the original vial was transferred into a 75cm^2 cell culture flask (Corning, NY); flask treated with oxygen to transform the hydrophobic surface to hydrophilic surface to let the cell attach the surface. The flask was placed in an incubator set at 37°C with and 5.2% CO_2 content.

When approximately 80% of the growth area was covered with a monolayer of cells, the media was decanted and 7 mL of 0.25% trypsin (GIBCO™; Grand Island, NY), was added to detach the cells from the flask. After approximately 8 minutes, the cells and trypsin were removed from the flask and transferred in a 15mL centrifuge that was centrifuged at 200xg

for 10 minutes. When removed from the centrifuge, cells formed a pellet and trypsin remained as a supernatant. Trypsin was decanted and 10 mL of α-MEM was added to the tube in order to resuspend the pellet of cells. Resuspension was achieved by aspiration, which was the repeated action of drawing up and dispensing liquid using the pipettor. An aliquot of 10 µL was taken aside, and combined with 10 µL of 0.4% Trypan Blue dye (Invitrogen; Grand Island, NY), then used to perform a cell count using an automated cell counter (Countess™ automated cell counter; Invitrogen). The cell-resuspension solution was fractionated and an aliquot was transferred into each of the 63 culture flasks with 75 cm^2 growth area, each previously filled with 30 mL of α-Minimum Essential Media (α-MEM), to get a cell density of 2.5 $X10^4$cells/mL. All flasks were placed in the incubator set at 37°C and 5.2% CO_2 and allowed to proliferate for 24 hours.

7.2 Propagation of MCF-7 Cell Line

MCF 7 cell line (Human breast cancer cells; ATCC Manassas, VA) uses the Eagle's Minimum Essential Media (EMEM; ATCC) as a growth medium. Eagle's Minimum Essential Media will be supplemented with 10% fetal bovine serum, 7.5% sodium bicarbonate, 1% insuline from bovine pancreas (Sigma-Aldrich, St. Louis, MO). Penicillin/streptomycin was added to EMEM to avoid bacterial contamination.

The contents of the vial purchased from ATCC containing MCF 7 cell line was placed in a 25 cm^2 flask (Corning); the flask was treated with oxygen to transform the hydrophobic surface to hydrophilic surface to let the cell attach the surface. The 25cm^2 flask had 7 mL of EMEM previously added to put MCF7 cell line. The cells were fed with 2 mL of EMEM until

they covered 80% of the growth area. After reaching 80% of covered growth area by cells, they were trypsinized. After recollecting cells in trypsin, the solution was centrifuged for 5 minutes at 200xg, a cell pellet was formed and the trypsin was decanted. The cell pellet was resuspended in EMEM and an aliquot of 10µL was taken and mixed with 10µL of 0.4% trypan blue, from this solution a cell count was performed in an automated cell counter. Experimental flasks (63) had 7mL of EMEM and an aliquot of the resuspension solution was added to get a 100,000 cell/mL density. The flasks were placed in the incubator and allowed to proliferate for 48 hours.

7.3 Preparation of Flavonoid Media for Hepa-1c1c7 cell line and MCF7 cell line

The flavonoid-enriched media was prepared with 9µM Quercetin (Sigma-Aldrich, St. Louis, MO), 12µM or 120µM Caffeine (Sigma-Aldrich) and a second flavonoid in two different concentrations: a) Hesperedin in 6.25 and 12.5µM (Sigma-Aldrich), b) Ginger (extract) in 12.5 and 25µM , c) Resveratrol in 12.5 and 25µM (LKT, Laboratories, Inc, St. Paul, MN), and d) β-Carotene in 10 and 20µM (Sigma-Aldrich). These flavonoids were chosen, because the literature suggests that they are GST inducers. Additionally a control positive 4-bromoflavone (LKT, Laboratories, Inc, St. Paul, MN) was prepared, this positive control was chosen because it is a synthetic flavonoid that the literature suggests to be a strong GST inducer.

Flavonoid-enriched solutions were prepared from flavonoid powders; all phytochemicals except ginger extract were weighted out and dissolved in 5 mL of dimethyl-sulfoxide (DMSO) to prepare the flavonoid-enriched solutions at the desired millimolar concentrations. The flavonoid-enriched medias were prepared with the base growth medium for each cell line plus the

flavonoid-enriched solutions in order to obtain 1% of DMSO (99.99%, Sigma-Aldrich) in the flavonoid-enriched media (final concentration μM).

Pilot studies were done in order to test a broad range of concentrations of phytochemicals with Quercetin and caffeine. Based on the results of pilot experiments the concentrations of the final experiment were chosen.

7.4 Treatment of cells with Flavonoid-Enriched Media

The cell treatment with Flavonoid-Enriched Media was based on the pilot study that was used as a baseline to choose the final concentrations of the caffeine concentration and the second flavonoid. The cells were treated with the flavonoid-enriched media; the treated cells were analyzed to compare GST and QR specific activity with the cellular control, in order to detect any statistical significant difference.

Twenty four hours after seeding Hepa1c1c7's flasks, the original media was decanted and the appropriate phytochemical-induced media was added to the flasks. Hepa1c1c7's flasks were separated in the following groups: Cellular Control (3 flasks), 50μM 4-Bromoflavone as positive control (3 flasks), 9μM Quercetin Control (3 flasks), 12μM Caffeine Control (3 flasks), 120μM Caffeine Control (3 flasks), 6.25μM Hesperedin Control (flasks), 12.5μM Hesperedin Control (3 flasks), 12.5μM Ginger Control (3 flasks), 25μM Ginger Control (3 flasks), 12.5μM Resveratrol Control (3 flasks), 25μM Resveratrol Control (3 flasks), 10μM β-Carotene Control (3 flasks), 20μM β-Carotene Control (3 flasks), 9μM Quercetin +120μM Caffeine + 6.25μM Hesperedin (3 flasks), 9μM Quercetin +120μM Caffeine + 12.5μM Hesperedin (3 flasks), 9μM Quercetin +120μM Caffeine + 12.5μM Ginger (3 flasks), 9μM Quercetin +120μM Caffeine + 25μM

Ginger (3 flasks), 9µM Quercetin +12µM Caffeine + 12.5µM Resveratrol (3 flasks), 9µM Quercetin +12µM Caffeine + 25µM Resveratrol (3 flasks), 9µM Quercetin +120µM Caffeine + 10µM β-Carotene (3 flasks), 9µM Quercetin +120µM Caffeine + 20µM β-Carotene (3 flasks). The Hepa-1c1c7 experimental flasks were incubated for 48 hours at 37^{o}C and 5.2% CO_2 before the samples were collected for lysis.

Treatment of MCF7's cells with the phytochemicals was carried out for 48 hours instead of twenty four. However, the concentrations of the phytochemicals remain the same as it was in the heap1c1c cells. Following the treatment, the flasks were incubated for 72 hours at 37^{o}C and 5.2% CO_2 before the cells were harvested for lysis.

7.5 Lysis of Cells for Protein Isolation

After their final incubation periods the experimental flasks were trypsinized to detach the cells, detached cells were transferred into a conical tube and placed into the centrifuge for 5 minutes at 200xg. The supernatant was decanted and the remaining pellet of cells was re-suspended in 7mL of the respective plain media (α-MEM for Hepa 1c1c7 and EMEM for MCF-7). An aliquot of 10 µL was taken aside and combined with 10 µL of 0.4% Trypan Blue dye and used to perform a cell count in an automated cell counter. The remaining cells were centrifugated again to decant the media. The cells were washed two times with phosphate buffer saline (PBS) solution to remove traces of media which contained unwanted proteins. After the final wash, the PBS was decanted and the pellet of cells was re-suspended in 1 mL of 10mM phosphate buffer without β-mercaptoethanol (B-OH). The cells were lysed using a Sonic© VibraCell sonicator. The resulting lysate solution was transferred into microcentrifuge tubes and

spun for 10 minutes at 1,500xg to pellet down the unwanted cellular debris. Without disturbing the pellet, 200 µL of the protein-rich supernatant were transferred into each of 2 duplicates microcentrifuge tubes. The remaining supernatant was transferred into storage tubes and place in the freezer for further testing.

7.6 Determination of GSH Concentration

The amount of GSH in unknown samples was measured using a Beckman DU 640 spectrometer via a colorimetric enzyme assay. The solutions required for the assay were: 0.1 mM GSH (must be fresh and kept cold; Sigma-Aldrich), precipitating solution, DTNB (5,5, Dithiobis (2-nitro-benzoic acid)); Sigma-Aldrich) reagent (must be fresh and kept cold), and 0.3 M Na_2HPO_4 (Sigma-Aldrich). This assay consisted of blank, standard, and experimental samples that were run in duplicates. Two hundred µL 10mM PO4 buffer and 200µL precipitating solution were added to the blank sample. Two hundred µL of 0.1mM GSH and 300µL precipitating solution were added to the standard sample. Finally, to the experimental duplicates, 200 µL of unknown sample and 200 µL precipitating solution were added. The contents of each sample were mixed and left incubated for five minutes at room temperature, and then they were centrifugated in a microcentrifuge tubes for five minutes at 1,500xg. Afterwards, 200 µL of each supernatant was pipetted into the corresponding glass cuvettes, and 800 µL of 0.3 M Na_2HPO_2 were added. The cuvettes contents were mixed and the absorbance was taken at 412 nm (absorbance number 1). Then, absorbance 2 was obtained by adding 100 µL of DTNB to each cuvette, mixing in thoroughly and reading the absorbance one more time. This procedure was

repeated for the 63 samples right after the sonication, to prevent GSH degradation in the samples (Beutler *et al.,* 1963).

7.7 GST activity determination using 1-chloro-2,4-dinitobenzene (CDNB) as a substrate

The activity of GST was determined according to the method described by Habig *et al., 1974.* The substrate used for this assay was 1-chloro-2, 4-dinitrobenzene (CDNB, sigma Aldrich) . The solutions used for this assay were: 20 mM CDNB, 10 mM GSH, Assay Buffer, and enzyme sample. Buffer A with BOH was used for samples that needed a dilution; Beckman DU 640 was used in this method, 850 µL Assay Buffer, 100 µL 10 mM GSH, and 50 µL 20 mM CDNB were added to the Blank. 830 µL Assay Buffer, 100 µL 10 mM GSH, 50 µL 20 mM CDNB, and 20 µL purified GST enzyme sample were added to the experimental sample, when the CDNB was added, the contents of the cuvettes were immediately shaken and the absorbance was read at 340 nm (Habig *et al.,1974).*

7.8 GST activity determination using 4-nitroquinoline 1-oxide (4NQO) as a substrate

The objective of this assay was to determine if GST activity was induced by various nitrates. After the assay, GST activity was measured against CDNB. Later, its activity against 4NQO (Sigma-Aldrich) was measured. This assay required several solutions: 5 mM 4NQO, 10 mM GSH, and 100 mM Phosphate Buffer, pH 6.5. The assay consisted measuring a blank and experimental sample duplicates; both were read at 350nm after adding 4NQO and shaking. 880 µL assay buffer, 100 µL GSH, and 20 µL 4NQO were added to the Blank. 860 µL assay buffer,

100 µL GSH, and 20 µL enzyme samples were added to the experimental cuvettes. The 4NQO was added to the blank and experimental samples simultaneously after the shaking. The samples were read at 350 nm by a Beckman Du 640 spectrometer (Stanley and Benson, 1988).

7.9 Determination of Quinone-oxidoreductase activity

Quinone-oxidoreductase activity was determined in protein samples by the NADPH quinone-reductase assay. A blank and a control were run with the experimental cuvettes. Each experimental cuvette contained 900 µL of 25mM Tris/HCl buffer, 20 µL of BSA, 20µL of 5µM FAD, 20µL of DCPIP (Sigma-Aldrich), 20 µL of protein sample and 20 µL of 0.2mM NADPH (Sigma-Aldrich); for a total volume of 1mL. The Blank did not include the protein sample and the NADPH, while the Control did not include the sample, but had the NADPH. Duplicate cuvettes were made and placed in the spectrometer where their absorbance was read in the wavelength of 600nm (Shaw, 1991).

7.10 Determination of Protein Concentration

The Lowry method was used to determine the amount of protein in each sample. The solutions needed for this assay are: Lowry's Reagent A, Lowry's Reagent B1, Lowry's reagent B2, Lowry's Reagent C, and Lowry's Reagent E. An appropriate volume of unknown samples (20-200 µL containing 20-200 µg protein) were used in this assay. The assay included one blank and unknown samples in duplicates. The test tubes were labeled in duplicates and then the appropriate amount distilled water was added to bring the final volume of each tube to 1.0mL.

After the water addition, the protein samples were pipetted into each tube. Five milliliters of Lowry's Reagent C (made fresh) were added to each tube (including blank). The tubes were mixed with a vortex and left to rest at room temperature for 10 minutes. Then 0.5mL of reagent E was added to all tubes and each tube was immediately vortexed. The tubes were left to rest at room temperature for at least 25 minutes. After standing, the samples were transferred into cuvettes and the absorbance were read in comparison to the blank at 600nm (Lowry *et al.,* 1951)

7.11 Statistical Analysis

Statistical analysis was performed using the specific activity data, which is the defined as unit of enzyme activity per milligram of protein. The enzyme activity units were taken from the three independent assays of each sample (GST/CDNB, GST/4-NQO and QR) and the milligrams of protein was obtained by Lowry Method. T-test was performed to compare if the variance of our experimental groups versus the cellular control were statically significant.

