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PLANT BASED POLYPHENOLS DECREASE CELL MIGRATION AND INHIBIT PROLIFERATION OF HUMAN PANCREATIC CANCER CELLS

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PLANT BASED POLYPHENOLS DECREASE CELL MIGRATION AND INHIBIT PROLIFERATION OF HUMAN PANCREATIC CANCER CELLS

An Abstract of the Thesis by
Mary Gathoni

Pancreatic cancer is an aggressive and often fatal malignancy. The disease has a poor prognosis with a dismal relative 5-year survival rate of 10% and a median overall survival of only 6 months for patients with metastatic pancreatic cancer. Pancreatic cancer-related mortality can be mainly attributed to lack of early diagnosis, tumor metastasis, lack of effective treatment strategies and drug resistance. Recently, there has been mounting interest in the use of plant-based compounds such as polyphenols for cancer treatment. This is mainly due to their low toxicity and a lack of side effects when compared to conventional cytotoxic chemotherapy. In this study, the anticancer properties of the polyphenols curcumin, resveratrol and rutin were evaluated using human pancreatic cancer cell lines capan-1 and panc-1. Effects on cell morphology, cell migration and cell viability were investigated by treating cells with various concentrations of the polyphenols. A wound healing assay was used to study cell migration and the MTT assay was utilized to investigate cell viability. None of the three polyphenols significantly altered the morphology the cell lines although they did cause a dramatic decrease in cell density. The ability of the compounds to reduce cell viability and cell migration were varied with resveratrol having the greatest effect on capan-1 cells in both the MTT and wound healing assays. Rutin exhibited the greatest effect on panc-1 cells in terms of lowering cell viability and inhibiting cell migration. These findings highlight the potential benefits of polyphenols in treatment of both primary and metastatic tumors.

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CHAPTER I

INTRODUCTION

1.1 What is Cancer

Cancer is a disease that is characterized by uncontrolled growth and proliferation of abnormal cells. It arises because of an imbalance between the production of new cells (mitosis) and cell death (apoptosis). Altered expression of tumor suppressor genes, oncogenes and apoptotic genes play a key role in the pathophysiological mechanism of cancer (Nouri,2020). Many types of cancer can result in death if not treated (CDC). Cancer is a global health concern and in the U.S alone, 1.9 million new cancer cases are expected to be diagnosed in 2022 with approximately 600,000 deaths as a result (Siegel, 2022). As recently pointed out in Cancer Facts & Figures 2022, cancer is the second most common cause of death after heart disease in the U.S.

1.2 Conversion of normal cells to cancerous cells

The transformation of a normal healthy cell into a cancerous cell is a multistep process that is initiated by DNA mutations. For a cell to become cancerous, it must typically accumulate multiple independent mutations and in numerous genes that influence cell proliferation, cell division and survival. Furthermore, the mutations must escape the cell's intrinsic defenses that repair DNA mutations and induce abnormal cells to die via

apoptosis. These cancerous cells exhibit qualities that distinguish them from normal cells. For example, cancerous cells have the ability to stimulate their own growth, ignore growth-inhibiting signals, avoid death by apoptosis, induce angiogenesis (develop a blood supply), metastasize, proliferate indefinitely, and evade the immune system (Parham,2021).

1.3 Causes of Cancer

There is a myriad of risk factors that can contribute to cancer development. These include both genetic and environmental factors. Hereditary cancers are those which arise due to an inherited predisposition caused by an inherited gene mutation (Kleinsmith,2006). One well studied example is familial retinoblastoma which is a rare childhood cancer of the eye that occurs in children who have inherited a defective *RB* gene from a parent. The cancer arises when the child sustains one additional mutation creating two mutant genes. Mutations of key proto-oncogenes and tumor suppressor genes contribute to the development of certain cancers. Mutations may arise spontaneously due to errors during DNA replication or may be induced by chemicals or ionizing radiation. These environmental carcinogens can contribute to cancer development by producing single-nucleotide substitutions in DNA or more extensive DNA damage such as DNA strand breaks and chromosomal translocations. It has also been well established that infections with certain pathogens such as the bacterium *Helicobacter pylori* or numerous viruses can lead to cancer development. These infections can increase cancer risk directly by causing tissue destruction and inducing a state of chronic inflammation or indirectly by interfering with the functions of the immune system (Kleinsmith,2006). Other risk factors such as age and obesity have been implicated in cancer development.

1.4 Cancer and the Immune System

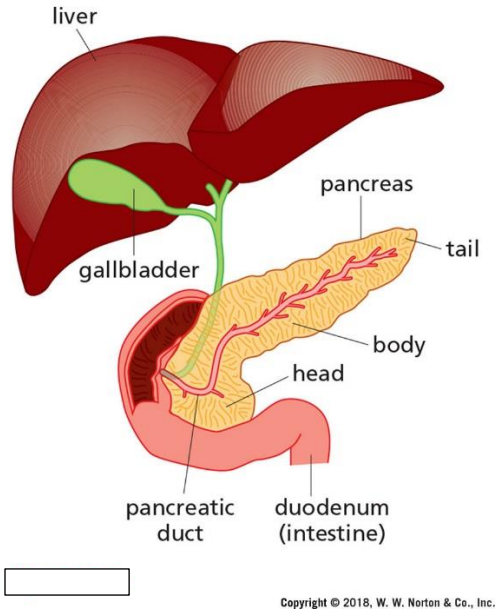
The immune system is responsible for defending the body from a wide range of pathogens. The cells of the immune system are constantly looking for and recognizing any foreign pathogens present in the body, a phenomenon referred to as immunosurveillance (Kleinsmith,2006). The immune system is finely tuned and highly specific. Cells of the immune system have the capability to distinguish between "self", "non-self" and "altered-self" then proceed to deal accordingly with the "non-self" and "altered-self" cells (Weinberg,2007). The immune response to cancerous cells is similar to that made against virus-infected cells. Most mutated cells and potentially malignant cells (recognized by the immune cells as "altered-self") are detected and eliminated at an early stage by the immune system. The cells of the immune system that are tasked with cancer eradication are usually T cells, although other cells like Natural Killer (NK) cells, macrophages and dendritic cells can also play a critical role. The most powerful effector cells for tumor control are the CD8 T cells. Tumors become detectable when the T cell response can no longer control the proliferating tumor cells due to T cell exhaustion. This exhaustion is brought about by the persistence of tumor antigen and inflammation (Parham). The development of cancer is a reflection of the immune system failing to mount an adequate immune response against the aberrant cells. Consequently, the immune system has its best chance of eliminating the cancer at its early stages when the population of tumor cells is small and is yet to adapt to the immune response.

Tumors that become detectable are those that successfully evade the immune system using various methods. First, through natural selection there is a continual selection of tumor cells that lack or produce fewer antigens and thus invoke less of an immune

response. Some tumor cells secrete cytokines such as TGF-β (transforming growth factor β) and interleukin-10. These cytokines create an immunosuppressive tumor environment, in addition to inducing apoptosis of T lymphocytes, dendritic cells, and macrophages. Some tumor cells lose their expression of HLA (human leukocyte antigen) class I which makes them resistant to T cell attack. However, this loss makes them susceptible to NK cell attack (Parham,2021). Other cancerous cells divide at a rapid pace such that the immune system cannot destroy them quickly enough to keep their proliferation in check.

1.5 Pancreatic Cancer

Pancreatic cancer is one of the most highly malignant tumors of the digestive tract (Li,2020). It is estimated that in 2022 62,210 new cases will be diagnosed in the U.S and approximately 50,000 people will die from the disease (Cancer Facts,2022). Pancreatic cancer can develop from two kinds of pancreatic cells: exocrine cells and neuroendocrine cells. About 90% of pancreatic cancers are adenocarcinomas which are malignant tumors formed from gland-forming epithelia tissue arising from the duct cells. Two-thirds of adenocarcinomas arise in the head of the pancreas whereas a third arise in the tail of the pancreas (see figure 1.5). Pancreatic neuroendocrine cell tumors (NETs) arise from cells within the pancreas that secrete hormones and comprise 5-10% of pancreatic malignancies (Lee,2018). Pancreatic NETs follow a less aggressive clinical course than the common pancreatic exocrine tumors.



Pancreatic cancer risk factors can be broadly categorized into two: inherited risk factors and modifiable risk factors. Some inherited gene mutations such as TP53 have been known to increase the risk of developing pancreatic cancer. Modifiable risk factors include tobacco use, chronic pancreatitis, cirrhosis, obesity, diabetes mellitus and prior radiation to the pancreas (Tao,2021). Obesity has been associated with a 20% higher risk of developing pancreatic cancer whereas smoking is implicated in 20-30% of pancreatic cancer cases (Lee,2018).

Pancreatic cancer has a poor prognosis with a 5-year relative survival rate of only 11%. Lack of early diagnosis (due to absence of specific symptoms in early-stage disease), tumor metastasis, few effective treatment strategies, and drug resistance contribute to pancreatic cancer- related mortality and poor prognosis (Gasic, Qin,2020). Most patients initially present with non-specific symptoms such as weight loss, progressive loss of appetite, fatigue, and painless jaundice. Jaundice presentation is due to the obstruction of the bile duct by a tumor in the pancreatic head. Advanced disease presents symptoms such

as early satiety, abnormal blood glucose control, nausea and vomiting, and abdominal pain. In some cases, the liver and spleen may be enlarged (Lee,2018).

Diagnosis involves physical assessment, blood tests and imaging tests such as computerized tomography (CT) scans. Tumor markers such as cancer antigen 19-9 (CA 19-19) may be elevated but not necessarily diagnostic due to its non-specificity. However, CA 19-9 is noted to be elevated in 70-90% of patients with advanced pancreatic cancer. Imaging tests may show pancreatic tumors as well as metastasis to typical regions such as the liver. Diagnosis is confirmed following a biopsy and analysis of the pancreatic lesion. The disease is then categorized as resectable, localized but unresectable, or metastatic (Lee,2018). Currently, surgical resection is considered the only curative treatment for pancreatic cancer, but only 20% of cases can potentially be resected (Tao,2021). The majority of pancreatic cancer deaths are accredited to secondary tumors resulting from metastasis (Li,2020) which may be attributed to the often-advanced stage of the disease at the time of presentation and diagnosis. Since the therapeutic options are limited, it is crucial to identify alternative tools that can improve treatment of pancreatic cancer.

1.6 Polyphenols in cancer research

The rapid increase in the cost of healthcare coupled with the limited effectiveness of single target cancer treatment therapies has led to a growing interest in naturally occurring plant-based phytochemicals for use in the prevention of human diseases including cancer (Shehzad,2013). Plant-based compounds have been investigated lately as possible chemopreventive and chemotherapeutic agents. Particular interest has focused on polyphenols which are secondary plant metabolites comprising over 4000 types of molecules which fall into one of nine subgroups: flavonoids, isoflavonoids, aurones,

chalconoids, flavonolignans, lignans, stilbenoids, curcuminoids and tannins (Gasic,2020). Polyphenols are structurally characterized by the presence of two or more phenol units. They act on multiple molecular targets in cellular pathways that influence carcinogenesis, tumor cell proliferation and death, inflammation, metastatic spread, angiogenesis, or drug and radiation resistance (Asensi,2011).

