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PLANT BASED COMPOUNDS INHIBIT PROLIFERATION, ALTER CYTOMORPHOLOGY AND DECREASE MIGRATION OF HUMAN ADENOCARCINOMA CELLS

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PLANT BASED COMPOUNDS INHIBIT PROLIFERATION, ALTER CYTOMORPHOLOGY AND DECREASE MIGRATION OF HUMAN ADENOCARCINOMA CELLS

A Thesis Submitted to the Graduate School
in Partial Fulfillment of the Requirements
for the Degree of
Master of Science

Devapriya Segaran

Pittsburg State University

Pittsburg, Kansas

April 2021

PLANT BASED COMPOUNDS INHIBIT PROLIFERATION, ALTER CYTOMORPHOLOGY AND DECREASE MIGRATION OF HUMAN ADENOCARCINOMA CELLS

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PLANT BASED COMPOUNDS INHIBIT PROLIFERATION, ALTER CYTOMORPHOLOGY AND DECREASE MIGRATION OF HUMAN ADENOCARCINOMA CELLS

An Abstract of the Thesis by
Devapriya Segaran

Adenocarcinoma is an aggressive form of lung cancer that has a high risk of recurrence with a survival rate of 33%. In recent years, there has been much interest in the ability of naturally occurring plant derived phenols to inhibit specific type of cancers. Compounds like curcumin derived from turmeric, rutin derived from citrus fruits and resveratrol derived from blueberries have been of particular interest. In this thesis I studied the anti-cancer effects of the above-mentioned compounds on a human A549 adenocarcinoma cell line. Inverted phase contrast microscopy was used to observe alterations to the cytomorphology of cells. An MTT assay was used to measure cell viability. Dose and time dependent A549 cell viability were observed following treatment with curcumin, rutin and resveratrol. The effects on cell migration after treatment with compounds was determined by wound-healing assay and MTT assay. I found that the survival rates of curcumin treated cells reduced at higher concentrations after 24 h treatment when compared to rutin and resveratrol treated cells, although resveratrol showed lower viability than rutin. After 48 hours of treatment the viability of curcumin treated cells dropped to 10% with somewhat higher survival with rutin (18% viability) and resveratrol (12% viability). Morphological analysis showed that compound treated cells became round and the normal spindle shape disappeared. Over a period of 6 h to 24 h, the number of bright circular dead floating cells increased. Treatment with curcumin and resveratrol strongly reduced wound repair and significantly inhibited the migration of A549 cells in a concentration dependent manner.

Rutin treatment reduced wound repair but had a very minimal effect on cell migration. These findings provide support to the potential utility of curcumin, resveratrol and rutin as natural molecules with anticancer activity against adenocarcinoma.

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CHAPTER I

INTRODUCTION

1.1 Cancer

Cancer is a disease caused by changes in the DNA that results in cells dividing uncontrollably and spreading into surrounding tissues. The human body is made up of trillions of cells that undergoes cell division to form new cells as required by the body. As a part of the ageing process, new cells are substituted for old, an event that never happens in cancer cells where old cells never die and new cells are formed without necessity. Cancer cells continue growing and form overgrowths in the body called tumors. They are usually solid or strong masses of tissues which can be malignant or benign [21].

Malignant tumors spread into surrounding tissues and as they develop, they move to different parts in the body either through the blood stream or lymph system and initiate new carcinogenic tumors a long way from the original site of emergence. In contrast, benign tumors lack the ability to either invade neighboring tissue or metastasize (spread throughout the body). When removed, benign tumors usually do not grow back, whereas malignant tumors often do. Cancer cells are also often able to evade the immune system, a network of organs, tissues, and specialized cells that protects the body from infections and other conditions [47]. Although the

immune system normally removes damaged or abnormal cells from the body, some cancer cells can hide from the immune system.

1.2 Difference between normal cells and cancer-causing cells in the human body

Cancer cells differ from normal cells in many respects, causing them to proliferate uncontrollably and become invasive. Cancer cells are less specialized than normal cells, which is a significant distinction. That is, while normal cells develop into very different cell types with unique roles, cancer cells do not [21]. This is one of the reasons that, unlike normal cells, cancer cells continue to differentiate indefinitely. Cancer cells are a perversion of normal, functional differentiated cells. They differentiate into an abnormal and aggressive cell that no longer performs the functions of the healthy cell **(Fig.1.1)**. Furthermore, cancer cells may miss signals that usually warn cells to avoid dividing or that trigger a mechanism known as programmed cell death, or apoptosis, which the body uses to kill unneeded cells. Cancer cells often affect the normal cells, chemicals, and blood vessels that accompany and feed a tumor, a region known as the microenvironment [12].