CHAPTER VIII

RESULTS AND DISCUSSION

Different assays were performed to obtain raw data; from this raw data, statistical analysis was performed to determine differences between experimental and control groups to compare experimental samples with the cellular control and their corresponding flavonoid control. Statistics were completed in order to detect statistical differences between experimental groups and controls. Relevant differences in specific activities in experimental groups reflected the statistically significant effect of the phytochemicals. An enzyme induction or suppression by the phytochemicals was observed based on the results of experimental groups: a) the positive effect was seen on the induction of GST or QR-specific activity in the cells (Hepa 1c1c7 or MCF 7), and b) the negative effect was observed when some experimental groups produced a decrease in GST or QR-specific activity, or as in the case of the other experimental groups that were cytotoxic to the cells.

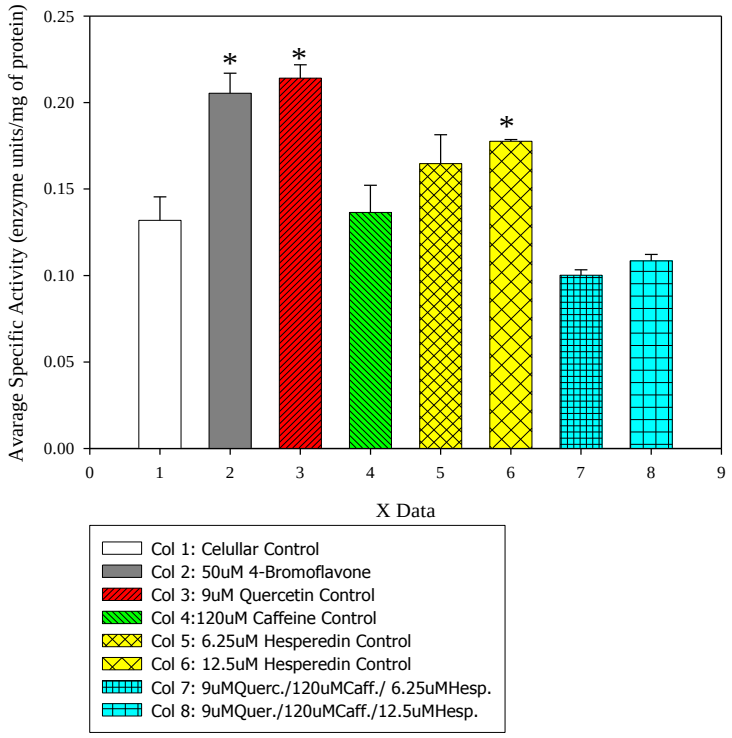
In general phytochemicals did not show induction of GST specific activity using CDNB as a substrate on Hepa 1c1c7 cells when compared to the controls. On the other hand, when GST specific activity was measured using 4NQO as a substrate, some phytochemicals showed GST induction when compared with the control. The specific activity of QR was also not induced in presence of the phytochemicals on Hepa 1c1c7 cells when compared with the control. Experimental groups that showed significant induction of GST were compared with their

using 4NQO as a substrate, some experimental groups showed GST induction when compared with the cellular control's specific activity. The specific activity of QR was not induced using the experimental groups as a treatment on Hepa 1c1c7 cells when compared with the cellular control's specific activity. Experimental groups that showed significant induction of GST (compared to the cellular control) were compared with their corresponding flavonoid controls, and it was observed that the experimental groups did not have higher induction compared to them.

In the case of MCF7 cell line, only the QR assay was performed due to consistently low and sometimes negative results obtained during the pilot study in this cell line. This decision was reinforced with a previous experiment run in the lab; this previous project proved that GST was not induced when breast cancer cells were treated with Quercetin or when cells were treated with pools of flavonoids (with Quercetin). The results in QR assay were promising in this cell line, when the experimental samples were compared with the cellular control's specific activity of QR; but when the experimental samples were compared with their corresponding flavonoid control most of the experimental groups had lower QR activity. Some results proved that the objective of caffeine as protector was correct, and avoided cell death in front of the flavonoid pool and in some cases induction was observed.

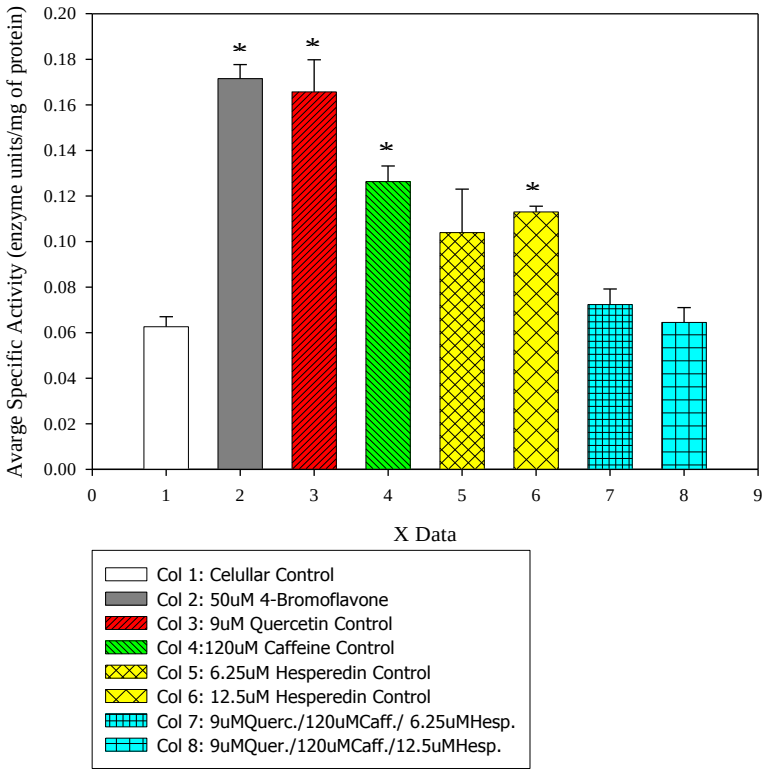
8.1a

Hepa1c1c7 treated with 9uMQuerc./ 120uMCaff./ 6.25uM and 12.5uM Hesp.:
GST/CDNB Avarge Specific Activity (enzyme units/mg of protein)

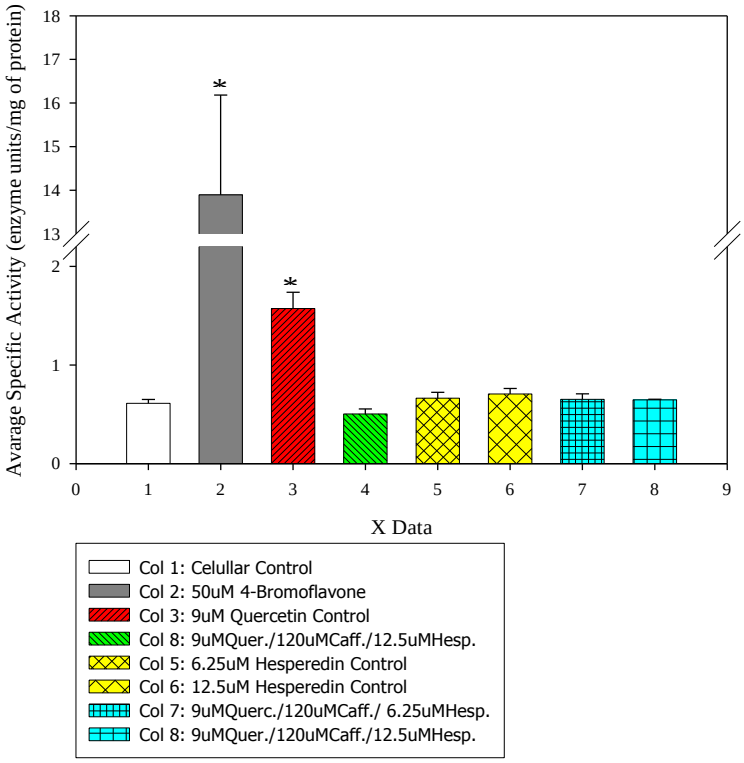


8.1b

Hepa1c1c17 treated with 9uMQuerc./ 120uMCaff./ 6.25uM and 12.5uM Hesp.:
GST/4NQO Avarage Specific Activity (enzyme units/mg of protein)



8.1c Hepa1c1c17 treated with 9uMQuerc./ 120uMCaff./ 6.25uM and 12.5uM Hesp.:
QR Avarage Specific Activity (enzyme units/mg of protein)



8.1d MCF7 treated with 9uMQuerc./ 120uMCaff./ 6.25uM and 12.5uM Hesp.:
QR Avarage Specific Activity (enzyme units/mg of protein)

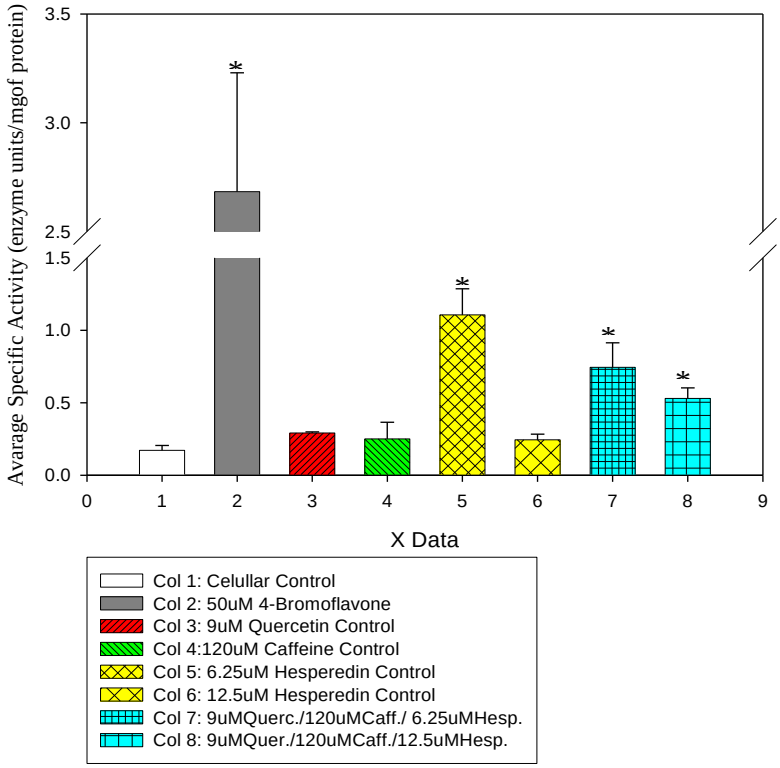


Figure 8.1: GST and QR Specific Activity on Hepa1c1c7 cell line/ QR Specific Activity on MCF7 cell line by 9µM Quercetin/ 120µM Caffeine/ 6.25 and 12.5µM Hesperidin experimental groups: a) GST analysis using CDNB as a substrate, b) GST analysis using 4NQO as a substrate and c) QR: NADPH analysis using DCPIP as substrate. QR Average Specific Activity on MCF 7 cell line d) QR using DCPIP as a substrate.

Figure 8.1 illustrates experimental groups 9µM Quercetin/ 120µM Caffeine/6.25 Hesperedin and 9µM Quercetin/120µM Caffeine/12.5µM Hesperedin. Sections a, b and c on this figure shows the results of three different assays done on Hepa1c1c7 cells:

8.1a Evaluated the average specific activity of GST using CDNB as a substrate from both experimental groups versus the cell control; in this analysis both experimental groups did not show induction of the GST enzyme activity, but it did not affect the GST activity of Hepa1c1c7 since $p>0.05$ (Table9). From the flavonoids contained in the experimental group Quercetin 9µM and Hesperedin 12.5µM showed induction of GST activity when they were compared to the cell control since $p<0.05$ (Table 9)

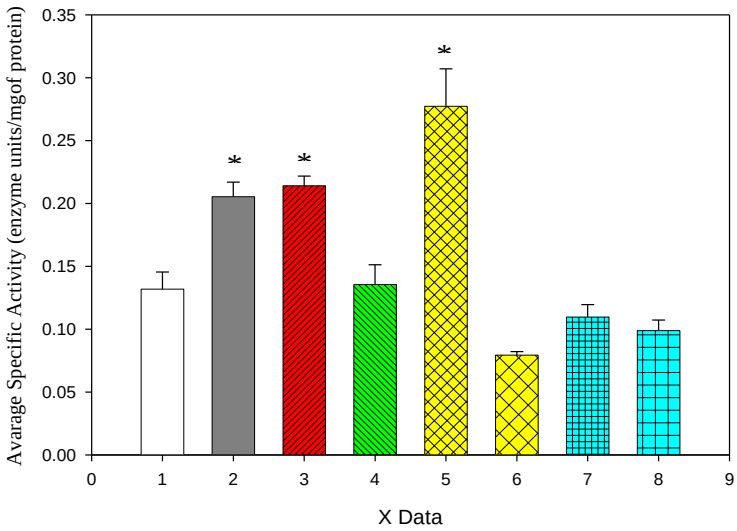
8.1b Evaluated the average specific activity of GST using 4NQO as a substrate, in this graph induction on GST activity by the experimental group induction was not revealed and an adverse effect is not observed when these experimental groups were compared to the cell control since $p>0.05$ (Table 10). From the flavonoids contained in the experimental group Quercetin 9µM, Caffeine 120µM and Hesperedin 12.5µM showed induction of GST activity when compared to the cell control since $p<0.05$ (Table 10).

8.1c Evaluated the average specific activity of QR when DCPIP is used as substrate revealing no induction of GST activity by the experimental groups. There was not a negative effect in the cells by the experimental groups since $p>0.05$. From the flavonoids contained in the experimental groups just Quercetin showed induction compared to the cell control since $p<0.05$ (Table 11).