Conventional treatments of cancer with chemotherapeutic agents and ionizing radiation have two main problems: time dependent development of tumor resistance to therapy and nonspecific toxicity towards normal cells (Asensi,2011). Tumor cells exploit several strategies to hinder drug efficacy such as enhancement of drug efflux, limited uptake of drugs to the cell, mutation of drug targets and alteration of drug metabolism (Panda,2017). All these strategies contribute to tumor resistance to therapy. Besides cancerous cells, other healthy dividing hematopoietic cells, epithelial cells, and hair follicles are also affected severely by exposure to chemotherapeutic agents**.** Some of the most common adverse effects of chemotherapy are fatigue, nausea, pain, headache, and infection (Panda,2017). Polyphenols are an attractive alternative to conventional treatment options mainly due to their low toxicity and lack of side effects. In recent works, natural polyphenols were used to supplement traditional therapies as they have been found to sensitize tumor cells to chemotherapeutic agents and radiation therapy by affecting pathways that lead to treatment resistance (Asensi,2011). A recent study by Segaran(2021) evaluating the effect of the polyphenols curcumin, resveratrol and rutin on lung adenocarcinoma cells found that these three compounds at low concentrations could effectively inhibit proliferation and decrease migration of A549 cells.

1.7 Curcumin

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also called diferuloylmethane, is the main natural polyphenol found in the roots of *Curcuma longa* commonly known as turmeric (Panda,2017). Curcumin has been long used as a traditional remedy in Asian countries due to its antioxidant, anti-inflammatory, antimutagenic, antimicrobial, anti-inflammatory, and anticancer properties (Gasic,2020). It is a highly pleiotropic molecule that interacts with numerous molecular targets. Curcumin mediates the modulation of enzymes, transcription factors, growth factors and their receptors as well as cytokines and various kinase proteins which control cell proliferation and cell cycle progression. Its anti-inflammatory mechanism has been correlated with the inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression (Shehzad,2013). In a recent study investigating antiviral properties of curcumin, it was found to have a strong antiviral effect against SARS-CoV-2 in cell culture at low subtoxic concentrations (Bormann,2021).

In suppressing pancreatic cancer growth, curcumin modulates a great number of signaling pathways including: NFκB, STAT-3, Notch-1, and ATM/Chk1 (Gasic,2020). Studies showed that curcumin treatment downregulated the expression of NFκB and Notch-1 in pancreatic cancer cells leading to inhibition of cell growth and induction of apoptosis (Li,2004 Wang,2006). Curcumin has been used in various combinatorial therapies as an adjuvant due to its nontoxic nature. Doxorubicin is a chemotherapeutic agent that suffers from serious limitations due to its cardiotoxicity and drug resistance. However, curcumin in combination with doxorubicin was highly effective in sensitizing cancer cells that were resistant to the drug in addition to reducing the survival of these

cancer cells. Additionally, curcumin has been shown to induce the hepato- and cardioprotective properties of doxorubicin (Panda,2017). In another study, curcumin was shown to significantly increase the radiation killing of lung carcinoma cells in a dose dependent manner but did not increase the killing of healthy pulmonary microvascular endothelial cells (Lee,2010).

1.8 Resveratrol

Resveratrol (3,5,4'-trihydroxystilbene) is a stilbenoid present in grapes, berries, peanuts, and food products such as red wine. It exhibits several pharmacologic properties including, anti-inflammatory, anticancer, antiangiogenic and antimetastatic (Han,2016). Resveratrol was found to inhibit proliferation, induce apoptosis, and overcome chemoresistance in human myeloma cells (Bhardwaj,2007). Opipari et al (2004) demonstrated that resveratrol was able to induce autophagy-mediated cell death in ovarian cancer cells. In varied studies resveratrol has been found to sensitize tumor cells to chemotherapeutic agents and radiation therapy by affecting pathways that lead to treatment resistance (Asensi,2011). Studies by Cheng et. al indicated that the combination of Gemcitabine (antineoplastic chemotherapy agent) and the polyphenol resveratrol significantly promoted the rate of apoptosis in pancreatic cancer cell lines. The study demonstrated that resveratrol has a potent effect in enhancing the sensitivity of pancreatic cancer cells to Gemcitabine by inhibiting NAF-1 expression (Cheng,2018). Other studies examining the effect of resveratrol on apoptosis induced by Paclitaxel found that pretreatment with 10 μM resveratrol for three days significantly enhanced subsequent antiproliferative effect of Paclitaxel (Garg,2005). In combination with curcumin,

resveratrol was shown to induce apoptosis, inhibit angiogenesis and reduce growth of MDA-MB-231 tumor xenografts (Panda,2017).

1.9 Rutin

Rutin (3, 3′, 4′, 5, 7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonol glycoside found in a wide variety of vegetables, fruits, and beverages, including passionflower, grapes, green asparagus, apples, tea, and wine. Many medicinal plants also contain rutin, such as buckwheat. Rutin is also called vitamin P or rutoside (Nouri,2020). Rutin has exhibited diverse pharmacological properties including antimicrobial, antioxidant, cardioprotective, anti-diabetic and anticarcinogenic properties (Khan,2021). It has been demonstrated to hamper inflammation, oxidative insults, and platelet aggregation. In countering inflammation, rutin targets several inflammatory mediators such as NFκB and TNF-α. Rutin counteracts cancer by inhibiting malignant cell growth, inducing cell cycle arrest and apoptosis, modulation of angiogenesis and multiple cellular signaling pathways. In triple-negative breast cancer (TNBC) cell lines rutin interfered with c-met/HGF axis and its downstream cascades thereby reducing the average tumor volume of the TNBC in nude mice (Nouri,2020). A study by Khan et. al showed that treatment of cervical cancer cells with rutin, led to dose dependent antiproliferative effects by triggering apoptosis, G0/G1 phase arrest, and downregulating the level of *Notch-1* and *Hes-1* in the Notch signaling pathway (Khan,2021). As an adjuvant agent rutin was shown to synergistically increase the antiproliferative effect of tamoxifen on estrogen-receptor-α (ER-α) positive breast cancer MCF-7 cells (Nouri,2020).

Despite the encouraging pharmacological properties of these plant-based polyphenols, there is a difficulty when it comes to clinical translation of these benefits

mainly due to their low solubility and bioavailability. The chemical structure of polyphenols along with their molecular weight, glycosylation, and esterification reduce their bioavailability (Gasic,2020). Many of the compounds including curcumin, resveratrol and rutin have poor aqueous solubility or cannot be effectively retained in circulation. Low gastrointestinal absorption, high metabolism, rapid clearance, and chemical degradation limit their capability of achieving effective concentration in tumor tissues (Ricardo,2020). Efforts have been employed to overcome these drawbacks and increase solubility and bioavailability of these compounds in addition to improving their targeting of tumor cells. With the advent of nanotechnology, it has become possible to encapsulate these compounds into various forms of nanosized delivery vehicles such as polymer nanoparticles and liposomes (Panda,2017). This strategy provides nano compounds with enhanced bioavailabilities and targeted drug-delivery systems thereby providing maximum therapeutic activities of these compounds (Nouri,2020).

This present study endeavors to investigate the effect of the polyphenols curcumin, resveratrol and rutin on pancreatic cancer cell lines. Three experiments will be carried out to examine their effect on cell morphology, cell viability and cell migration. A healthy pancreatic epithelial cell will be used as a control in the experiments. The study will provide insight on how the effects of these polyphenols differ in treating primary tumor cells (panc-1) versus metastatic tumor cells (capan-1) as well as how they affect normal healthy cells (Htert-Hpne).

CHAPTER II

MATERIALS AND METHODS

2.1 Preparing cell culture medium

All procedures for preparing culture medium were carried out in the tissue culture hood under sterile conditions. All media solutions were filter-sterilized using Stericup® Quick Release (cat # S2GPU02RE) and stored at recommended conditions. Capan-1 cell line required modified Iscove's Modified Dulbecco's Medium (IMDM) with 20% Fetal Bovine Serum (FBS). To prepare 300 ml of Capan-1 cell media, 240 ml of filter-sterilized IMDM was placed in a sterile container. 60 ml of sterile FBS was added to the 240 ml sterile IMDM. The solution was mixed well and stored in the refrigerator at 4℃. To make 1L of media for Htert-Hpne cells, the following contents were combined: 700 ml Dulbecco's Modified Eagle Medium (DMEM), 250 ml medium M3 base, 1 ml human recombinant Epidermal Growth Factor (EGF) (10 ug/ml), 75 µl Puromycin stock (10mg/ml) and 50 ml FBS. The solution was stored at 4℃. Panc-1cell line required modified DMEM with 10% FBS. To make 600 ml Panc-1 stock media, 540 ml of filter sterilized DMEM was added to 60 ml sterile FBS. The solution was mixed well and stored at 4℃.

2.2 Culturing cell lines Capan-1, Htert-Hpne and Panc-1

Capan-1 and Panc-1 cell lines were obtained from ATCC (Manassas, VA). The vials were thawed in a 37℃-water bath. Under aseptic conditions the contents of the vial were transferred into a 15ml tornado tube containing 9 ml of the appropriate media for each cell line. The contents of the tornado tube were centrifuged at 125 x g for 5 minutes at 4℃. The cell pellet was resuspended in appropriate media that was prewarmed to 37℃ and cells were seeded at appropriate ratios in a 90 mm petri dish. The petri dishes were incubated at 37℃, 5% CO_2 in a humidified incubator. Htert-Hpne cells were donated from Wallace lab at Kansas State University and similar procedures were followed to culture the first plate. The cells were passaged after they had achieved confluency (typically every 4 to 5 days) and media was changed every other day. To passage the cells, old media was removed via suction and discarded. The cell monolayer was then gently washed with 10 ml Dulbecco's phosphate-buffered saline (DPBS) and the wash solution was removed via suction. 3ml of sterile 1x Trypsin EDTA (ATCC, Manassas VA) that was previously thawed and warmed to 37℃ was added to the petri dish. The plates were incubated at 37℃ for 7 minutes. After the cells had completely detached, Trypsin EDTA was neutralized by adding 7 ml of the appropriate media for each cell line. The cell suspension was transferred into a 15 ml tornado tube and centrifuged at 125 x g for 5 minutes at 4℃. The supernatant was discarded, and the cell pellet was resuspended in appropriate media and new plates were seeded. All the plates were incubated at 37℃, 5% CO_2 in a humidified incubator.