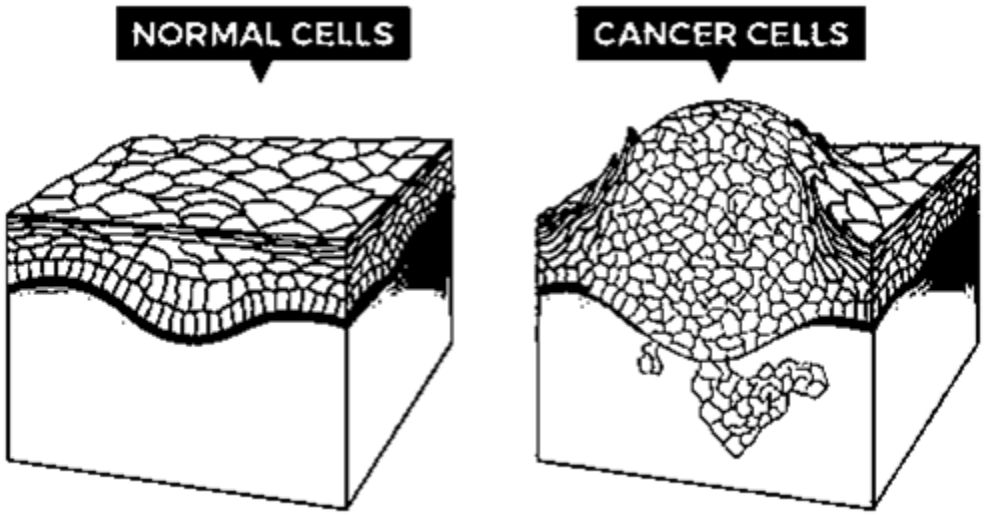


Figure 1.1: Uncontrollable division of cancer cells and spread into surrounding tissues vs regular division of normal cells [21].

1.3 Lung cancer and types

Lung cancer occurs as a result of unchecked growth of unusual cells either in one or in both the lungs. These anomalous cells do not perform the functions of healthy human cells and may block the function of normal cells. This abnormality interferes with the lung's normal role of providing oxygen to the human body through blood. Despite numerous advancements in treatment methods, lung cancer is often not curable, even when detected early. Lung cancer screening is a demanding, fast-paced, and interdisciplinary area [47]. Lung cancer is usually classified based on its appearance in microscope as either small cell lung cancer (SCLC) or Non-small cell lung cancer (NSCLC).

Since these two forms of lung cancer have distinct traits for development and distribution, their treatment is very different [42]. As a result, discriminating between small cell lung cancer and non-small cell lung cancer is clinically important.

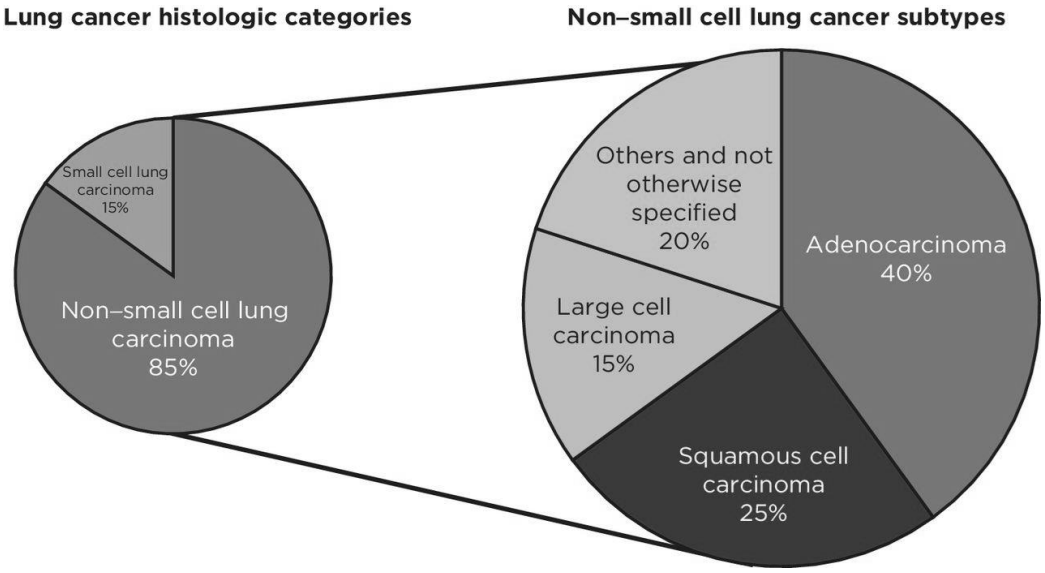


Figure 1.2: Histologic classification of lung cancer. The two major lung cancer histologic categories (NSCLC and small cell lung carcinoma) and the most common histologic subtypes among NSCLC (adenocarcinoma, squamous cell carcinoma, and large cell carcinoma) [42].