Section d) on figure 8.1 shows the results from QR assay done on MCF7 cells:

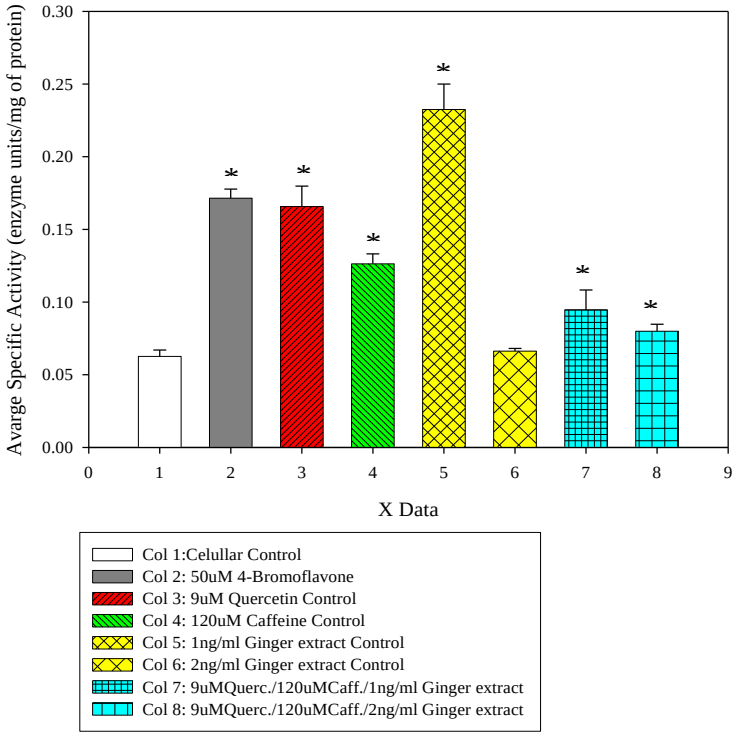
8.1d Evaluated the average specific activity of QR using DCPIP as a substrate; this analysis revealed induction of QR activity when the cells were treated with these experimental groups on the MCF7 cells, since the specific activity average was higher than the cell control , this was confirmed with $p<0.05$. From the flavonoids contained in the experimental group, Quercetin and 6.25µM Hesperedin Control showed induction on QR activity compared to the cell control (Table 12).

8.2a Hepa1c1c7 treated with 9uMQuerc./120uMCaff./1ng/ml and 2ng/ml of Ginger extract: GST/CDNB Avarage Specific Activity (enzyme units/mg of protein)

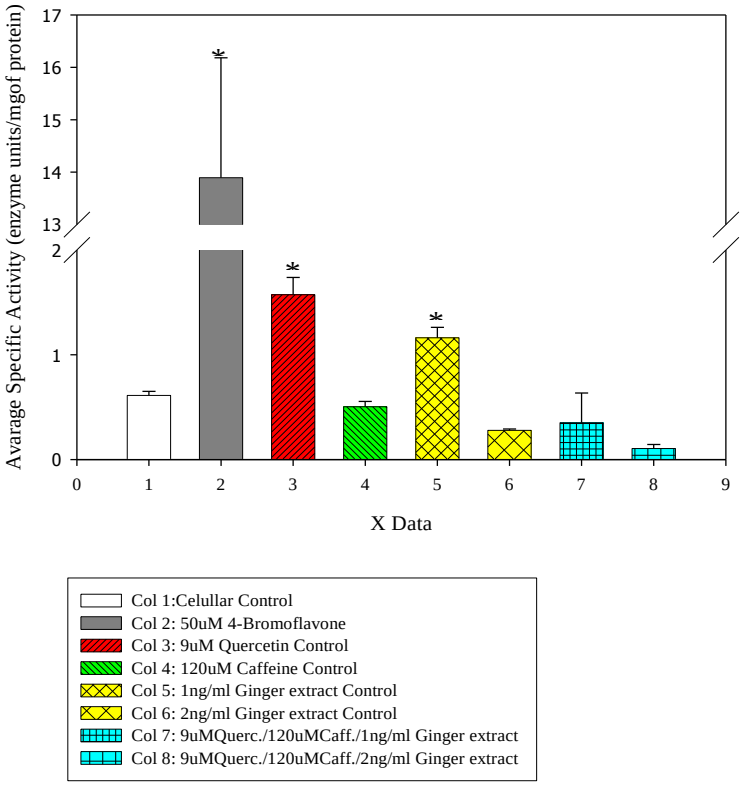


Col 1:Celullar Control
Col 2: 50uM 4-Bromoflavone
Col 3: 9uM Quercetin Control
Col 4: 120uM Caffeine Control
Col 5: 1ng/ml Ginger extract Control
Col 6: 2ng/ml Ginger extract Control
Col 7: 9uMQuerc./120uMCaff./1ng/ml Ginger extract
Col 8: 9uMQuerc./120uMCaff./2ng/ml Ginger extract

8.2b Hepa1c1c7 treated with 9uMQuerc./120uMCaff./1ng/ml and 2ng/ml of Ginger extract: GST/4NQO Avarage Specific Activity (enzyme units/mg of protein)



8.2c Hepa1c1c7 treated with 9uMQuerc./120uMCaff./1ng/ml and 2ng/ml of Ginger extract: QR Avarage Specific Activity (enzyme units/mg of protein)



8.2d MCF7 treated with 9uMQuerc./120uMCaff./1ng/ml and 2ng/ml of Ginger extract:
QR Avarage Specific Activity (enzyme units/mg of protein)

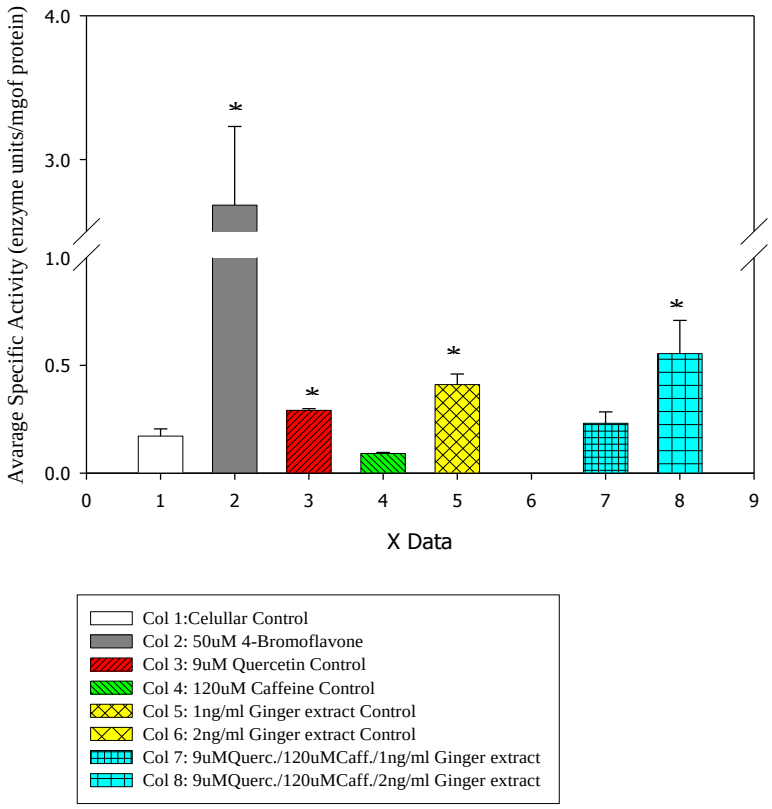


Figure 8.2: GST and QR Specific Activity on Hepa1c1c7 cell line/ QR Specific Activity on MCF7 cell line by 9µM Quercetin/ 120µM Caffeine/ 1ng/mL and 2ng/mL Ginger extract experimental groups: a) GST analysis using CDNB as a substrate, b) GST analysis using 4NQO as a substrate and c) QR: NADPH analysis using DCPIP as substrate. QR Average Specific Activity on MCF 7 cell line d) QR: NADPH using DCPIP as a substrate.

Figure 8.2 illustrates experimental groups 9µM Quercetin/ 120µM Caffeine/1ng/mL Ginger extract and 9µM Quercetin/120µM Caffeine/2ng/mL Ginger extract. Sections a, b and c in this figure shows the results of three different assays done on Hepa1c1c7 cells:

8.2a Evaluated the average specific activity of GST using CDNB as a substrate from both experimental groups versus the cell control; experimental groups did not display induction of the

GST enzyme activity, adverse effect on the GST activity of Hepa1c1c7was not observed since $p>0.05$when compared to the cell control (Table 13). The flavonoids contained in the experimental group 9µM Quercetin and 1ng/mL Ginger extract showed induction of GST activity when were compared to the cell control, since $p<0.05$ (Table 13). From the flavonoids controls, only 2ng/mL Ginger extract control had an adverse effect on GST specific activity revealing lower specific activity average compared to the cell control and $p<0.05$.

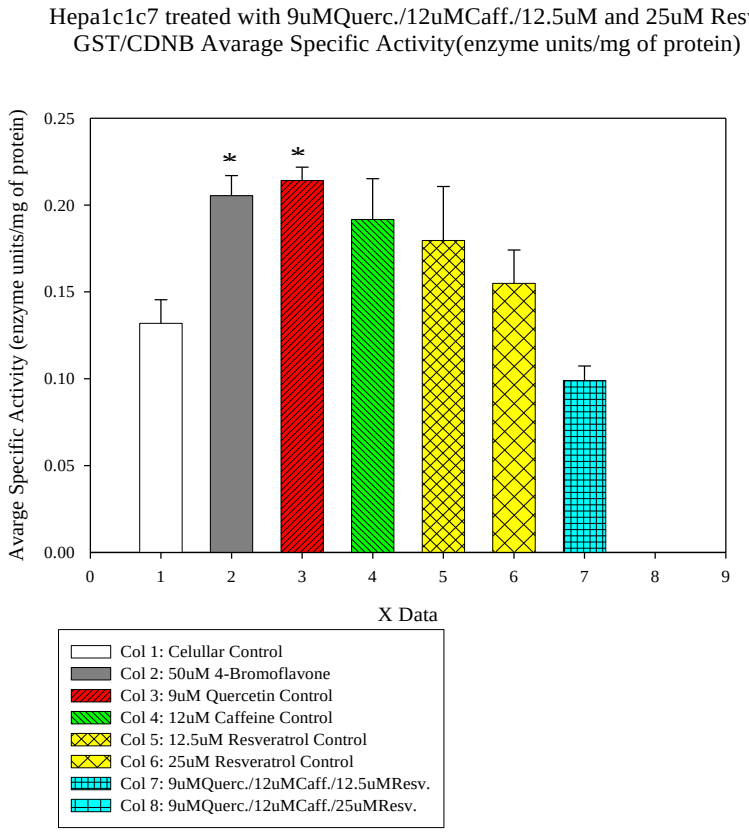
8.2b Evaluated the average specific activity of GST using 4NQO as a substrate, this graph displayed induction of GST when compared to the cell control, even though $p>0.05$, the higher specific activity average and the lower standard error suggest us the induction from both experimental groups (Table 14). From the components which form the experimental groups, only Quercetin 9µM, Caffeine 120µM and 1ng/mL Ginger extract controls exhibit induction of GST activity compared to the cell control.

8.2c Evaluated the average specific activity of QR when DCPIP was used as substrate, was not evidence of GST induction by the experimental group with 1ng/ml Ginger extract, an adverse effect in one experimental group with 2ng/mL Ginger extract since average specific activity was less than cell control and p value confirm this adverse effect was observed since $p<0.05$. From the flavonoids contained in the experimental groups, 9µM Quercetin and 1ng/mL Ginger extract controls showed induction compared to the cell control since specific activity average was higher than the cell control and $p<0.05$ (Table 15). An adverse effect in 2ng/mL Ginger extract control was observed since it had lower specific activity than the cell control and $p<0.05$ (Table 15).

Section d) on figure 8.2 shows the results from QR assay done on MCF7 cells:

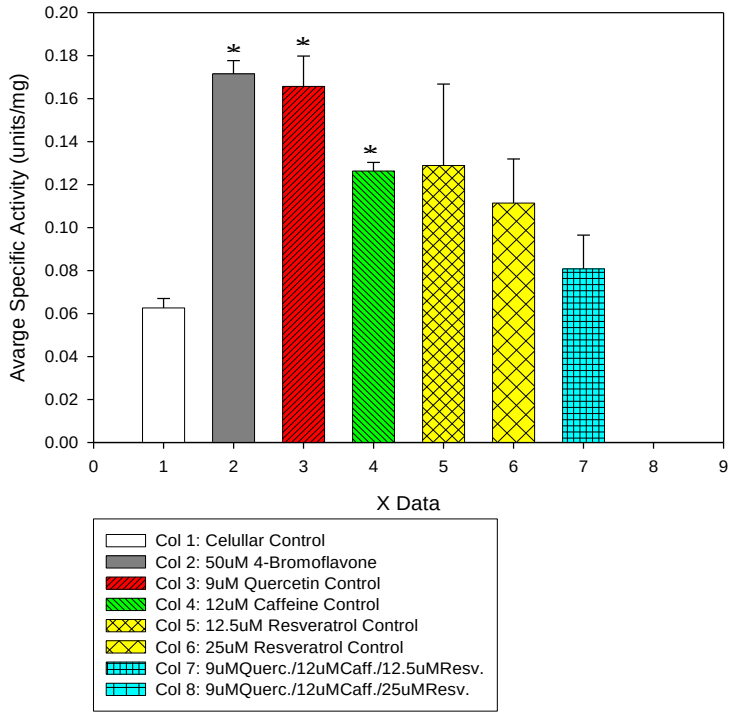
8.2d Evaluated the average specific activity of QR using DCPIP as a substrate; in this analysis was observed induction of QR activity by the experimental group with 2ng/mL Ginger extract when compared with the specific activity average of the cell control, but p-value was higher than 0.05, this was the result of the variation presented in the triplicate; on the other hand if the triplicate was analyzed individually result from each sample of the triplicate was higher than the specific activity average of the cell control. On the other hand the experimental group with 1ng/mL Ginger extract did not affect the specific activity of QR in the MCF7 cells. Of the flavonoids that form these experimental groups Quercetin, 1ng/mL Ginger extract control showed induction of QR since $p<0.05$ (Table 16) when compared to the cell control and 2ng/mL Ginger extract had an adverse effect since did not reflect any GST induction on the cells.