2.3 Preparing stock solutions for treatment inoculum

Resveratrol 99%, Curcumin 98%, and Rutin Hydrate 98% were purchased from Thermo Fisher scientific and were in powder form. An analytical balance was used to weigh appropriate amounts of each inhibitor to make 10 ml of 1 mM stock solution for each compound. For curcumin preparation 0.0037g of curcumin powder was first dissolved in 1 ml of dimethyl sulfoxide (DMSO) then 9ml DPBS was added to make 10 ml stock solution. For rutin preparation 0.006g of rutin powder was first dissolved in 1 ml of DMSO then 9 ml DPBS was added to make 10 ml stock solution. For resveratrol preparation 0.002g of resveratrol powder was first dissolved in 1 ml of DMSO then 9 ml DPBS was added to make 10 ml stock solution. All the treatment compounds were filter-sterilized with 0.22-micron syringe filter. Treatment compounds were prepared and used on the same day.

2.4 Cell counting

INCYTO C-Chip disposable hemacytometers purchased from Thermo Fisher scientific were used for manual cell counting. Trypan Blue (Thermo Fisher Scientific) was used in the dye exclusion procedure of viable cell counting. This is because viable cells do not take up the dye whereas nonviable cells stain blue. 0.5 ml of cell solution was mixed with 0.5 ml trypan blue. After mixing the solution well and allowing to sit for 2 minutes, 10 µL of the solution was loaded into the ports on the chamber slide. All viable cells in the four corner squares were counted. An average count per square was obtained by dividing the total number of cells counted from all four squares by four. The number of cells per unit volume (cells/ml) was calculated using the formula: $Cells\ per\ ml =$

$Average\ count\ \ per\ square * dilution\ factor * 10{,}000$. After establishing the number of cells/ml, cells were seeded in their respective growth medium.

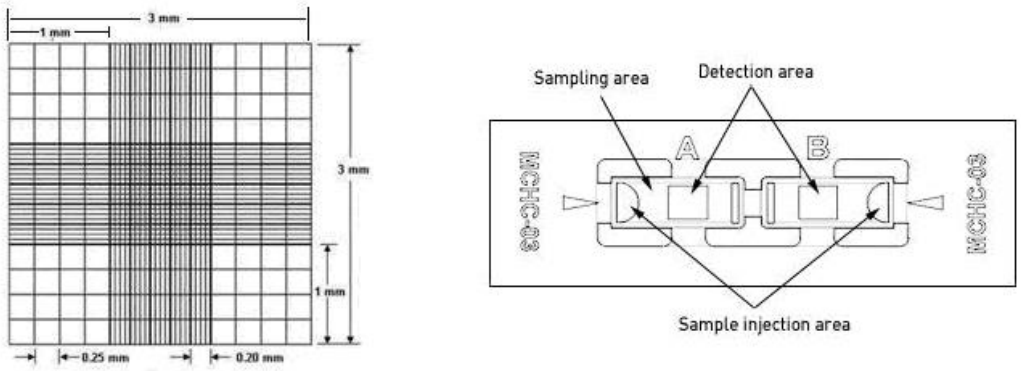


Figure: Schematic illustration of INCYTO C-chip hemacytometer

Image obtained from: http://www.incyto.com/shop/item.php?it_id=1482380591

2.5 Morphology observations

For each cell line, three 6-well plates were seeded with 200,000 cells/well. The plates were incubated at 37 ℃ and 5% CO_2 in a humidified incubator for 24 hours until the cells had formed a monolayer. After 24 hours, the plates were treated with 40 µM of resveratrol, curcumin or rutin. Each treatment was done in triplicate including the control. Treated plates were incubated at 37℃ and 5% CO_2 in a humidified incubator. Microscopic pictures of the plates were taken using an inverted phase contrast microscope at the 24- and 48-hour mark

2.6 Wound Healing Assay

This is an *in vitro* procedure for studying the migration of cells in addition to evaluating cell-to-cell interactions. The technique involves making a linear thin scratch or "wound" which creates a gap in a confluent cell monolayer and subsequent imaging at

defined timepoints after the scratch was made in order to assess the amount of cell migration into the scratch (Grada,2017). All three cell lines were seeded into 12-well plates at a density of 100,000 cells/well with each cell line having 3 12-well plates. Plates were incubated 37°C, 5% CO_2 for 24 hours. After 24 hours, media was removed via suction and a scratch was made on the cell monolayer using a 20 µL sterile pipette tip. 2 ml DPBS was added to each well to gently wash the cell monolayer and remove detached cells. Appropriate media was added to the wells with treatment concentrations of 40µM and 80µM. Microscopic pictures were taken of the scratch at 0-hour mark. Plates were returned to the incubator. More images were captured at 12-hours and 24-hours.

2.7 Cell viability assay

The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The reduction of tetrazolium salts is widely accepted as a reliable way to examine cell viability. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means (ATCC). The MTT kit was purchased through ATCC (catalog #30-1010K™) and stored at 4°C in the dark until ready to use. After trypsinization, resuspension and counting of cells, 9 96-well plates were seeded with 10,000 cells/well for all three cell lines. Each cell line had 3 96-well plates. These plates were incubated at 37°C, 5% CO_2 and were allowed to grow for a period of 48 hours. After 48 hours, treatment with the three compounds was added to the plates at concentrations of 0 µM, 5 µM, 10 µM, 20

µM, 40 µM and 80 µM. Each treatment condition had 6 replicates. The treated plates were incubated for another 24 hours. According to the protocol, 10 µL MTT reagent was added to all treatment wells and control wells. The plates were incubated for 4 hours. Purple crystals were visualized under the microscope in wells that had MTT reagent. Detergent reagent (100 µL) was added to all the wells with MTT reagent. The plates were placed in the dark at room temperature for 2 hours. A microplate spectrophotometer (Epoch) was used to record absorbance at 570 nm.

2.8 Statistical Analysis

The statistical software program R was used to perform statistical analysis for the data obtained from the MTT assay(R,2020). One-way Analysis of Variance (ANOVA) and two-way ANOVAs were used to analyze the data. To meet assumptions for these tests some data was transformed using Log_{10} data transformation.

CHAPTER III

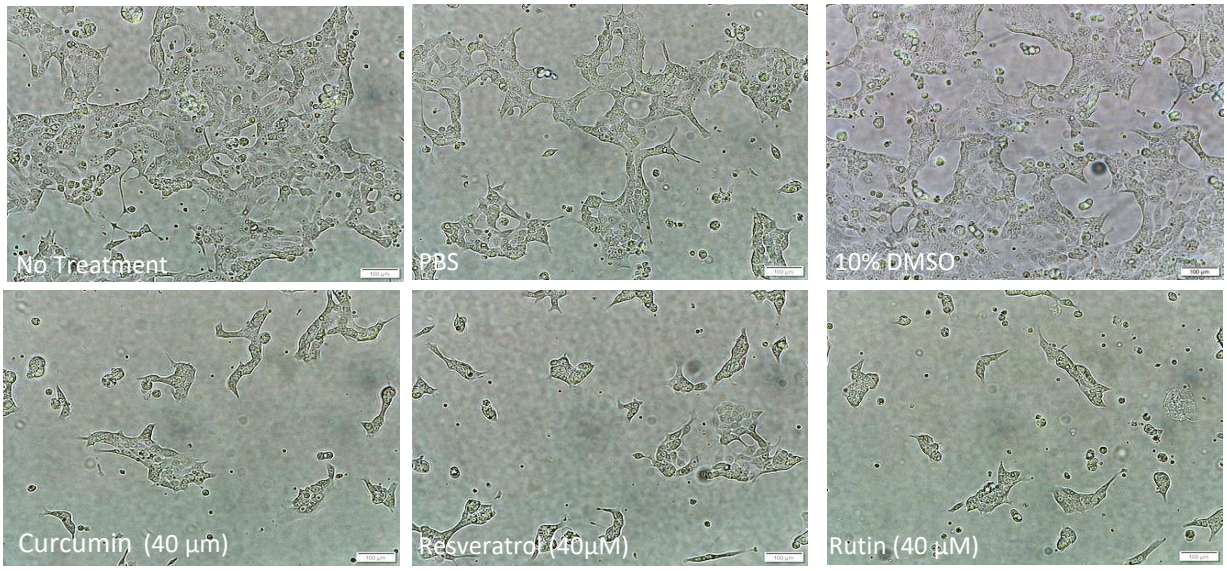
RESULTS AND FINDINGS

3.1 Cell morphology

Morphology of the cells was evaluated using Olympus inverted phase contrast microscope (IX73). All three cell lines (capan-1, panc-1 and Htert-Hpne) were exposed to 40 µM of curcumin, resveratrol and rutin for 24 hours. Images of cells were captured at 24- and 48-hours incubation with treatment. For all three cell lines there was no distinguishable difference in cell morphology pre-treatment and post-treatment with all three compounds. However, there was a notable decrease in cell density as illustrated in the figures below.

Figure 3.1A

CAPAN-1 (24 hours)



CAPAN-1 (48 hours)

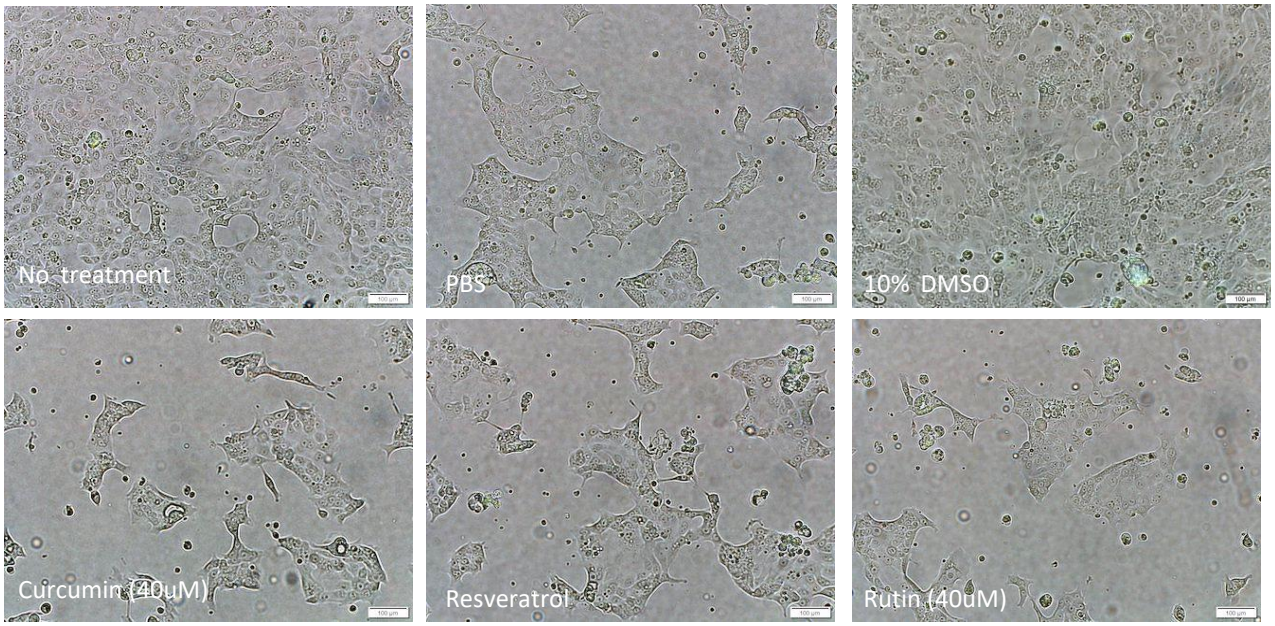
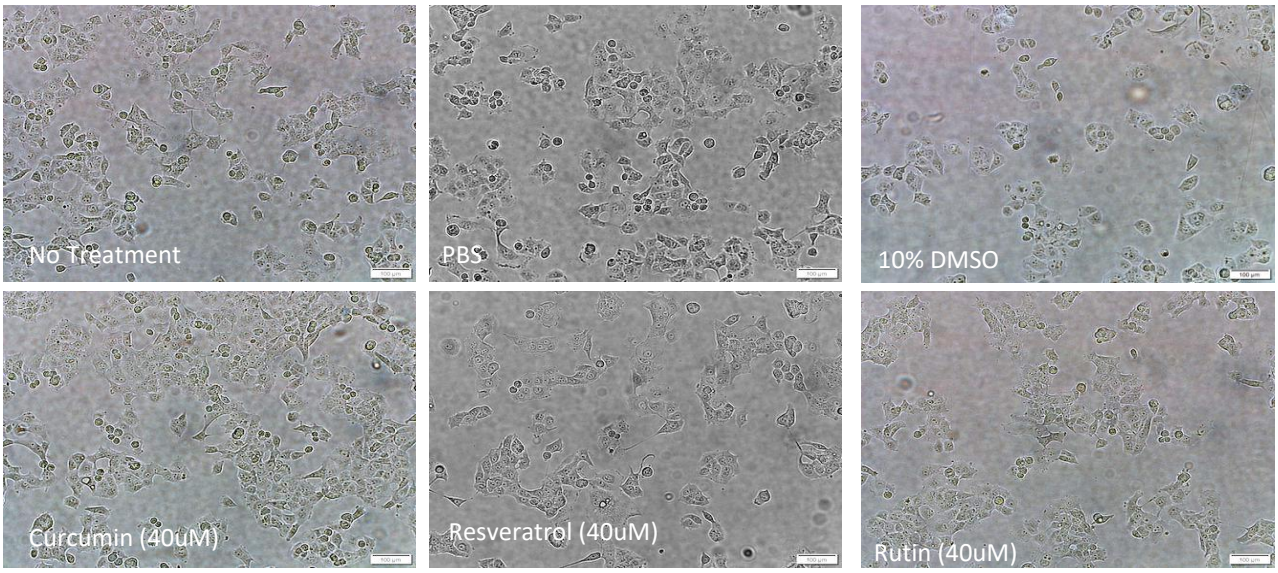


Figure 3.1: Morphology of capan-1 cells as visualized under an inverted phase contrast microscope. 3.1A(i) illustrates images taken after 24 hours of treatment exposure and 3.1(ii) shows the cells after 48 hours treatment exposure. Since treatment compounds were dissolved in both 10% DMSO and PBS, these were used as a controls in addition to the 'no treatment' control. Scale bar = 100 µm

Figure 3.1B

PANC-1 (24 hours)



PANC-1 (48 hours)

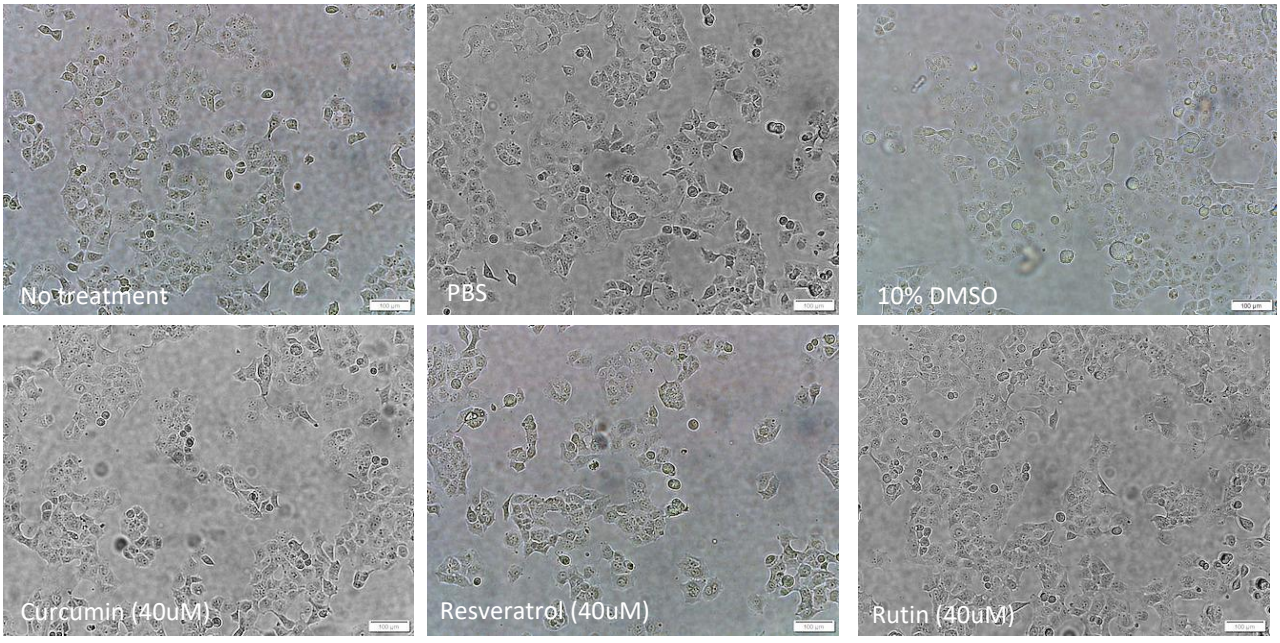
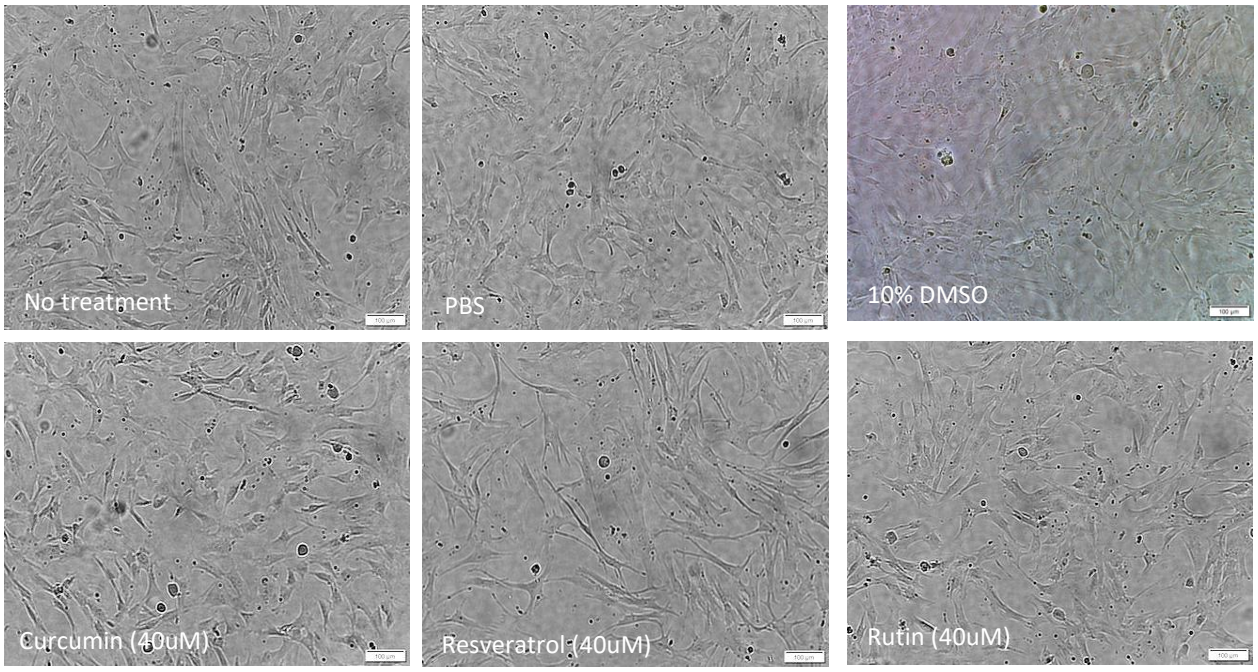


Figure 3.1B: Morphology of panc-1 cells as visualized under an inverted phase contrast microscope. Figure 3.1B(i) shows images of cells after 24-hour treatment exposure and figure 3.1B(ii) shows images of cells after 48-hour treatment exposure. Scale bar = 100 µm.

Figure 3.1C

HTERT (24 hours)



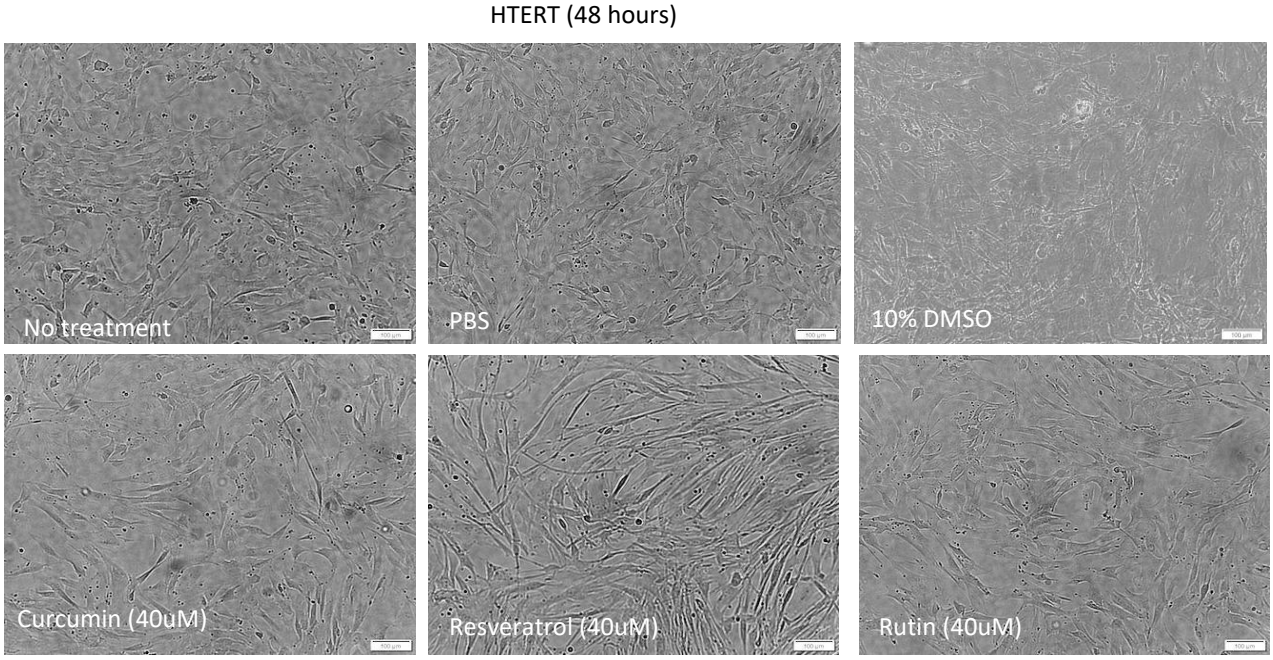


Figure 3.1C: Morphology of Htert-Hpne cells as visualized under an inverted phase contrast microscope. Figure 3.1C(i) shows images of cells after 24-hour treatment exposure and figure 3.1C(ii) shows images of the cells after 48 hours of treatment exposure. Scale bar = 100 µm.