8.3a



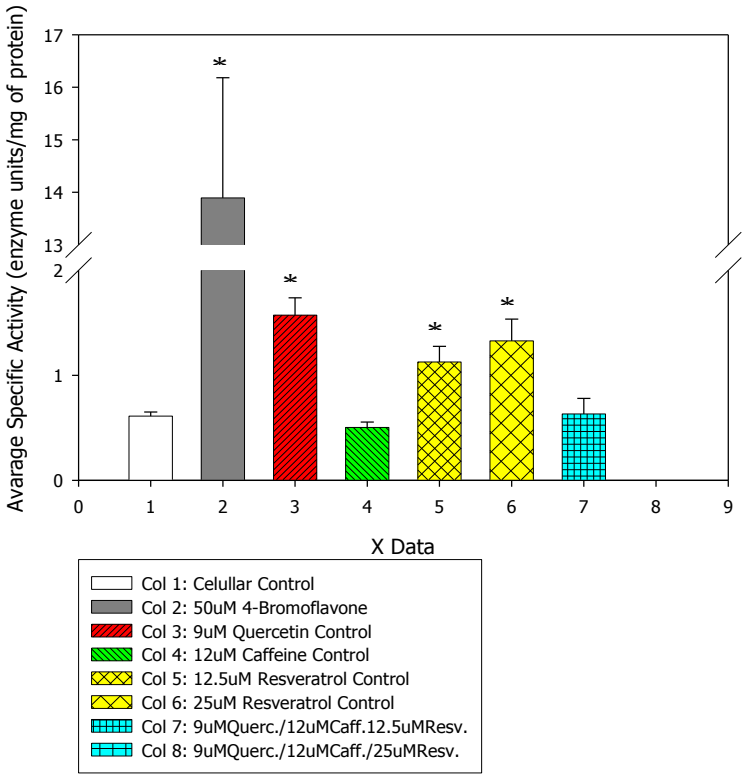
8.3b

Hepa1c1c7 treated with 9uMQuerc./12uMCaff./12.5uM and 25uM Resv.
GST/4NQO Avarage Specific Activity(units/mg)



8.3c

Hepa1c1c7 treated with 9uMQuerc./12uMCaff./12.5uM and 25uM Resv.
QR Avarage Specific Activity (enzyme units/mg of protein)



8.3d

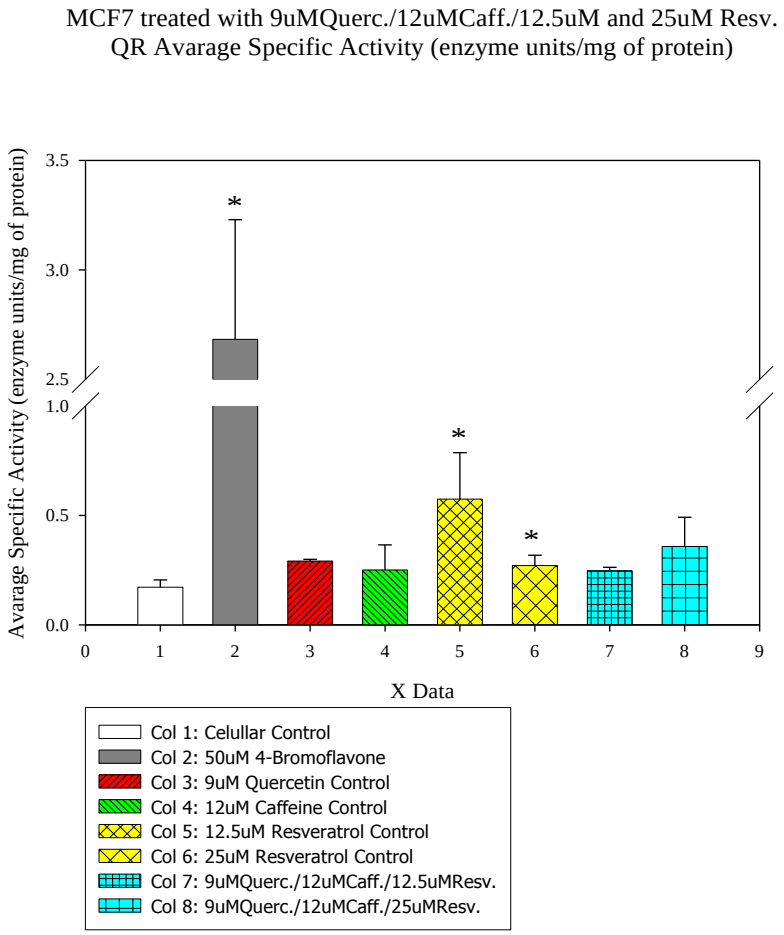


Figure 8.3: GST and QR Specific Activity on Hepa1c1c7 cell line/ QR Specific Activity on MCF7 cell line by 9µM Quercetin/ 12µM Caffeine/ 12.5 and 25µM Resveratrol experimental groups: a) GST analysis using CDNB as a substrate, b) GST analysis, using 4NQO as a substrate and c) QR: NADPH analysis using DCPIP as substrate. QR Average Specific Activity on MCF 7 cell line d) QR: NADPH using DCPIP as a substrate.

Figure 8.3 illustrates experimental groups 9µM Quercetin/ 120µM Caffeine/12.5 Resveratrol and 9µM Quercetin/120µM Caffeine/25µM Resveratrol. Sections a, b and c on this figure shows the results of three different assays done on Hepa1c1c7 cells:

8.3a Evaluated the average specific activity of GST using CDNB as a substrate from both experimental groups versus the cell control; experimental groups did not showed induction of the GST activity; the experimental group with 25µM Resveratrol exhibited an adverse effect since

GST activity was not evident. Only 9µM Quercetin control show induction on GST activity compared with the cell control.

8.3b Evaluated the average specific activity of GST using 4NQO as a substrate, this graph did not display induction of GST activity, but an adverse effect was observed on the experimental with 25µM Resveratrol since any GST activity was displayed. Of the components of the experimental groups, only 9µM Quercetin and 120µM Caffeine controls exhibited induction of GST activity, since specific activity average was higher than cell control and $p<0.05$ (Table 18).

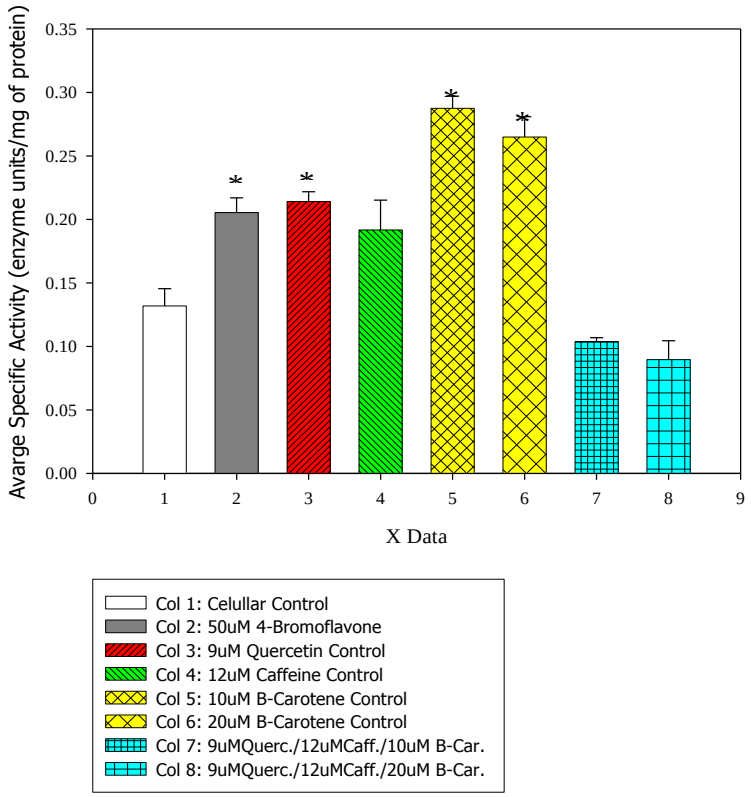
8.3c Evaluated the average specific activity of QR when DCPIP was used as substrate, no evidence of QR induction by the experimental groups was observed. But experimental group with 25µM Resveratrol did not produce any GST activity. The controls 9µM Quercetin, 12µM Caffeine, 12.5µM and 25µM Resveratrol showed induction compared to the cell control since specific activity average was higher than the cell control and $p<0.05$ (Table 19)

Section d) on figure 8.3 shows the results from QR assay done on MCF7 cells:

8.3d Evaluated the average specific activity of QR activity using DCPIP as a substrate, in this analysis QR activity was induced by the experimental groups when compared with the specific activity average of the cell control, but p-value was higher than 0.05, this was the result of the variation presented in the triplicate (Table 20). From the flavonoids that form these experimental groups, only 9µM Quercetin control showed induction of QR since $p<0.05$ and specific average activity was higher when compared to the cell control (Table 20).

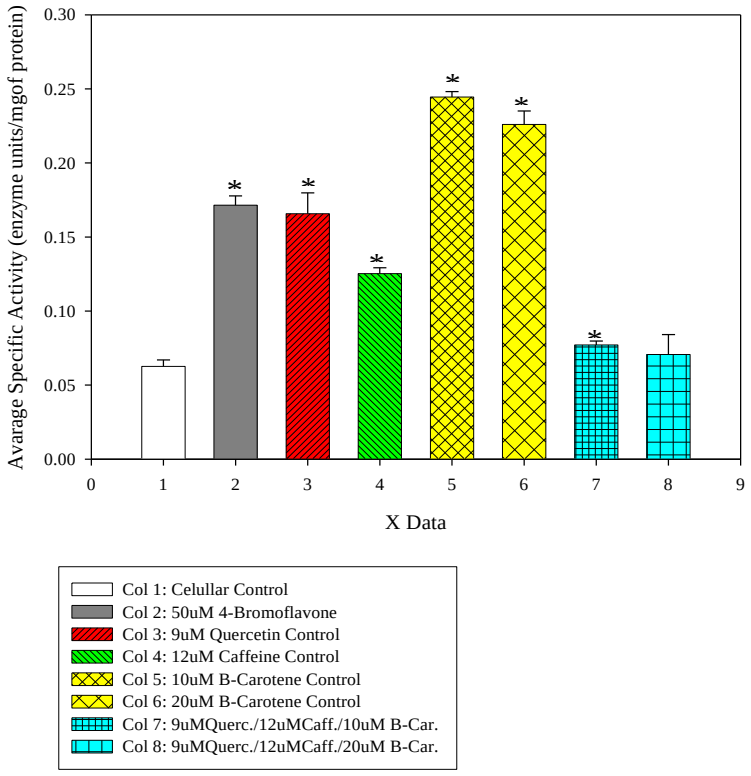
8.4a

Hepa1c1c7 treated with 9uMQuerc./12uMCaff./ 10uM and 20uM B-Car.:
GST/CDNB Avarage Specific Activity(enzyme units/mg of protein)



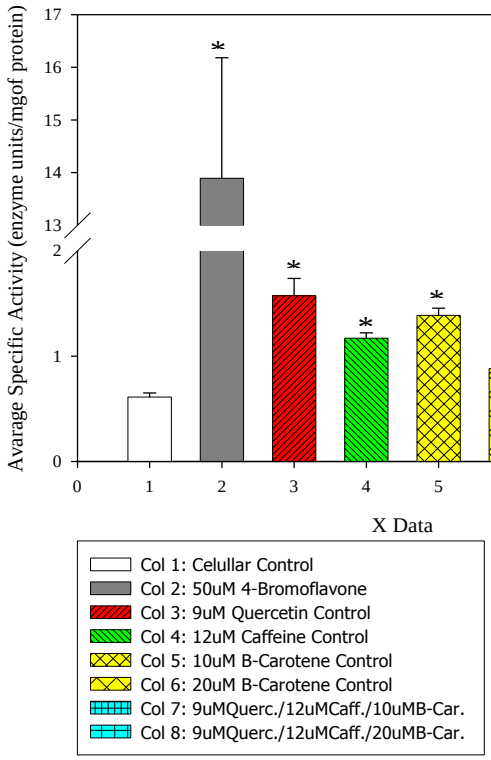
8.4b

Hepa1c1c7 treated with 9uMQuerc./12uMCaff./ 10uM and 20uM B-Car.:
GST/4NQO Avarage Specific Activity (enzyme units/mg of protein)



8.4c

Hepa1c1c7 treated with 9uMQuerc./12uMCaff./ 10uM and 20uM B-Car.:
QR Avarage Specific Activity (enzyme units/mg of protein)



8.4d

MCF7 treated with 9uMQuerc./12uMCaff./ 10uM and 20uM B-Car.:
QR Avarage Specific Activity (enzyme units/mg of protein)