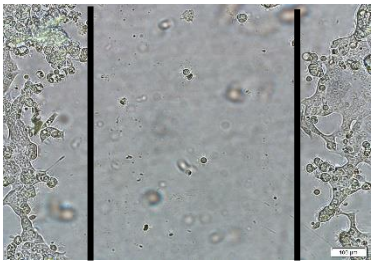
3.2 Wound Healing Assay

Cell migration is a hallmark of wound repair, cancer invasion and metastasis. Wound healing is a complex cellular and biochemical process which involves dynamic interactions and processes that lead to cell migration, proliferation, and differentiation (Grada,2017). Analysis of cell migration *in vitro* is a useful assay to study alterations in cell migratory capacity in response to experimental manipulation. To assess the effect of the plant-based polyphenols on cell migration, scraped cell monolayers were incubated in the presence of 40 µM and 80 µM curcumin, resveratrol and rutin. Images of cell

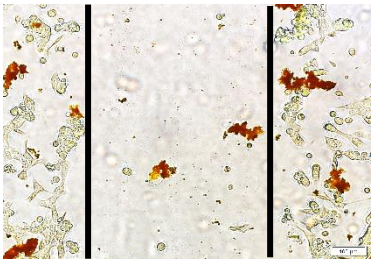
monolayers were captured using an inverted phase contrast microscope (Olympus IX73) at 0, 12 and 24 hours. Images shown in the figures below were of the monolayers treated with 80 µM of treatment compounds. This is because lower concentrations (≤40 µM) did not show any significant effects on cells except for 40 µM curcumin on capan-1 cell monolayer.

Figure 3.2A

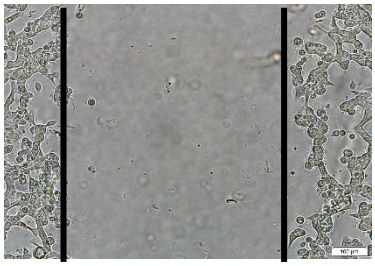
CAPAN-1 Resveratrol 0HR



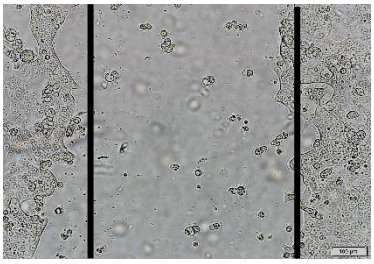
CAPAN-1 Curcumin 0HR



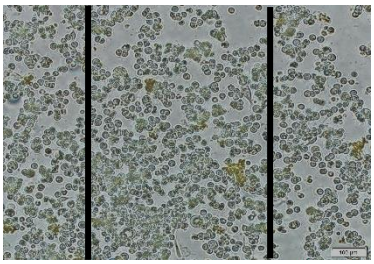
CAPAN-1 Rutin 0HR



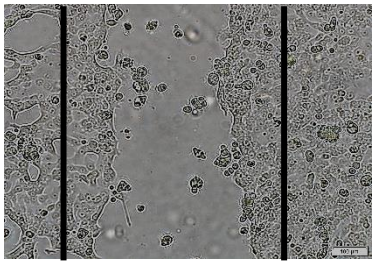
CAPAN-1 Resveratrol 24HR



CAPAN-1 Curcumin 24HR



CAPAN-1 RUTIN 24HR



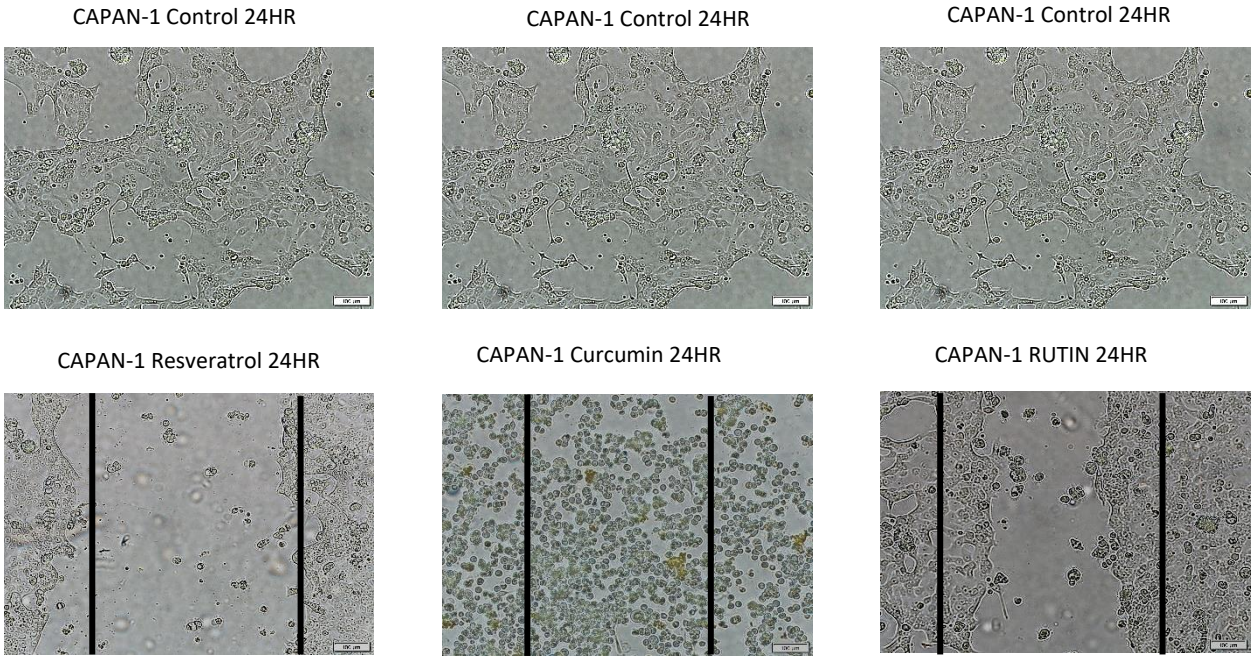
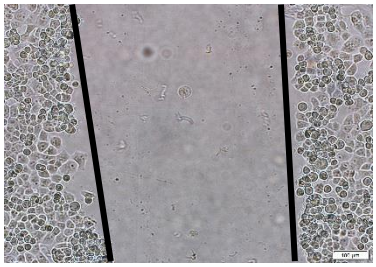


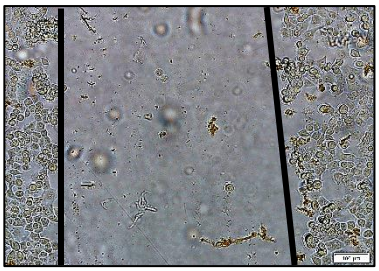
Figure 3.2A: 3.2A(i) shows representative images of capan-1 cell monolayers treated with resveratrol, curcumin and rutin (80 µM) at 0 and 24-hours following initiation of the scratch. The scratch is indicated by the black parallel lines. Scale bar = 100 µm. Figure 3.2A(ii) contrasts the control capan-1 monolayers that were not treated with any compound with the treated monolayers at the 24-hour mark. Black lines indicate the borders of the scratch. Scale bar = 100 µm.

Figure 3.2B

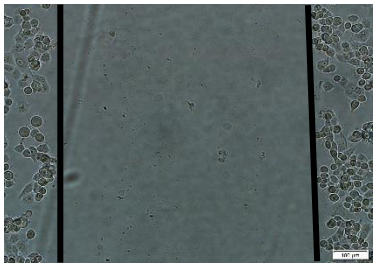
PANC-1 Resveratrol 0HR



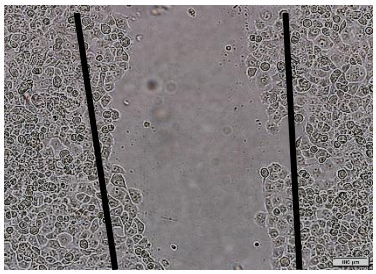
PANC-1 Curcumin 0HR



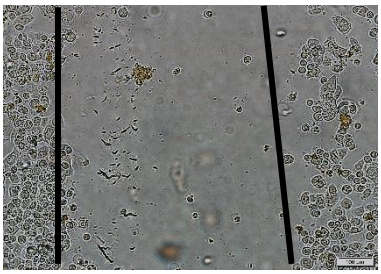
PANC-1 Rutin 0HR



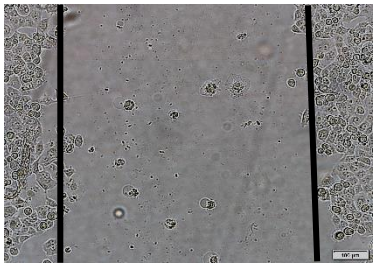
PANC-1 Resveratrol 24 HR



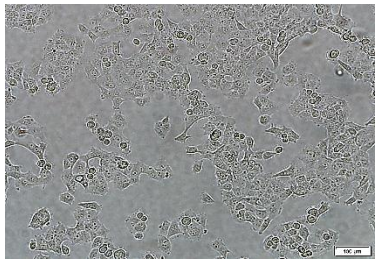
PANC-1 Curcumin 24HR



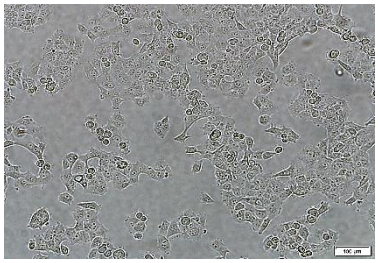
PANC-1 Rutin 24 HR



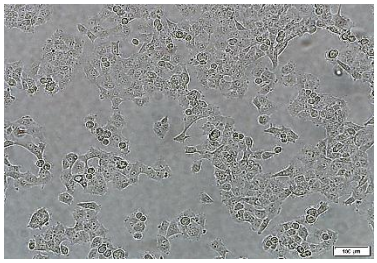
PANC-1 Control 24 HR



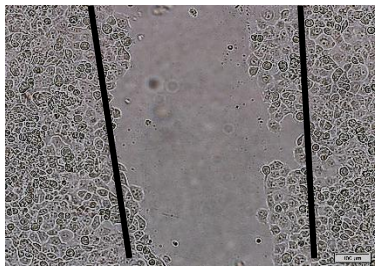
PANC-1 Control 24 HR



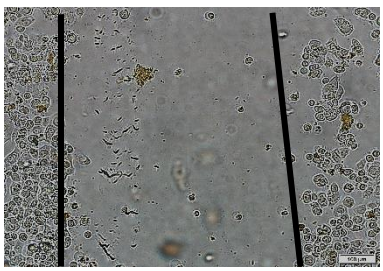
PANC-1 Control 24 HR



PANC-1 RESVERATROL 24



PANC-1 Curcumin 24HR



PANC-1 Rutin 24 HR

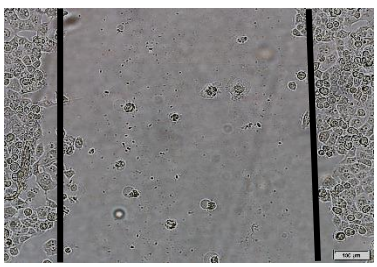
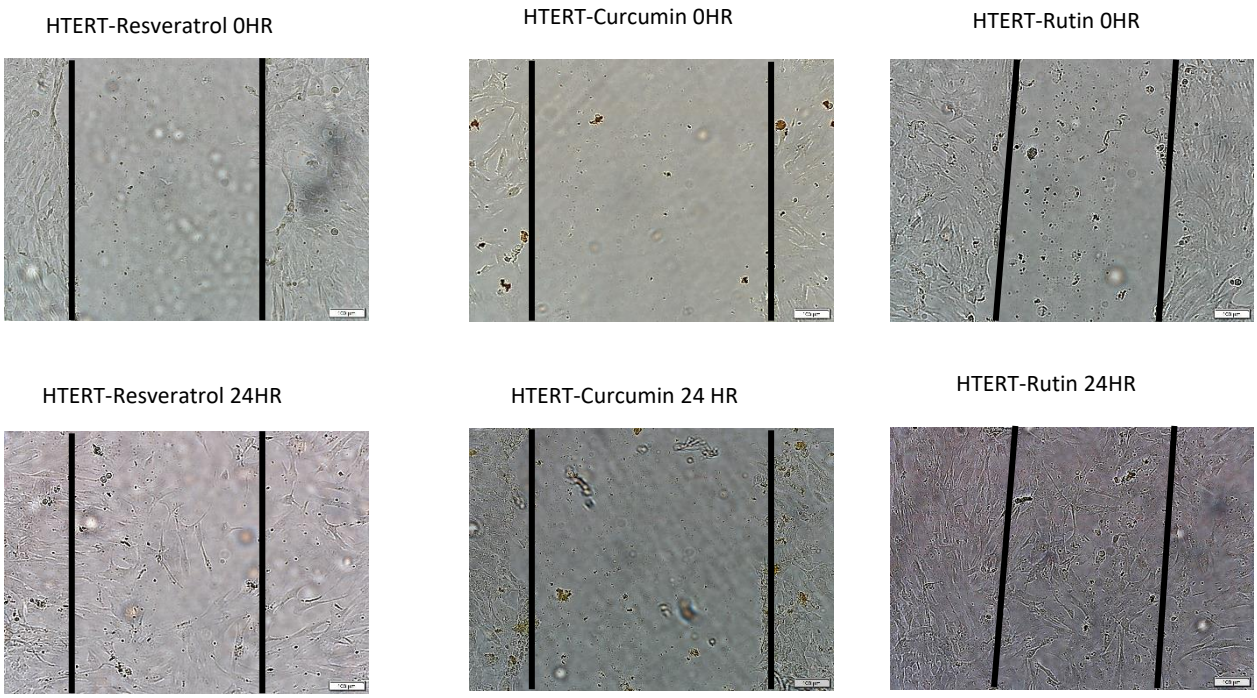


Figure 3.2B: 3.2B(i) shows images of panc-1 monolayers treated with 80 µM resveratrol, curcumin and rutin imaged at 0 and 24 hours. Figure 3.2B(ii) contrasts between the control panc-1 monolayers that were untreated against those that were treated at the 24-hour mark. The scratch is indicated by the black parallel lines. Scale bar = 100 µm.

Figure 3.2C



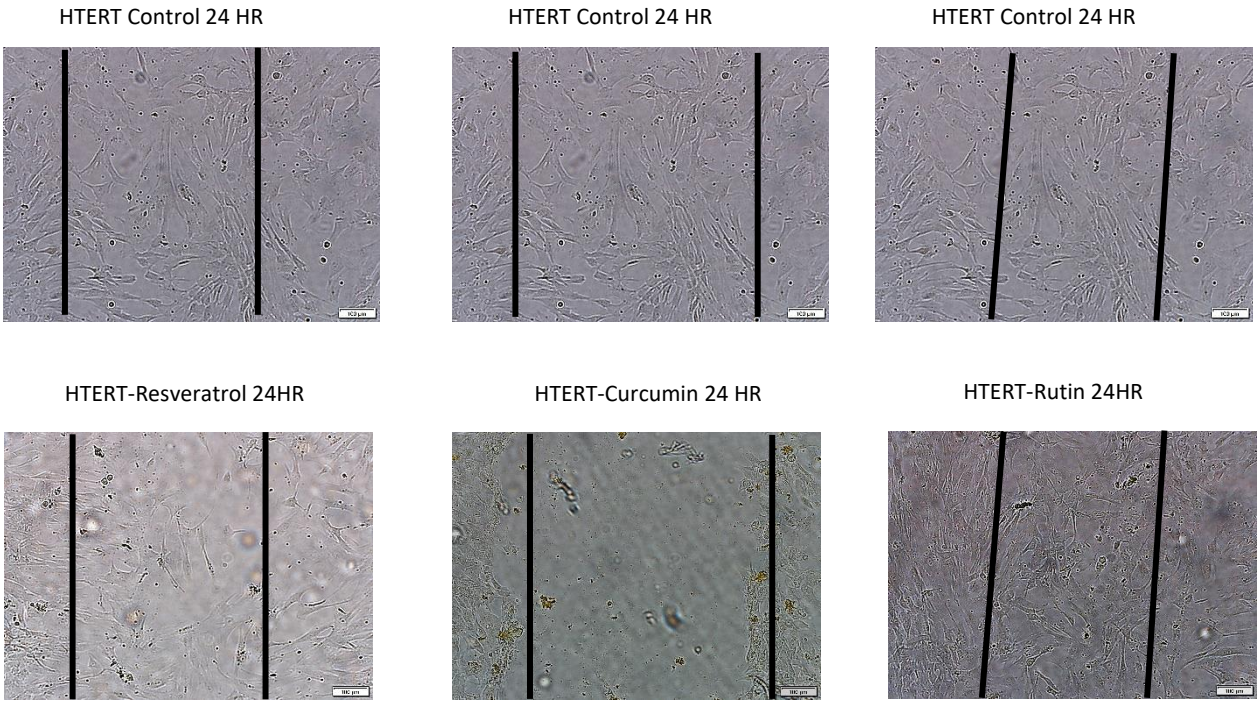


Figure 3.2C: 3.2C(i) shows representative images of Htert-Hpne cell monolayers at 0- and 24-hours following initiation of the scratch. Figure 3.2B(ii) contrasts between the control Htert-Hpne monolayers that were untreated against those that were treated at the 24-hour mark. The scratch is indicated by the black parallel lines. Scale bar = 100 µm.

3.3 Cell Viability

The MTT assay is a calorimetric assay that is widely used to quantify cell viability. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer. Viable cells with active metabolism convert MTT into a purple formazan product which accumulates as an insoluble precipitate. When cells die, they lose

their ability to convert MTT into formazan thus color formation serves as a convenient marker for viable cells (Riss,2013). To assess the effect of resveratrol, curcumin and rutin on cell viability, all three cell lines were seeded into 3 96 well plates at a density of 10,000 cells/well. Various concentrations of these treatment compounds (0, 5, 10, 20, 40, and 80 µM) were added to the 96 well plates and MTT assays were performed after 24 hours of treatment. Each treatment condition had 6 replicates. The percentage cell viability was calculated by first obtaining the mean optical densities (OD) of treatment replicates then applying the following formula (Satar,2015):

$$control\ \%\ viability = \frac{mean\ OD\ control}{\text{mean OD control}} * 100$$

$$Treatment\ \%\ viability = \frac{mean\ OD\ treatment}{\text{mean OD control}} * 100$$

Figure 3.3A

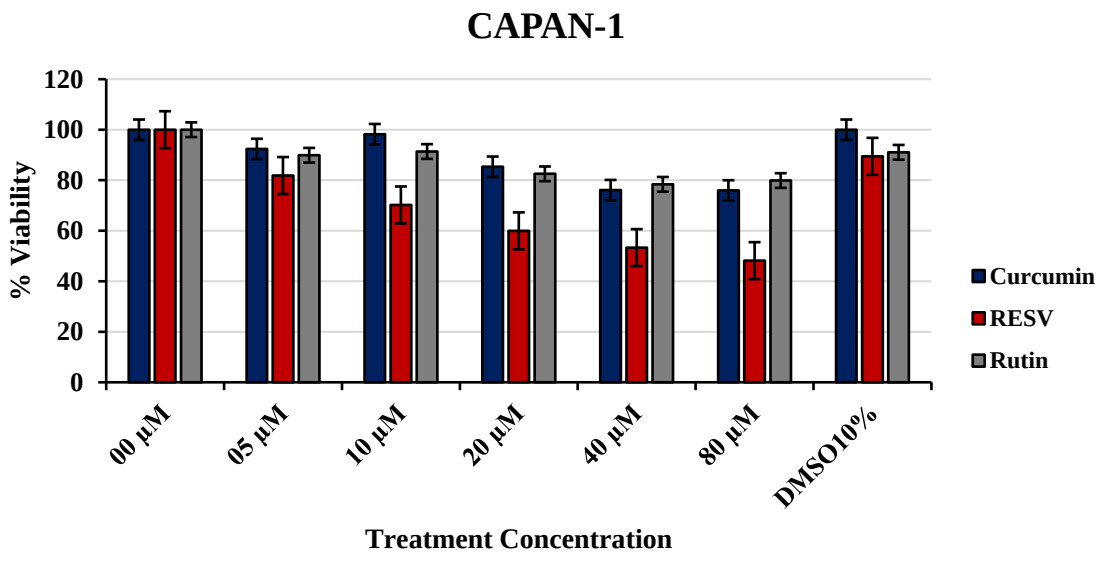


Figure 3.3A illustrates the percentage cell viability of capan-1 cells that have been treated with curcumin, resveratrol and rutin at 0, 5, 10, 20, 40, and 80 µM concentrations. Error bars in the graph represent standard error.