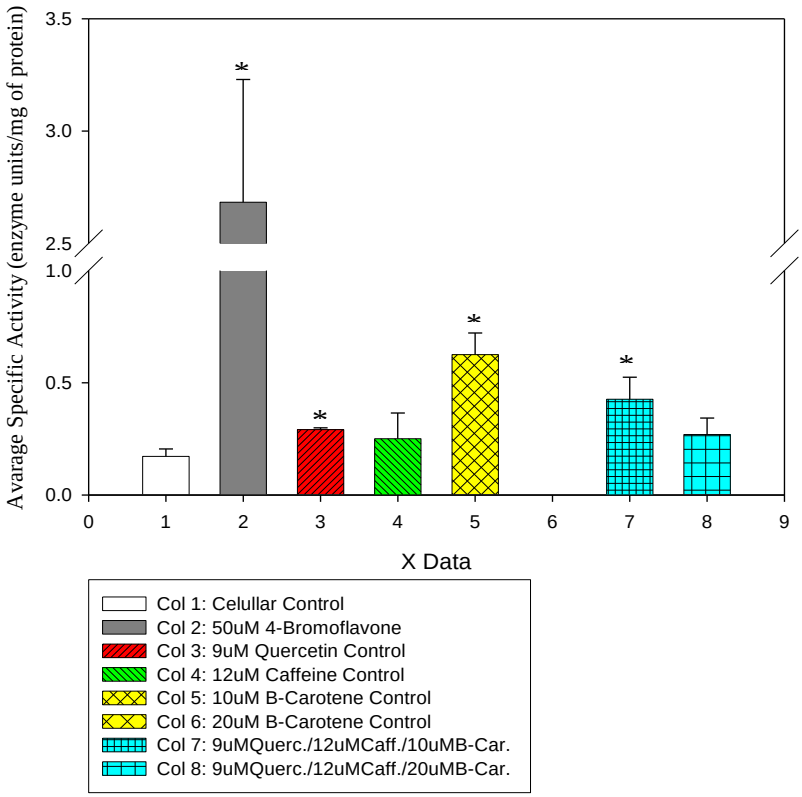


Figure 8.4 GST and QR Specific Activity on Hepa1c1c7 cell line/ QR Specific Activity on MCF7 cell line by 9µM Quercetin/ 12µM Caffeine/ 10 and 20µM β-Carotene experimental groups: a) GST analysis using CDNB as a substrate, b) GST analysis, using 4NQO as a substrate and c) QR: NADPH analysis using DCPIP as substrate. QR Average Specific Activity on MCF 7 cell line d) QR: NADPH using DCPIP as a substrate.

Figure 8.4 illustrates experimental groups 9µM Quercetin/ 12µM Caffeine/10 β-Carotene and 9µM Quercetin/12µM Caffeine/20µM β-Carotene. Sections a, b and c on this figure shows the results of three different assays done on Hepa1c1c7 cells:

8.4a Evaluated the average specific activity of GST using CDNB as a substrate from both experimental groups versus the cell control; experimental groups did not showed induction or an adverse effect of the GST activity when compared to the specific activity average of cell control. 9µM Quercetin, 12µM Caffeine, 10µM and 20µM β-Carotene control show induction on GST activity compared with the specific activity average of the cell control and $p<0.05$ confirmed it (Table 21).

8.4b Evaluated the average specific activity of GST using 4NQO as a substrate, this graph displayed GST activity induction by experimental group with 10µM of β-Carotene since the specific average was higher than the cell control as well as $p<0.05$ (Table 22), this induction was not relevant when compared by the individual induction that produced each flavonoid that make up the experimental group.

8.4c Evaluated the average specific activity of QR when DCPIP was used as substrate, not evidence of QR induction by the experimental groups was not observed; but experimental group with 20µM β-Carotene produced an adverse effect since it had less specific activity average than the cell control and the statistical difference was confirmed

by $p<0.05$ (Table 23). 9µM Quercetin, 12µM Caffeine, 10µM and 20µM β-Carotene controls produced induction of GST activity since they had higher specific activity average than cell control and the statistical difference is confirmed by $p<0.05$ (Table 23).

Section d) on figure 8.4 shows the results from QR assay done on MCF7 cells:

8.4d Evaluated the average specific activity of QR activity using DCPIP as a substrate, in this analysis QR activity was induced by the experimental groups when were compared with the specific activity average of the cell control, but p-value was higher than 0.05, this was the result of the variation presented in the triplicate (Table 24). Of the flavonoids that form these experimental groups, 9µMQuercetin and 10µM β-Carotene control, showed induction of QR since $p<0.05$ and specific average activity was higher when compared to the cell control; the 20µM β-Carotene control displayed an adverse effect since there was not GST activity in this experimental group (Table 24).

CHAPTER IX

CONCLUSION

Hepa-1c1c7 cell line was treated with the experimental groups (Quercetin in a constant concentration+ Caffeine in a constant concentration+ Second phytochemical in different concentrations), and the specific activity average of cellular control was compared between them. The comparison reflected the lack of GST induction by the experimental groups on the cell line when CDNB was used as substrate. On the other hand, when 4NQO was used as a substrate, the experimental groups with 1ng/mL Ginger extract, 2ng/mL Ginger (second phytochemical in the pool, Figure 8.2b), and 10μM β-Carotene (Figure 8.4 d) showed GST induction, when compared to the cellular control's specific activity average. Induction of QR activity on Hepa-1c1c7 cells was not observed by the experimental groups in this study. Flavonoids controls were created in order to visualize if the flavonoids that formed the experimental groups displayed induction of GST and QR activity.

When MCF 7 cell line was treated with the experimental groups, promising results were observed when QR determination was analyzed, the results showed induction on MCF7 cell line using experimental group with Hesperedin in both concentrations (Figure 8.1d). These groups have higher specific activity average than the cellular control, Quercetin control and 12.5μM

Hesperedin control; QR induction was observed using experimental group with 2ng/mL Ginger extract. This induction was higher than the cellular control and flavonoids controls. QR induction was observed in cells treated with the experimental group with 10µM β-Carotene (Figure 8.4 d), this group produced higher induction than the cellular control and the Quercetin Control. 20µM β-Carotene displayed a higher specific activity average compared to all flavonoids controls, but statistically it was not relevant in this sample due to the significant variance between the samples that formed the triplicate in this group. It is also important to mention that the protective properties that caffeine could be observed in experimental group with 2ng/mL of Ginger extract.

The results in this study were not promising by almost all the phytochemicals using this experimental model, except with the 2ng/ml of Ginger, since the specific activity was higher than the cell control as well as the phytochemicals control. Nevertheless, it is expected that in an *in vivo* study of these results could provide a better and more consequential results, because the absorption of caffeine in the blood plasma is established in literature which may have a better response to the induction of Phase II enzymes.

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APPENDIX A

APPENDIX A

TABULAR DATA FROM ASSAYS PART I

Table 1: MTT Assay- Day 1 Elasthane™ 55D NF (595nm)

MTT Assay- Day 1 Elasthane™ 55D NF (595nm):

TTest: Cell Control- NIH 3T3 cells (fibroblasts) incubated for 1 Day

	Blank	**Std 1**	**Std 2**	**Std 3**	**Std 4**	**Std 5**	**Std 6**	**NF**
1	0.086	0.133	0.242	0.502	0.915	0.960	1.269	0.482
2	0.070	0.123	0.241	0.524	0.839	0.969	1.283	0.725
3	0.088	0.142	0.228	0.503	0.830	0.973	1.283	0.611
average	0.080	0.128	0.237	0.514	0.858	0.981	1.301	0.495
std. deviation	0.008	0.013	0.005	0.018	0.048	0.045	0.071	0.226
(std. dev.^2)/ n	2.40E-05	5.40E-05	8.17E-06	1.04E-04	7.71E-04	6.62E-04	1.70E-03	1.70E-02
std. error	0.004	0.006	0.002	0.009	0.024	0.022	0.036	0.113

TTest: Cell Control- NIH 3T3 cells (fibroblasts) exposed to Elasthane™ 55D NF for Day 1

	Blank	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
1	0.101	0.082	0.692	0.781	0.718	1.36	1.119
2	0.071	0.072	0.684	0.42	1.112	1.522	1.055
3	0.078	0.068	0.736	0.353	0.956	0.933	1.953
4	0.076	0.064	0.434	0.583	0.482	0.87	1.245
average	0.082	0.072	0.637	0.534	0.817	1.171	1.343
std. deviation	0.0177	0.0127	0.1369	0.1400	0.1669	0.3465	0.0891
(std. dev.^2)/ n	6.25E-05	5.40E-05	6.25E-03	6.53E-03	9.28E-03	4.00E-02	2.65E-03
std. error	0.009	0.006	0.068	0.070	0.083	0.173	0.045

Table 2: MTT Assay- Day 1 Elasthane™ 75D NF (595nm)

MTT Assay- Day 1 Elasthane™ 75D NF (595nm):

TTest: Cell Control- NIH 3T3 cells (fibroblasts) incubated for 1 Day

	Blank	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	NF
1	0.095	0.504	1.092	1.672	2.100	2.160	2.554	0.217
2	0.094	0.547	1.169	1.884	2.109	2.395	2.561	0.360
3	0.087	0.509	1.104	2.074	1.981	2.056	2.545	0.217
average	0.087	0.525	1.123	1.848	2.054	2.213	2.531	0.228
std. deviation	0.016	0.026	0.025	0.064	0.054	0.057	0.065	0.071
(std. dev.^2)/ n	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
std. error	0.008	0.013	0.012	0.032	0.027	0.029	0.033	0.035

TTest: Cell Control- NIH 3T3 cells (fibroblasts) exposed to Elasthane™ 75D NF for 1 Day

	Blank	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
1	0.082	0.066	0.921	1.572	1.719	3.251	2.036
2	0.080	2.409	0.907	1.456	2.087	1.999	2.316
3	0.073	0.582	0.703	1.386	1.583	1.990	1.919
4	0.099	0.387	0.437	1.143	2.172	1.956	1.742
average	0.084	0.861	0.742	1.390	1.891	2.299	2.004
std. deviation	0.012	0.227	0.226	0.303	0.320	0.688	0.208
(std. dev.^2)/ n	2.89E-05	1.03E-02	1.03E-02	1.84E-02	2.05E-02	9.47E-02	8.64E-03
std. error	0.006	0.114	0.113	0.152	0.160	0.344	0.104

Table 3: MTT Assay- Day 1 Biospan® NF (595nm)

MTT Assay- Day 1 Biospan® NF (595nm):

TTest: Cell Control- NIH 3T3 cells (fibroblasts) incubated for 1 Day

	Blank	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	NF
1	0.080	0.134	0.289	0.074	0.149	0.268	0.637	0.585
2	0.064	0.144	0.257	0.079	0.124	0.210	0.400	0.260
3	0.075	0.137	0.238	0.112	0.140	0.201	0.383	0.312
average	0.073	0.138	0.261	0.088	0.138	0.226	0.473	0.386
std. deviation	0.004	0.002	0.036	0.027	0.006	0.047	0.180	0.193
(std. dev.^2)/ n	2.50E-06	9.00E-07	2.60E-04	1.44E-04	8.10E-06	4.49E-04	6.45E-03	7.45E-03
std. error	0.002	0.001	0.021	0.016	0.004	0.027	0.104	0.111

TTest: Cell Control- NIH 3T3 cells (fibroblasts) exposed to Biospan® NF for 1 Day

	Blank	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
1	0.077	0.349	0.290	0.501	0.902	0.820	1.482
2	0.067	0.327	0.364	0.494	0.944	0.841	1.354
3	0.067	0.334	0.291	0.483	0.964	0.900	1.374
average	0.070	0.337	0.315	0.493	0.937	0.854	1.403
std. deviation	0.007	0.011	0.001	0.013	0.044	0.057	0.076
(std. dev.^2)/ n	1.00E-05	2.25E-05	1.00E-07	3.24E-05	3.84E-04	6.40E-04	1.17E-03
std. error	0.004	0.006	0.000	0.007	0.025	0.033	0.044

Table 4: MTT Assay- Day 1 Hydrothane™ NF (595nm)

MTT Assay- Day 1 Hydrothane™ NF (595nm):

TTest: Cell Control- NIH 3T3 cells (fibroblasts) incubated for 1 Day

	Blank	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	NF
1	0.101	0.640	1.024	1.703	2.203	2.196	2.590	0.514
2	0.098	0.666	1.139	1.591	1.924	2.030	2.549	0.684
3	0.120	0.695	1.187	1.596	2.116	1.965	2.476	0.631
average	0.105	0.657	1.099	1.607	2.087	2.059	2.501	0.656
std. deviation	0.010	0.010	0.014	0.118	0.071	0.107	0.143	0.198
(std. dev.^2)/ n	2.04E-05	1.96E-05	4.00E-05	2.79E-03	1.00E-03	2.28E-03	4.08E-03	7.84E-03
std. error	0.005	0.005	0.007	0.059	0.035	0.053	0.071	0.099

TTest: Cell Control- NIH 3T3 cells (fibroblasts) exposed to Hydrothane™ NF for 1 Day

\	**Blank**	**Std 1**	**Std 2**	**Std 3**	**Std 4**	**Std 5**	**Std 6**
1	0.107	0.686	1.330	1.043	1.271	1.548	2.149
2	0.088	0.728	0.878	1.807	2.015	1.931	1.830
3	0.101	0.776	1.244	1.307	1.908	1.640	1.871
4	0.085	0.723	1.905	0.981	1.914	2.605	2.552
average	0.095	0.728	1.339	1.285	1.777	1.931	2.101
std. deviation	0.016	0.026	0.425	0.044	0.455	0.747	0.285
(std. dev.^2)/ n	4.84E-05	1.37E-04	3.61E-02	3.84E-04	4.13E-02	1.12E-01	1.62E-02
std. error	0.008	0.013	0.213	0.022	0.227	0.374	0.142

Table 5: MTT Assay- Day 1 Lycra® NF (595nm)

MTT Assay- Day 1 Lycra® NF (595nm):

TTest: Cell Control- NIH 3T3 cells (fibroblasts) incubated for 1 Day

	Blank	**Std 1**	**Std 2**	**Std 3**	**Std 4**	**Std 5**	**Std 6**	**NF**
1	0.085	0.141	0.219	0.477	0.937	1.047	1.364	0.122
2	0.069	0.102	0.201	0.509	0.965	1.140	1.375	0.141
3	0.085	0.074	0.228	0.523	0.970	1.196	1.458	0.136
average	0.076	0.100	0.217	0.521	0.973	1.124	1.401	0.137
std. deviation	0.013	0.042	0.001	0.068	0.059	0.045	0.029	0.018
(std. dev.^2)/ n	3.61E-05	3.60E-04	1.00E-07	9.22E-04	7.06E-04	4.10E-04	1.68E-04	6.25E-05
std. error	0.007	0.021	0.000	0.034	0.030	0.023	0.014	0.009

TTest: Cell Control- NIH 3T3 cells (fibroblasts) exposed to Lycra® NF for 1 Day

	Blank	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
1	0.077	0.202	0.327	0.972	0.989	1.053	1.237
2	0.072	0.224	0.320	0.543	1.041	1.244	1.093
3	0.072	0.277	0.332	0.557	0.901	1.111	1.939
4	0.071	0.203	0.562	0.533	0.777	0.970	1.142
average	0.073	0.227	0.385	0.651	0.927	1.095	1.353
std. deviation	0.004	0.001	0.118	0.310	0.150	0.059	0.067
(std. dev.^2)/ n	3.60E-06	1.00E-07	2.78E-03	1.93E-02	4.49E-03	6.89E-04	9.03E-04
std. error	0.002	0.000	0.059	0.155	0.075	0.029	0.034

Table 6: T-Test One Day-MTT determination on EPNs at 595nm

T-Test One Day-MTT determination on EPNs at 595nm: 25,000 cells/mL starting solution

Absorbances	Media Control	55	75	B	H	L
1	0.478	0.536	0.525	0.374	0.471	0.456
2	0.422	0.462	0.406	0.404	0.436	0.509
3	0.524	0.489	0.468	0.383	0.464	0.451
average	0.475	0.496	0.466	0.387	0.457	0.472
std. deviation	0.051	0.037	0.060	0.015	0.019	0.032
(std. dev.^2)/ n	5.22E-04	2.80E-04	7.08E-04	4.74E-05	6.86E-05	2.07E-04
p-value						
t-value						
std. error	0.029	0.022	0.034	0.009	0.011	0.019

	E55	E75	B	H	L
P Value	0.5993	0.8629	0.0461	0.5968	0.9427
T Value	0.0212	0.0086	0.0948	0.0187	0.0027

BLANKS

Absorbances	Media	55	75	B	H	L
1	0.104	0.103	0.176	0.276	0.206	0.285
2	0.105	0.142	0.146	0.247	0.189	0.283
3	0.113	0,153	0.159	0.271	0.153	0.249
average	0.107	0.123	0.160	0.265	0.183	0.272

Table 7: T-Test Third Day-MTT determination on EPNs at 595nm

T-Test Third Day-MTT determination on EPNs at 595nm: 25,000 cells/mL starting solution

Absorbances	Media Control	55	75	B	H	L
1	0.606	0.597	0.473	0.795	0.749	1.051
2	0.577	0.616	0.462	0.847	0.769	0.899
3	0.621	0.541	0.449	0.713	0.751	0.994
average	0.601	0.585	0.461	0.785	0.756	0.981
std. deviation	0.022	0.039	0.012	0.068	0.011	0.077
(std. dev.^2)/ n	1.00E-04	3.04E-04	2.89E-05	9.13E-04	2.43E-05	1.18E-03
p-value						
t-value						
std. error	0.013	0.023	0.007	0.039	0.006	0.044

Exp.	55	75	B	H	L
P Value	0.5557	0.0007	0.0111	0.0004	0.0012
T Value	0.0153	0.1358	0.1557	0.1330	0.3018

BLANKS

Absorbances	Media	55	75	B	H	L
1	0.139	0.183	0.224	0.305	0.206	0.201
2	0.158	0.202	0.219	0.270	0.190	0.195
3	0.143	0.202	0.234	0.187	0.206	0.153
average	0.147	0.196	0.226	0.254	0.201	0.183

Table 8: T-Test Seventh Day-MTT determination on EPNs at 595nm

T-Test Seventh Day-MTT determination on EPNs at 595nm: 25,000 cells/mL starting

Absorbances	Media Control	55	75	B	H	L
1	1.251	1.021	1.148	1.103	0.899	0.854
2	1.345	1.043	1.125	1.208	0.856	0.702
3	1.312	1.085	1.064	1.208	0.940	0.797
average	1.303	1.049	1.113	1.173	0.899	0.785
std. deviation	0.048	0.033	0.043	0.061	0.042	0.077
(std. dev.^2)/ n	4.55E-04	2.11E-04	3.77E-04	7.35E-04	3.53E-04	1.18E-03
p-value						
t-value						
std. error	0.028	0.019	0.025	0.035	0.024	0.044

Exp.	55	75	B	H	L
P Value	0.0016	0.0070	0.0436	0.0004	0.0006
T Value	0.1652	0.1223	0.0824	0.2723	0.3585

BLANKS

Absorbances	Media	55	75	B	H	L
1	0.113	0.151	0.132	0.129	0.219	0.318
2	0.083	0.196	0.187	0.153	0.178	0.391
3	0.093	0.120	0.116	0.187	0.173	0.278
average	0.096	0.156	0.145	0.156	0.190	0.329

TABULAR DATA FROM ASSAYS PART II

Table 9: T-Test GST/CDNB Hepa1c1c7-

9µM Quercetin/120µM Caffeine/6.25µM and 12.5µM Hesperedin

T-Test GST/CDNB Hepa1c1c7- 9µM Quercetin/120µM Caffeine/6.25µM and 12.5µM Hesperedin:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	**Media Control**	**4-BF Control**	**Querc. Control**	**Caff. Ctrl. 120**	**Hesp. Ctrl. 6.25**	**Hesp. Ctrl. 12.5**	**Q/C/H-1**	**Q/C/H-2**
1	0.1311	0.2244	0.2296	0.1394	0.1501	0.1793	0.0992	0.1106
2	0.1087	0.1843	0.2066	0.1080	0.1981	0.1759	0.1060	0.1135
3	0.1558	0.2073	0.2062	0.1621	0.1459	0.1777	0.0953	0.1014
average	0.1319	0.2054	0.2141	0.1365	0.1647	0.1776	0.1002	0.1085
std. deviation	0.0235	0.0201	0.0134	0.0272	0.0290	0.0017	0.0054	0.0063
(std. dev.^2)/ n	1.84E-04	1.35E-04	5.96E-05	2.47E-04	2.80E-04	9.48E-07	9.75E-06	1.34E-05
p-value								
t-value								
std. error	0.0136	0.0116	0.0077	0.0157	0.0167	0.0010	0.0031	0.0037

Exp.	**4-Bromo**	**Querc**	**Caff. 120**	**Hesp. 6.25**	**Hesp. 12.5**	**Q/C/H-1**	**Q/C/H-2**
P Value	0.0147	0.0062	0.8340	0.2021	0.0283	0.0853	0.1721
T Value	0.1265	0.1399	0.0090	0.0603	0.0822	0.0658	0.0476

Table 10: T-Test GST/4NQO Hepa1c1c7-

9µM Quercetin/120µM Caffeine/6.25µM and 12.5µM Hesperedin

T-Test GST/4NQO Hepa1c1c7- 9µM Quercetin/120µM Caffeine/6.25µM and 12.5µM Hesperedin:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	**Media Control**	**4-BF Control**	**Querc. Control**	**Caff. Ctrl. 120**	**Hesp. Ctrl. 6.25**	**Hesp. Ctrl. 12.5**	**Q/C/H-1**	**Q/C/H-2**
1	0.0652	0.1743	0.1935	0.1136	0.0714	0.1152	0.0802	0.0564
2	0.0541	0.1805	0.1562	0.1279	0.1376	0.1159	0.0782	0.0772
3	0.0686	0.1598	0.1475	0.1375	0.1027	0.1080	0.0586	0.0599
average	0.0626	0.1715	0.1657	0.1263	0.1039	0.1130	0.0723	0.0645
std. deviation	0.0076	0.0107	0.0244	0.0120	0.0331	0.0044	0.0119	0.0112
(std. dev.^2)/ n	1.16E-05	2.28E-05	1.19E-04	2.88E-05	2.19E-04	3.85E-06	2.84E-05	2.50E-05
p-value								
t-value								
std. error	0.0044	0.0062	0.0141	0.0069	0.0191	0.0025	0.0069	0.0065

Exp.	**4-Bromo**	**Querc**	**Caff. 120**	**Hesp. 6.25**	**Hesp. 12.5**	**Q/C/H-1**	**Q/C/H-2**
P Value	0.0001	0.0022	0.0015	0.1034	0.0006	0.3010	0.8229
T Value	0.2251	0.2157	0.1466	0.1011	0.1202	0.0264	0.0052

Table 11: T-Test QR Hepa1c1c7-

9µM Quercetin/120µM Caffeine/6.25µM and 12.5µM Hesperedin

T-Test QR Hepa1c1c7- 9µM Quercetin/120µM Caffeine/6.25µM and 12.5µM Hesperedin:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	Media Control	4-BF Control	Querc. Control	Caff. Ctrl. 120	Hesp. Ctrl. 6.25	Hesp. Ctrl. 12.5	Q/C/H-1	Q/C/H-2
1	0.6167	18.3002	1.8759	0.5944	0.5483	0.6258	0.5794	0.6575
2	0.5407	10.6302	1.5317	0.4180	0.7463	0.6781	0.6169	0.6487
3	0.6762	12.7530	1.3117	0.4977	0.6977	0.8137	0.7607	0.6324
average	0.6112	13.8945	1.5731	0.5034	0.6641	0.7059	0.6523	0.6462
std. deviation	0.0679	3.9604	0.2844	0.0883	0.1032	0.0970	0.0957	0.0128
(std. dev.^2)/ n	9.226E-04	3.137E+00	1.618E-02	1.560E-03	2.129E-03	1.881E-03	1.833E-03	3.264E-05
p-value								
t-value								
std. error	0.0392	2.2865	0.1642	0.0510	0.0596	0.0560	0.0553	0.0074

Exp.	4-Bromo	Querc	Caff. 120	Hesp. 6.25	Hesp. 12.5	Q/C/H-1	Q/C/H-2
P Value	0.0044	0.0047	0.1690	0.4991	0.2383	0.5765	0.4297
T Value	3.4877	0.6508	0.1021	0.0469	0.0825	0.0366	0.0312

Table 12: T-Test QR MCF 7-

9µM Quercetin/120µM Caffeine/6.25µM and 12.5µM Hesperedin

T-Test QR MCF 7- 9µM Quercetin/ 120µM Caffeine/ 6.25µM and 12.5µM Hesperedin:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	Media Control	4-BF Control	Querc. Control	Caff. Ctrl. 120	Hesp. Ctrl. 6.25	Hesp. Ctrl. 12.5	Q/C/H-1	Q/C/H-2
1	0.1607	2.0700	0.2285	0.0992	0.7737	0.2243	0.3882	0.6060
2	0.2342	2.2050	0.3248	0.0913	1.3951	0.1879	0.6278	1.0814
3	0.1199	3.7731	0.3199	0.0833	1.1484	0.3202	0.5724	0.5441
average	0.1716	2.6827	0.2911	0.0913	1.1057	0.2441	0.5295	0.7438
std. deviation	0.0580	0.9467	0.0542	0.0080	0.3129	0.0683	0.1255	0.2940
(std. dev.^2)/ n	6.72E-04	1.79E-01	5.88E-04	1.27E-05	1.96E-02	9.33E-04	3.15E-03	1.73E-02
p-value								
t-value								
std. error	0.0335	0.5466	0.0313	0.0046	0.1806	0.0394	0.0724	0.1697

Exp.	4-Bromo	Querc	Caff. 12	Hesp 6.25	Hesp 12.5	Q/C/H-1	Q/C/H-2
P Value	0.0101	0.0596	0.0761	0.0071	0.2336	0.0109	0.0297
T Value	1.4863	0.1756	0.1567	0.8265	0.1125	0.4274	0.5981

Table 13: T-Test GST/CDNB Hepa1c1c7-
9µM Quercetin/120µM Caffeine/1ng/mL and 2ng/mL Ginger extract