Figure 3.3B

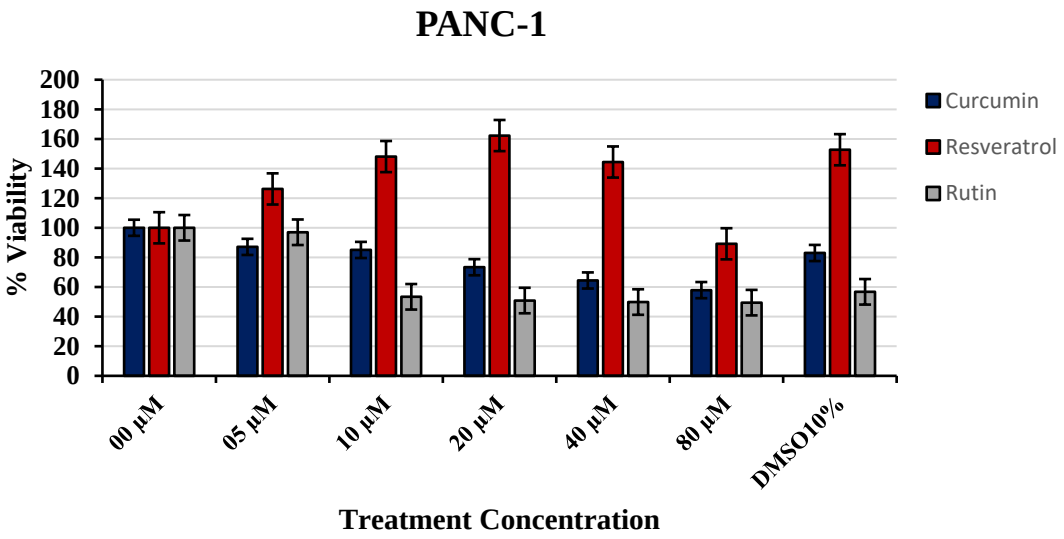


Figure 3.3B shows the percentage cell viability of panc-1 cells that have been treated with curcumin, resveratrol and rutin at 0, 5, 10, 20, 40, and 80 µM concentrations. Error bars in the graph represent standard error.

Figure 3.3C

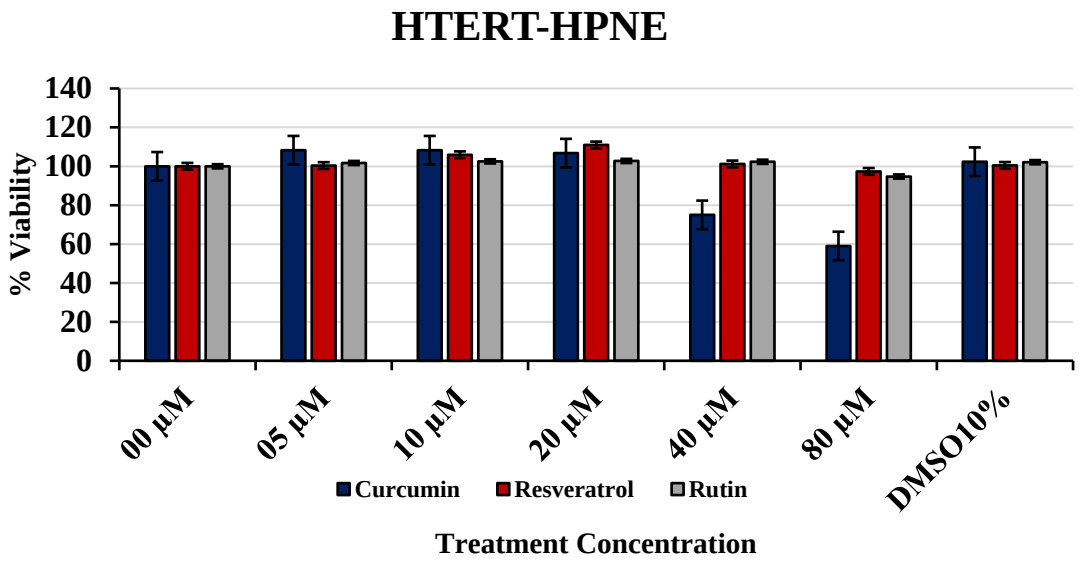


Figure 3.3C illustrates the percentage cell viability of Htert-Hpne cells that have been treated with curcumin, resveratrol and rutin at 0, 5, 10, 20, 40, and 80 µM concentrations. Error bars in the graph represent standard error.

3.4 Statistical Analysis

Absorbance values obtained from the MTT assay was used to study the interactions between the three cell lines and the three treatment compounds and determine whether that interaction was statistically significant. Preceding the analysis some of the data was $\log_{10}$ transformed to meet the assumptions of normality and homoscedasticity. The mean differences were examined using two-way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) post hoc analyses. For all data analyses $\alpha = 0.05$ was used. Nine one-way ANOVAs were conducted (one for each compound and cell line) and they all yielded statistically significant effects $p < 0.001$ except for the effect of rutin on Htert-Hpne. The one-way ANOVA of this interaction yielded results that were statistically

non-significant ($F_{6,14}$ 1.85 p = 0.16). Three two-way ANOVAs were performed to analyze the effect of treatment compounds and their various concentrations on viability of the three different cell lines. All two-way ANOVAs revealed that there was a statistically significant interaction between the effect of treatment compounds and their concentrations on cell viability.

Figure 3.4A

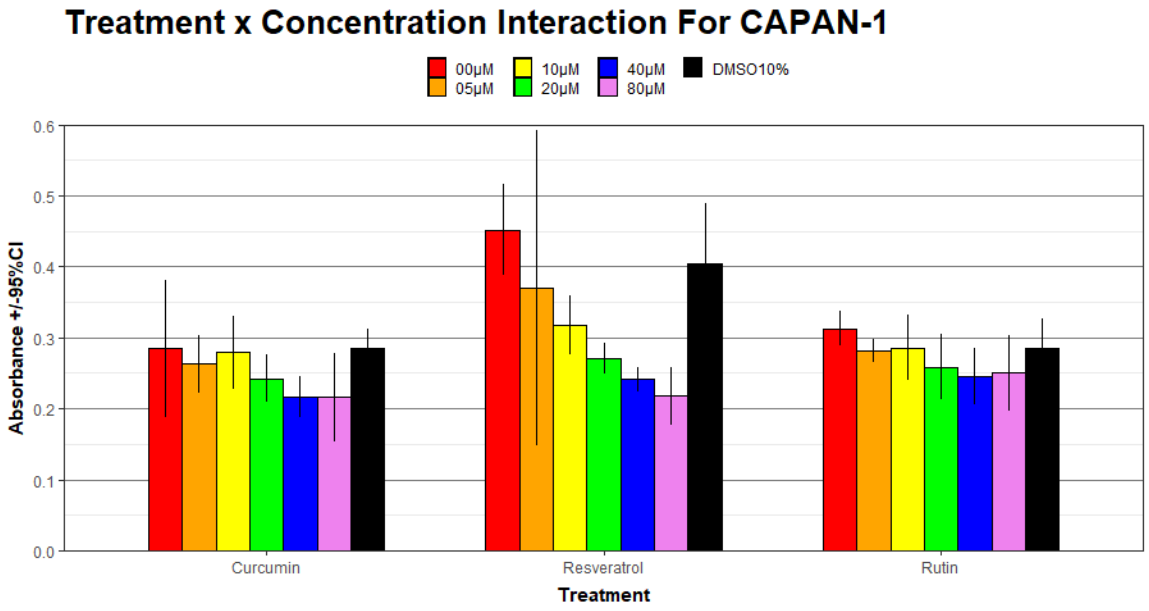


Figure 3.4A illustrates interactive effect of all three treatments compound at concentrations 0 to 80 µM on capan-1 cells. This interaction is statistically significant $F_{12,42} = 5.5601$ p = 1.406^{-5}

Figure 3.4B

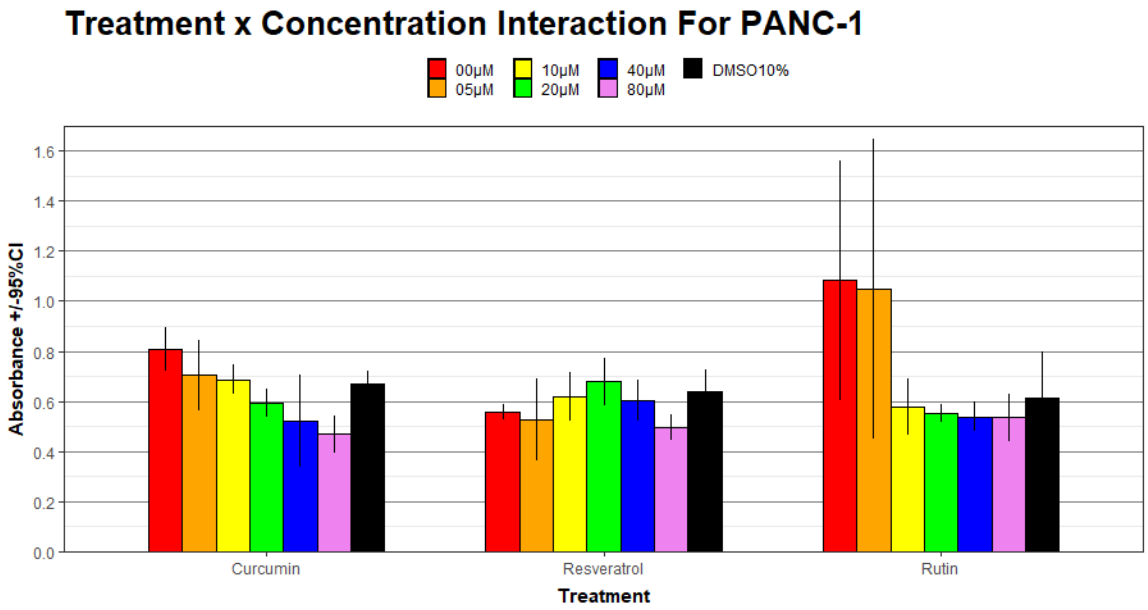


Figure 3.4B shows the interactive effect of all three treatments compound at concentrations 0 to 80 µM on panc-1 cells. This interaction is statistically significant $F_{12,42} = 13.298$ $p = 1.076^{-10}$