T-Test GST/CDNB Hepa1c1c7- 9µM Quercetin/120µM Caffeine/1ng/mL and 2ng/mL Ginger extract:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	**Media Control**	**4-BF Control**	**Querc. Control**	**Caff. Ctrl. 120**	**Ginger Ctrl. 12.5**	**Ginger Ctrl. 25**	**Q/C/G-1**	**Q/C/G-2**
1	0.1311	0.2244	0.2296	0.1394	0.2042	0.0753	0.1293	0.0848
2	0.1087	0.1843	0.2066	0.1080	0.3111	0.0767	0.1028	0.0979
3	0.1558	0.2073	0.2062	0.1621	0.3164	0.0861	0.0971	0.1140
average	0.1319	0.2054	0.2141	0.1365	0.2773	0.0794	0.1097	0.0989
std. deviation	0.0235	0.0201	0.0134	0.0272	0.0633	0.0059	0.0172	0.0146
(std. dev.^2)/ n	1.84E-04	1.35E-04	5.96E-05	2.47E-04	1.33E-03	1.16E-05	9.83E-05	7.13E-05
p-value								
t-value								
std. error	0.0136	0.0116	0.0077	0.0157	0.0365	0.0034	0.0099	0.0084

	4-Bromo	**Querc**	**Caff. 120**	**Ging 12.5**	**Ging 25**	**Q/C/G-1**	**Q/C/G-2**
P Value	0.0147	0.0062	0.8340	0.0203	0.0200	0.2581	0.1084
T Value	0.1265	0.1399	0.0090	0.2273	0.1142	0.0451	0.0686

Table 14: T-Test GST/4NQO Hepa1c1c7 –
9µM Quercetin/120µM Caffeine/1ng/mL and 2ng/mL Ginger extract

T-Test GST/4NQO Hepa1c1c7- 9µM Quercetin/120µM Caffeine/1ng/mL and 2ng/mL Ginger extract:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	**Media Control**	**4-BF Control**	**Querc. Control**	**Caff. Ctrl. 120**	**Ginger Ctrl. 12.5**	**Ginger Ctrl. 25**	**Q/C/G-1**	**Q/C/G-2**
1	0.0652	0.1743	0.1935	0.1136	0.2667	0.0639	0.1218	0.0702
2	0.0541	0.1805	0.1562	0.1279	0.2376	0.0639	0.0830	0.0845
3	0.0686	0.1598	0.1475	0.1375	0.1932	0.0709	0.0788	0.0849
Average	0.0626	0.1715	0.1657	0.1263	0.2325	0.0662	0.0946	0.0799
std. deviation	0.0076	0.0107	0.0244	0.0120	0.0370	0.0040	0.0237	0.0084
(std. dev.^2)/ n	1.9E-05	3.8E-05	2.0E-04	4.8E-05	4.6E-04	5.4E-06	1.9E-04	2.3E-05
p-value								
t-value								
std. error	0.0044	0.0062	0.0141	0.0069	0.0214	0.0023	0.0137	0.0048

	4-Bromo	**Querc**	**Caff. 120**	**Ging 12.5**	**Ging 25**	**Q/C/G-1**	**Q/C/G-2**
P Value	0.0001	0.0022	0.0015	0.0015	0.5071	0.0904	0.0575
T Value	0.2251	0.2157	0.1466	0.3127	0.0101	0.0805	0.0456

Table 15: T-Test QR Hepa1c1c7-
9µM Quercetin/120µM Caffeine/1ng/mL and 2ng/mL Ginger extract

T-Test QR Hepa1c1c7- 9µM Quercetin/120µM Caffeine/1ng/mL and 2ng/mL Ginger extract:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	Media Control	4-BF Control	Querc. Control	Caff. Ctrl. 120	Ginger Ctrl. 12.5	Ginger Ctrl. 25	Q/C/G-1	Q/C/G-2
1	0.6167	18.3002	1.8759	0.5944	1.0602	0.2603	0.4898	0.2177
2	0.5407	10.6302	1.5317	0.4180	1.4050	0.2642	0.4185	0.2852
3	0.6762	12.7530	1.3117	0.4977	1.0191	0.3113	0.1443	0.3493
average	0.6112	13.8945	1.5731	0.5034	1.1614	0.2786	0.3508	0.2841
std. deviation	0.0679	3.9604	0.2844	0.0883	0.2119	0.0284	0.1824	0.0658
(std. dev.^2)/ n	1.538E-03	5.228E+00	2.696E-02	2.599E-03	1.497E-02	2.682E-04	1.109E-02	1.444E-03
p-value								
t-value								
std. error	0.0392	2.2865	0.1642	0.0510	0.1223	0.0164	0.1053	0.0380

	4-Bromo	Querc	Caff. 120	Ging 12.5	Ging 25	Q/C/G-1	Q/C/G-2
P Value	0.0044	0.0047	0.1690	0.0128	0.0014	0.0814	0.0039
T Value	3.4877	0.6508	0.1021	0.4133	0.3526	0.2654	0.3457

Table 16: T-Test QR MCF7-
9µM Quercetin/120µM Caffeine/1ng/mL and 2ng/mL Ginger extract

T-Test GST/CDNB MCF7- 9µM Quercetin/120µM Caffeine/1ng/mL and 2ng/mL Ginger extract:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	Media Control	4-BF Control	Querc. Control	Caff. Ctrl. 12	Ginger Ctrl. 12.5	Ginger Ctrl. 25	Q/C/G-1	Q/C/G-2
1	0.1607	2.0700	0.2285	0.4761	0.3988	0.0000	0.1457	0.3858
2	0.2342	2.2050	0.3248	0.1704	0.5195	0.0000	0.3276	0.8614
3	0.1199	3.7731	0.3199	0.1046	0.3137	0.0000	0.2196	0.4151
average	0.1716	2.6827	0.2911	0.2504	0.4107	0.0000	0.2310	0.5541
std. deviation	0.0580	0.9467	0.0542	0.1982	0.0844	0.0000	0.0915	0.2666
(std. dev.^2)/ n	6.717E-04	1.793E-01	5.883E-04	7.859E-03	1.425E-03	0.000E+00	1.674E-03	1.421E-02
p-value								
t-value								
std. error	0.0335	0.5466	0.0313	0.1145	0.0487	0.0000	0.0528	0.1539

	4-Bromo	Querc	Caff. 12	Ging 12.5	Ging 25	Q/C/G-1	Q/C/G-2
P Value	0.0101	0.0596	0.5452	0.0251	0.0068	0.3961	0.0721
T Value	1.4863	0.1756	0.1212	0.3133	0.4143	0.0936	0.4490

Table 17: T-Test GST/CDNB Hepa1c1c7-
9µM Quercetin/12µM Caffeine/12.5µM and 25µM Resveratrol

T-Test GST/CDNB Hepa1c1c7- 9µM Quercetin/12µM Caffeine/12.5µM and 25µM Resveratrol:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	**Media Control**	**4-BF Control**	**Querc. Control**	**Caff. Ctrl. 12**	**Resv. Ctrl. 12.5**	**Resv. Ctrl. 25**	**Q/C/R-1**	**Q/C/R-2**
1	0.1311	0.2244	0.2296	0.1718	0.1203	0.1480	0.0848	0.0000
2	0.1087	0.1843	0.2066	0.2385	0.1926	0.1256	0.0979	0.0000
3	0.1558	0.2073	0.2062	0.1648	0.2258	0.1910	0.1140	0.0000
average	0.1319	0.2054	0.2141	0.1917	0.1796	0.1549	0.0989	0.0000
std. deviation	0.0235	0.0201	0.0134	0.0407	0.0539	0.0332	0.0146	0.0000
(std. dev.^2)/ n	0.0002	0.0001	0.0001	0.0006	0.0010	0.0004	0.0001	0.0000
p-value								
t-value								
std. error	0.0136	0.0116	0.0077	0.0235	0.0311	0.0192	0.0084	0.0000

	4-Bromo	**Querc**	**Caff. 12**	**Resv. 25**	**Resv. 12.5**	**Q/C/R-1**	**Q/C/R-2**
P Value	0.0147	0.0062	0.0922	0.3835	0.2328	0.1084	0.0006
T Value	0.1265	0.1399	0.1052	0.0429	0.0855	0.0686	0.3631

Table 18: T-Test GST/4NQO Hepa1c1c7-
9µM Quercetin/12µM Caffeine/12.5µM and 25µM Resveratrol

T-Test GST/4NQO Hepa1c1c7- 9µM Quercetin/12µM Caffeine/12.5µM and 25µM Resveratrol:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	**Media Control**	**4-BF Control**	**Querc. Control**	**Caff. Ctrl. 12**	**Resv. Ctrl. 12.5**	**Resv. Ctrl. 25**	**Q/C/R-1**	**Q/C/R-2**
1	0.0652	0.1743	0.1935	0.1323	0.0583	0.0939	0.0566	0.0000
2	0.0541	0.1805	0.1562	0.1279	0.1400	0.0879	0.0756	0.0000
3	0.0686	0.1598	0.1475	0.1187	0.1884	0.1523	0.1102	0.0000
average	0.0626	0.1715	0.1657	0.1263	0.1289	0.1114	0.0808	0.0000
std. deviation	0.0076	0.0107	0.0244	0.0069	0.0657	0.0356	0.0272	0.0000
(std. dev.^2)/ n	1.2E-05	2.3E-05	1.2E-04	9.5E-06	8.6E-04	2.5E-04	1.5E-04	0.0E+00
p-value								
t-value								
std. error	0.0044	0.0062	0.0141	0.0040	0.0379	0.0205	0.0157	0.0000

	4-Bromo	**Querc**	**Caff. 12**	**Resv. 12.5**	**Resv. 25**	**Q/C/R-1**	**Q/C/R-2**
P Value	0.0001	0.0022	0.0004	0.1577	0.0811	0.3273	0.0001
T Value	0.2251	0.2157	0.1465	0.1514	0.1168	0.0480	0.2503

Table 19: T-Test QR Hepa1c1c7-

9µM Quercetin/12µM Caffeine/12.5µM and 25µM Resveratrol

T-Test QR Hepa1c1c7- 9µM Quercetin/12µM Caffeine/12.5µM and 25µM Resveratrol:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	**Media Control**	**4-BF Control**	**Querc. Control**	**Caff. Ctrl. 12**	**Resv. Ctrl. 12.5**	**Resv. Ctrl. 25**	**Q/C/R-1**	**Q/C/R-2**
1	0.6167	18.3002	1.8759	0.5944	0.9694	1.1295	0.5705	0.0000
2	0.5407	10.6302	1.5317	0.4180	0.9864	1.1121	0.4127	0.0000
3	0.6762	12.7530	1.3117	0.4977	1.4252	1.7429	0.9136	0.0000
average	0.6112	13.8945	1.5731	0.5034	1.1270	1.3282	0.6323	0.0000
std. deviation	0.0679	3.9604	0.2844	0.0883	0.2584	0.3592	0.2561	0.0000
(std. dev.^2)/ n	9.226E-04	3.137E+00	1.618E-02	1.560E-03	1.335E-02	2.581E-02	1.312E-02	0.000E+00
p-value								
t-value								
std. error	0.0392	2.2865	0.1642	0.0510	0.1492	0.2074	0.1479	0.0000

	4-Bromo	**Querc**	**Caff. 12**	**Resv. 12.5**	**Resv. 25**	**Q/C/R-1**	**Q/C/R-2**
P Value	0.0044	0.0047	0.1690	0.0287	0.0274	0.8971	0.0001
T Value	3.4877	0.6508	0.1021	0.3912	0.5148	0.0189	0.7818

Table 20: T-Test QR MCF7-

9µM Quercetin/12µM Caffeine/12.5µM and 25µM Resveratrol

T-Test QR MCF7- 9µM Quercetin/12µM Caffeine/12.5µM and 25µM Resveratrol:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	**Media Control**	**4-BF Control**	**Querc. Control**	**Caff. Ctrl. 12**	**Resv. Ctrl. 12.5**	**Resv. Ctrl. 25**	**Q/C/R-1**	**Q/C/R-2**
1	0.1607	2.0700	0.2285	0.4761	0.9213	0.3427	0.2153	0.6013
2	0.2342	2.2050	0.3248	0.1704	0.6123	0.2881	0.2603	0.3301
3	0.1199	3.7731	0.3199	0.1046	0.1884	0.1810	0.2648	0.1429
average	0.1716	2.6827	0.2911	0.2504	0.5740	0.2706	0.2468	0.3581
std. deviation	0.0580	0.9467	0.0542	0.1982	0.3679	0.0822	0.0274	0.2305
(std. dev.^2)/ n	6.717E-04	1.793E-01	5.883E-04	7.859E-03	2.708E-02	1.353E-03	1.500E-04	1.063E-02
p-value								
t-value								
std. error	0.0335	0.5466	0.0313	0.1145	0.2124	0.0475	0.0158	0.1331

Control Exp.	**MC 4-Bromo**	**MC Querc**	**MC Caff. 12**	**MC Resv 12.5**	**MC Resv 25**	**MC Q/C/R-1**	**MC Q/C/R-2**
P Value	0.0101	0.0596	0.5452	0.1346	0.1636	0.1120	0.2458
T Value	1.4863	0.1756	0.1212	0.4660	0.1488	0.1162	0.2883

Table 21: T-Test GST/CDNB Hepa1c1c7-

9µM Quercetin/12µM Caffeine/10µM and 20µM β-Carotene

T-Test GST/ CDNB Hepa1c1c7- 9µM Quercetin/12µM Caffeine/10µM and 20µM β-Carotene:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	Media Control	4-BF Control	Querc. Control	Caff. Ctrl. 12	β- Car. Ctrl. 10	β- Car. Ctrl 20	Q/C/β-Car-1	Q/C/β-Car-2
1	0.1311	0.2244	0.2296	0.1718	0.2666	0.2337	0.0986	0.1106
2	0.1087	0.1843	0.2066	0.2385	0.3074	0.2607	0.1043	0.0609
3	0.1558	0.2073	0.2062	0.1648	0.2885	0.3005	0.1089	0.0974
average	0.1319	0.2054	0.2141	0.1917	0.2875	0.2650	0.1039	0.0896
std. deviation	0.0235	0.0201	0.0134	0.0407	0.0204	0.0336	0.0052	0.0257
(std. dev.^2)/ n	1.84E-04	1.35E-04	5.96E-05	5.52E-04	1.39E-04	3.77E-04	8.95E-06	2.21E-04
p-value								
t-value								
std. error	0.0136	0.0116	0.0077	0.0235	0.0118	0.0194	0.0030	0.0149

	4-Bromo	Querc	Caff. 12	β-Car. 10	β-Car. 20	Q/C/β-Car 1	Q/C/β-Car 2
P Value	0.0147	0.0062	0.0922	0.0010	0.0049	0.1147	0.1037
T Value	0.1265	0.1399	0.1052	0.2403	0.2113	0.0576	0.0898

Table 22: T-Test GST/4NQO Hepa1c1c7-

9µM Quercetin/12µM Caffeine/10µM and 20µM β-Carotene

T-Test GST/ 4NQO Hepa1c1c7- 9µM Quercetin/12µM Caffeine/10µM and 20µM β-Carotene:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	Media Control	4-BF Control	Querc. Control	Caff. Ctrl. 12	β- Car. Ctrl. 10	β- Car. Ctrl 20	Q/C/β-Car-1	Q/C/β-Car-2
1	0.0652	0.1743	0.1935	0.1323	0.2533	0.2289	0.0802	0.0835
2	0.0541	0.1805	0.1562	0.1248	0.2421	0.2436	0.0716	0.0440
3	0.0686	0.1598	0.1475	0.1187	0.2383	0.2055	0.0794	0.0846
average	0.0626	0.1715	0.1657	0.1253	0.2445	0.2260	0.0771	0.0707
std. deviation	0.0076	0.0107	0.0244	0.0068	0.0078	0.0192	0.0047	0.0231
(std. dev.^2)/ n	1.93E-05	3.79E-05	1.99E-04	1.53E-05	2.01E-05	1.23E-04	7.40E-06	1.78E-04
p-value								
t-value								
std. error	0.0044	0.0062	0.0141	0.0039	0.0045	0.0111	0.0027	0.0134

	4-Bromo	Querc	Caff. 12	β-Car. 10	β-Car. 20	Q/C/β-Car 1	Q/C/β-Car 2
P Value	0.0001	0.0022	0.0004	0.0000	0.0002	0.0491	0.5959
T Value	0.2251	0.2157	0.1445	0.3282	0.3041	0.0386	0.0221

Table 23: T-Test QR Hepa1c1c7-

9µM Quercetin/12µM Caffeine/10µM and 20µM β-Carotene

T-Test QR Hepa1c1c7- 9µM Quercetin/12µM Caffeine/10µM and 20µM β-Carotene:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	Media Control	4-BF Control	Querc. Control	Caff. Ctrl. 12	β- Car. Ctrl. 10	β- Car. Ctrl 20	Q/C/β-Car-1	Q/C/β-Car-2
1	0.6167	18.3002	1.8759	0.9690	1.3453	0.8354	0.5629	0.3368
2	0.5407	10.6302	1.5317	1.4733	1.5474	0.7781	0.8472	0.0637
3	0.6762	12.7530	1.3117	1.0679	1.2679	1.0273	0.8675	0.4142
average	0.6112	13.8945	1.5731	1.1701	1.3869	0.8803	0.7592	0.2715
std. deviation	0.0679	3.9604	0.2844	0.2672	0.1178	0.1065	0.1703	0.1841
(std. dev.^2)/ n	1.538E-03	5.228E+00	2.696E-02	2.380E-02	4.629E-03	3.784E-03	9.667E-03	1.130E-02
p-value								
t-value								
std. error	0.0392	2.2865	0.1642	0.1543	0.0680	0.0615	0.0983	0.1063

	4-Bromo	Querc	Caff. 12	β-Car. 10	β-Car. 20	Q/C/β-Car 1	Q/C/β-Car 2
P Value	0.0044	0.0047	0.0247	0.0011	0.0339	0.2346	0.0401
T Value	3.4877	0.6508	0.4188	0.5488	0.2203	0.1264	0.3615

Table 24: T-Test QR MCF7-

9µM Quercetin/ 12µM Caffeine/10µM and 20µM β-Carotene

T-Test QR MCF7- 9µM Quercetin/12µM Caffeine/10µM and 20µM β-Carotene:

5% Level of significance. So if p-value is less than 0.05, the null hypothesis is rejected. If the null hypothesis is rejected the result is said to be statically significant and a possible induction. Critical value= 2.57 with 5 degrees of freedom, 2.45 with 6 degrees of freedom, 2.36 with 7 degrees of freedom, 2.31 with 8 degrees of freedom and 2.26 with 9 degrees of freedom.

Specific Activity (units/mg)	Media Control	4-BF Control	Querc. Control	Caff. Ctrl. 12	β- Car. Ctrl. 10	β- Car. Ctrl 20	Q/C/β-Car-1	Q/C/β-Car-2
1	0.1607	2.0700	0.2285	0.4761	0.8563	0.0000	0.2319	0.2095
2	0.2342	2.2050	0.3248	0.1704	0.4695	0.0000	0.5035	0.1831
3	0.1199	3.7731	0.3199	0.1046	0.5505	0.0000	0.5442	0.4155
average	0.1716	2.6827	0.2911	0.2504	0.6254	0.0000	0.4265	0.2694
std. deviation	0.0580	0.9467	0.0542	0.1982	0.1666	0.0000	0.1698	0.1273
(std. dev.^2)/ n	1.119E-03	2.988E-01	9.806E-04	1.310E-02	9.247E-03	0.000E+00	9.611E-03	5.398E-03
p-value								
t-value								
std. error	0.0335	0.5466	0.0313	0.1145	0.0962	0.0000	0.0980	0.0735

Exp.	4-Bromo	Querc	Caff. 12	β-Car. 10	β-Car. 20	Q/C/β-Car 1	Q/C/β-Car 2
P Value	0.0101	0.0596	0.5452	0.0207	0.0068	0.0696	0.2927
T Value	1.4863	0.1756	0.1212	0.5083	0.4143	0.3296	0.1472

APPENDIX B

APPENDIX B

PROTOCOLS AND REAGENTS PART I

Phosphate Buffer Saline (PBS): 10mM Phosphate Buffer, 150mM NaCl, pH 7.0

Solution 1: Measure 10mL of 1M KH_2PO_4 and fill to 1L with distilled waster

Solution 2: Measure 10mL of 1M K_2HPO_4 and fill to 1L with distilled water

- Acidic solution is added to the basic solution until the pH is 7.0
- 17.5g of NaCl is added in 2L solution (0.15M)

MTT reagent 5mg/mL= weight 30mg of MTT dye and dissolve in 6 mL of distilled water.

EDTA (Biorad, Catalog Number: 161-0723) solution: 10mM EDTA pH 12.3

Weight 3.726g of EDTA in 10mL of distilled water; adjust pH with 1M NaOH

Hoechst Standard Curve:

For DNA 200µg/mL was weight 0.00194g of Deoxyribonucleicacid sodium salt from calf thymus (Sigma, D1501) and dissolved in 1mL of EDTA 10Mm

[Standard] (μg/mL)	EDTA 10mM (μL)	DNA- 200μg/mL (μL)
0	1000	0
25	875	125
50	750	250
75	625	375

Hoechst 33258 solution:

- Solution A: Tris 10 mM: weight 0.0012g in 100mL of distilled water.

- Solution B: NaCl 0.1M: weight 0.5844g in 100mL of distilled water.

Weight 0.001g and dissolved in 1mL of Solution C (Solution A + Solution B), from this solution was taken 8 μL and addedn in 8mL of solution C.

PROTOCOLS AND REAGENTS PART II

Buffer A:

Concentrated Acidic Stock Solution: 1M KH_2PO_4

- Weight 1.361g of KH_2PO_4 (Sigma-Aldrich) and dissolve in 10mL of distilled water. Add this 10 mL of 1M KH_2PO_4 in 990mL of distilled water

Concentrated Basic Stock Solution: 1M K_2HPO_4\

- Weight 1.742g of K2HPO4 and dissolve in 10mL of distilled water. Add this 10 mL of 1M K2HPO4 in 990mL of distilled water

Add the acidic solution to the basic solutions until get pH 7.0. 100mM of 2-Mercaptoethanol is added to this solution

GSH Determination- Reagents:

0.1mM GSH

- Weight 0.0031g GSH in 10mM phosphate buffer, pH 7.0- without mercaptoethanol, this solution is diluted 10 times.

Precipitating solution

- Weight 1.67g of m-phosphoric acid (Sigma-Aldrich), 30g of sodium chloride (Sigma-Aldrich) and 0.2f of EDTA (Bio-Rad) in 100mL of distilled water.

DTNB reagent

- Weight 0.002g of DTNB in 10 mL 1% sodium citrate (1g/100mL; Sigma-Aldrich) 0.3M NA2PO4 in 100 mL of distilled water.

Procedure- In micro-centrifuge tubes:

Blank: 200µL of 10mM Phosphate buffer + 300µL Precipitating solution

Standard: 100µL of 0.1mM GSH + 300µL Precipitating solution

Sample: 20µL of enzyme (sample) + 300µL Precipitating solution.

Mix the contents and led stand for five minute at room temperature, following for 5 minutes of centrifugation at 1,500xg. After centrifugation 200µL of the supernatant is transferred to the cuvette, then is added 800µL of 0.3N Na2HPO4 (Sigma-Aldrich). Cuvette's contents are

mixed and absorbance is read at 412. 100µL of DTNB is added to the solution and absorbance is read it again.

Glutathione S-Transferase (GST) Assay) using CDNB as a substrate- Reagents:

Assay Buffer

- 400mL of 100mM K2HPO4\+800mL of 100mM KH2PO4 , pH 6.5

20mM CDNB (1-chloro-2,4 dinitrobenzene)

- Weight 0.0203g of CDNB in 5mL of95% of ethanol (The solution is good for two weeks in ±4°C)

10mM GSH

- Weight 0.0154g of GSH in 5mL of Assay buffer (Stable for 3 hour on ice).

Procedure- The following is added into the cuvette

Blank: 850µL of Assay buffer + 100µL 10mM GSH + 50µL 20mM CDNB

Sample: 850µL of Assay buffer + 100µL 10mM GSH + 20µL Sample + 50µL 20mM CDNB

Mix contents and read at 340nm immediately

Glutathione S-Transferase (GST) Assay) using 4NQO as a substrate- Reagents:

5mM 4NQO (4-Nitroquinoline 1-oxide)

- Weight 0.011412g of 4NQO in 12mL of 95% of ethanol. ((The solution is good for two weeks in ±4°C)

10mM GSH

- Weight 0.0154g of GSH in 5mL of Assay buffer (Stable for 3 hour on ice).

Procedure- The following is added into the cuvette

Blank: 880µL of Assay buffer + 100µL 10mM GSH + 20µL 5mM 4NQO

Sample: 860µL of Assay buffer + 100µL 10mM GSH + 20µL Sample + 20µL 5mM 4NQO

Mix contents and read at 350nm immediately

NADPH: Quinone oxidoreductase- Reagents:

25mM Tris/HCl, pH7.5

- Weight 3.0285g of Tris(hydroxymethyl)-aminomethane (Bio-Rad) in 900mL of distilled water, adjust the pH at 7.5 with 6M HCl and fill to 1L of distilled water.

0.18mg/mL BSA

- Weight 0.9g of Bovine serum albumin (Sigma-Aldrich) in 10mL of distilled water.\

0.5μM FAD

- Weight FAD-flavin adenine dinucleotide (sigma- Aldrich) in 10mL of distilled water (This solution is stable for weeks if it is kept in dark and 0°C)

0.2 μM NADPH

- Weight 0.00355g of NADPH (Sigma- Aldrich) in 0.5mL of distilled water

40 μM 2,6 dichlorophenol-indophenol

- Weight 0.0058g of DCPIP in 10mL of distilled water.

Procedure- The following is added into the cuvette

Blank: 940μL of Tris-HCL Buffer + 20μL BSA + 20μL FAD + 20μL DCPIP

Control: 920μL of Tris-HCL Buffer + 20μL BSA + 20μL FAD + 20μL DCPIP + 20μL NADPH

Sample: 920μL of Tris-HCL Buffer + 20μL BSA + 20μL FAD + 20μL DCPIP + 20μL sample + 20μL NADPH

Mix contents and read at 600nm immediately

BIOGRAPHICAL SKETCH

Brenda S. Benavides, daughter of Ruben Ochoa and Modesta Ramos, and married to Carlos R. Benavides, was born in Monterrey, Nuevo Leon, Mexico. She graduated from Preparatoria 7-Puentes, associated to the UANL (Universidad Autonoma de Nuevo Leon). She was employed by UANL as an Undergraduate Researcher for the Analytical Chemistry Department. Brenda received her Bachelor of Science in 2005, with a major in Chemistry and a minor in Biology form UANL Medical School in Monterrey, Nuevo Leon, Mexico. After graduation she worked at the Pharmacology Department at her alma mater, and as a high school biology teacher in San Nicolas de los Garza. Brenda worked as an Analyst Chemist at IPharma S.A. de C.V. performing bioequivalence studies. In 2009 Brenda started her master's degree studies at the University of Texas-Pan American. In addition, Brenda also worked as a Teacher Assistant in the General Chemistry Lab and Research Assistant in the Chemistry Department. Brenda S. Benavides obtained her Master's Degree in Science at The University of Texas Pan-American on May 2011.

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