Figure 3.4C

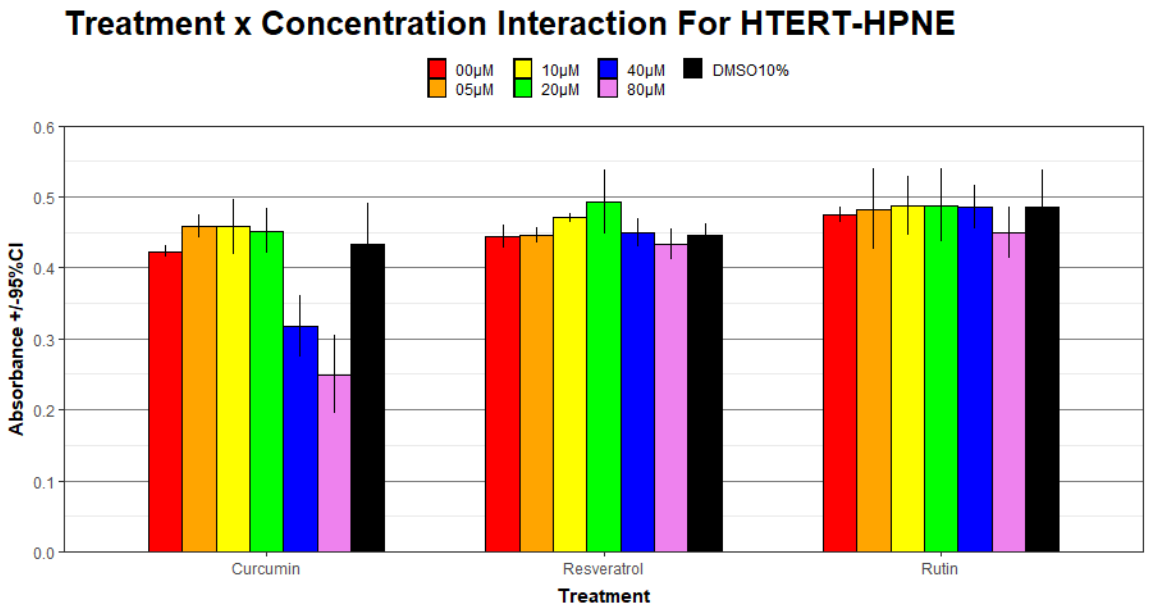


Figure 3.4C indicates interactive effect of all three treatments compound at concentrations 0 to 80 µM on Htert-Hpne cells. This interaction is statistically significant $F_{12,42} = 25.229$ $p = 2.241^{-15}$

CHAPTER IV

DISCUSSION

In this study human pancreatic adenocarcinoma (capan-1), human pancreatic epithelioid carcinoma (panc-1), and healthy epithelial pancreatic cells (Htert-Hpne) were treated with polyphenols and studied for effects on morphology, migration, and cell viability. Capan-1 was obtained from a liver metastasis whereas panc-1 was obtained from a primary tumor in the head of the pancreas (Deer,2010). None of the polyphenol treatments (curcumin, resveratrol, rutin) resulted in a significant change in morphology in any of the three cell lines (Figures 3.1A-3.1C). However, I did observe a dramatic decrease in cell density when capan-1 cells were treated with 40 µM of curcumin, resveratrol, or rutin (Figure 3.1A). For panc-1, treatment with 40 µM resveratrol resulted in a slight decrease in cell density (Figure 3.1B). None of the three compounds resulted in any effect on cell morphology or density of Htert-Hpne (Figure 3.1C).

The ability of the three polyphenols to inhibit cell migration and the initiation of cell proliferation was studied using the scratch assay. Out of the three polyphenols and the three cell lines, resveratrol displayed the highest inhibitory effect against capan-1 cell migration (Figure 3.2A). There were cells present in the scratch for rutin treatment, but the wound was still distinguishable at 24 hours after treatment. Curcumin showed a distinct effect in that the cells assumed a circular morphology and were scattered all over the plate

making the scratch indistinguishable. It is likely that curcumin caused capan-1 cells to detach from the surface of the petri dish which resulted in the cells floating all over the petri dish. Based on the rounded appearance of these floating cells, it seems probable that the detachment led to cell death. This observation was consistent in curcumin treated monolayers, at 40 and 80 µM. In the control (i.e. untreated) capan-1 monolayers, cells were able to migrate and close the gap which had been previously introduced by the scratch (Figure 3.2A ii). In panc-1 monolayers, curcumin and rutin seemed to have a greater inhibitory effect against cell migration compared to resveratrol (figure 3.2B). There were very few cells present within the scratch in the monolayers treated with curcumin and rutin. Conversely, in the monolayer treated with resveratrol there were many cells within the scratch, although they did not completely cover the scratch. In the untreated panc-1 cell monolayers, the cells migrated and completely covered the scratch (Figure 3.2B ii). In the treated panc-1 monolayers, the scratch was scratch failed to close completely. For Htert-Hpne cell monolayers, of all three compounds, curcumin seemed to have the greatest effect in inhibiting cell migration (figure 3.2C). There were many cells within the scratch treated with resveratrol, but the scratch was still mildly distinguishable. The monolayer treated with rutin displayed a lack of cell migration inhibition since the scratch was completely covered with cells. In the untreated Htert-Hpne cell monolayers, the cells migrated and completely covered the scratch (figure 3.2C ii). It was evident that these polyphenols were inhibiting cell migration in the cancerous cell lines but not in the healthy cell line.

Cell viability following treatment with polyphenols was assayed using the MTT assay as described in the Materials and Methods. Of all three polyphenols, the most dramatic effect was when capan-1 cells were treated with resveratrol (figure 3.3A). Cell

viability ranged from 82% with 5 µM to 48% with 80 µM resveratrol. This observed effect was dose dependent since there was a very low cell viability with higher concentrations of resveratrol. Curcumin caused a decrease in capan-1 cell viability as the treatment concentration increased. Interestingly, there was a slight increase in cell viability at 10 µM from 92% at 5 µM to 98% at 10 µM. The viability of capan-1 cells treated with curcumin ranged from 92% with 5 µM to 76% with 80 µM treatment. Rutin altered the viability of capan-1 cells in a manner similar to curcumin including a slight increase in viability at 10 µM rutin concentration. Overall, the cell viability of ruitin-treated capan-1 cells ranged from 90% to 78%.

Resveratrol seemed to have had a unique effect on panc-1 cells in that it caused an increase in percent cell viability in most treatment concentrations with the viability peaking at 20 µM. This increase in viability is also observed in the 10% DMSO control group. The only treatment concentration that showed a decrease in viability was 80 µM with an 89% viability. It is important to note that this unique effect (increase in viability >100% in panc-1 cells with higher reveratrol treatment concentrations) was also observed in a previous experiment during the course of this study. This was an unexpected finding which warrants further investigation to determine the reason for its occurrence. It is likely that this observed effect was as a result of increased proliferation of panc-1 cells rather than an increase in panc-1 cell viability. Curcumin resulted in a decrease in cell viability in a dose dependent manner with lower viability occurring with higher treatment concentrations. The cell viability ranged from 87% with 5 µM to 57% with 80 µM. Rutin had the greatest effect on cell viability of panc-1 cell line of all three compounds. Viability decreased with increased treatment concentrations. There was a significant decrease between 5 µM and 10 µM with

viability dropping from 97% to 53%. The variability in % viability among concentrations of 20 µM to 80 µM was minimal. This might indicate a maximum therapeutic concentration for rutin in regards to panc-1 cells. Overall, the viability of panc-1 cells treated with rutin ranged from 97% at 5 µM to 49 % at 80 µM.

In the control cell line Htert-Hpne all three treatment compounds seemed to cause a slight increase in cell viability at lower concentrations and a decrease in cell viability with higher concentrations. Curcumin caused an increase in cell viability which peaked at 10 µM with 108% cell viability which decreased to 75% at 40 µM and terminated at 59% at 80 µM treatment concentration. Resveratrol resulted in an increased cell viability which peaked to 111% with 20 µM treatment concentration. At the highest treatment concentration, cell viability decreased to 97%. Rutin caused a slight increase in cell viability to 103% at 20 µM and a slight decrease in viability to 95% at 80 µM.

The effect of the polyphenols on each cell line was statistically significant ($p<0.001$) except for the interaction between rutin and Htert-Hpne cell line. Despite the compound causing a slight increase in % cell viability to 103%, this effect was not statistically significant. When the interaction between all three compounds was analyzed for each cell line using two-way ANOVA, it was found to be highly significant $p < 0.0001$ (figures 10, 11, 12). The results of this study indicated that curcumin, resveratrol and rutin have varied effects on these cell lines. As already pointed out, resveratrol seemed to have had the greatest effect in inhibiting capan-1 cell migration and causing the greatest decrease in capan-1 percent viability. Rutin had the greatest inhibitory effect against panc-1 cell migration and also caused the greatest decline in panc-1 percent cell viability. Curcumin

showed the greatest effect on Htert-Hpne cells both in inhibiting cell migration and reducing percent cell viability.

Given some of the issues with solubility of polyphenols that were encountered during the experiments, future studies with these three polyphenols could explore the use of nano prepared formulations of these compounds which would have improved solubility and bioavailability. In addition to improving solubility and bioavailability nano prepared compounds would also provide a more targeted drug delivery system. Performing Western blot analyses on the cell lines could detect proteins and glycoproteins which could be useful pharmacological targets. As noted previously, polyphenols modulate various signaling pathways and protein molecules in exerting their anticancer effects. Further studies are required to elucidate the underlying molecular mechanisms by which curcumin, resveratrol and rutin produced their noted effects on the cell lines. Also, by determining the cell receptors that allow for cellular uptake of these treatment compounds, studies can be done to determine what types of cancers would be most sensitive to treatment compounds based on their expression of these cell receptors.

CHAPTER V

CONCLUSIONS

As the search for improved treatments for pancreatic cancer continues, this study presents evidence that plant-based polyphenols can inhibit pancreatic cell migration and significantly decrease pancreatic cancer cell viability. Different polyphenols exerted varied effects on the different cell lines. Most notably resveratrol exhibited the greatest effect on the metastatic cells (capan-1) whereas rutin had the greatest effect on the primary tumor cells (panc-1). These compounds did not seem to have any detrimental effects on the health control cell line (Htert-Hpne) except at high treatment concentrations (80 µM) This observation may give credence to the protective effect of polyphenols on normal cells.

This work was a follow up on a thesis study by Segaran (2021) which examined the effects of curcumin, resveratrol and rutin on lung adenocarcinoma (A549) cells. The study demonstrated that these three polyphenols at low concentrations could effectively inhibit proliferation and decrease migration of A549 cells. The present work adds to the previous study by demonstrating that curcumin, resveratrol, and rutin effectively inhibited cell migration and significantly reduced cell viability in pancreatic cancer cells. However, the mechanism through which these polyphenols exert their effects on the cancer cell lines remains to be understood. Further studies are required to understand why resveratrol caused an increase in panc-1 cell viability. Future studies could focus on elucidating these

mechanisms of action, improving solubility and bioavailability of these polyphenols through nano prepared compounds, and performing animal studies to see if the *in vitro* findings are upheld.

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