NSCLC is the most prevalent form accounting for 85% of lung cancers and is one of the main causes of death in humans **(Fig 1.2)**. The use of computer-aided diagnosis and survival estimation for NSCLC is very important in the care of lung

cancer patients. Lung cancer also has a poor prognosis, with a five-year survival rate of only 10% in many countries. Adenocarcinoma including bronchi alveolar carcinoma accounts for approximately 40% of NSCLC, while squamous cell carcinoma accounts for approximately 25% - 30% [17]. This study involves the use of adenocarcinoma cell lines (A546 cells), a part of NSCLC that accounts for the majority of lung cancers.

1.4 Adenocarcinoma

Adenocarcinoma is a form of cancer that develops in glands that secrete mucus or fluids in various parts of the body [8]. It is most common in the following cancer types:

- Lung cancer- Adenocarcinoma is the most common type of non-small cell lung cancer [8].
- Prostate cancer- This is the type of cancer that occurs in the prostate glands, adenocarcinoma being the cause of 99% of such cancers [31].
- Pancreatic cancer- This type of adenocarcinoma occurs in pancreatic ducts.
- Colorectal cancer- This adenocarcinoma occurs in the intestinal glands inside colon and rectum and is the cause of 95% of colon or rectal cancers [47].

1.5 Causes of cancer

Cancer has wide range of causes, including genetic, biochemical, occupational, and environmental exposure to benzene, heavy metals, pesticides, and mineral fibers.

These factors may function directly or indirectly, inducing the expression of the malignant phenotype, which controls the progression of the disease. Dietary causes are responsible for about 30-35% of cancer cases [35]. There is compelling evidence that diet plays a part in cancer prevention and/or development [13]. Combining this evidence, the Mediterranean diet represents one of the most popular dietary models currently under consideration in the field of cancer prevention and treatment outcomes [26].

Cancer prevalence is lower in countries where food habits include a low intake of beef, a high intake of dairy and alcohol, and regular ingestion of bioactive-nutrient-rich fruits and vegetables. In reality, fruits and vegetables contain thousands of phytochemicals with antioxidant, anti-inflammatory, anticarcinogenic, antiviral, and antiallergic properties; they are known as carotenoids, vitamins, alkaloids, nitrogen-containing, organo-sulfuric, and phenolic compounds [32]. In particular, *in vivo* and *in vitro* research, as well as human studies, have indicated that polyphenols may protect against chronic diseases and have shown an inverse relationship between polyphenol consumption and the risk of tumor growth [46]. In this study we will assess the ability of several polyphenols to inhibit the growth and spread of cancer cells.

1.6 Polyphenols against cancer

Polyphenols are a large family of naturally occurring organic compounds that are chemically distinguished by the presence of several phenol groups. They are common in plants and have a wide range of structural variations. Polyphenols

include flavonoids, tannic acid, and ellagitannin, all of which have traditionally been used as dyes and tanning agents for clothing. Decades of polyphenol studies has yielded many observations into the role of polyphenols on immune function [10]. Each polyphenol type targets and binds to one or more receptors on immune cells, triggering intracellular signaling pathways that eventually control the host immune response.

Polyphenol-containing dietary interventions can modulate immune responses by influencing epigenetic mechanisms such as regulatory DNA methylation, histone alteration, and microRNA-mediated posttranscriptional repression, all of which modify the expression of genes encoding key immune factors. Immune cells express many receptors that allow the transmission of external stimuli to activation processes within the cell *in vivo* [13]. Currently, researchers are studying a range of polyphenol receptors. Many studies have investigated the effects of polyphenols on various types of immune cells, such as primary macrophages, to identify potential targets [26].

Polyphenols are a significant group of phytochemicals that have received expanded research interest after it was discovered that they can influence cancer cell development. Epidemiologic trials provided the first proof that a diet high in polyphenols, such as fruits and vegetables, substantially decreases the incidence of multiple cancers [46]. Phenolic compounds influence carcinogenesis by inducing cell defense mechanisms, including detoxifying and antioxidant enzyme systems, as well as inhibiting anti-inflammatory and anti-cellular growth signaling pathways, which result in cell cycle arrest and/or cellular death [35]. Polyphenols

studied in this research include curcumin derived from turmeric, rutin derived from citrus fruits and resveratrol derived from blueberries.

1.7 Curcumin

Curcumin(diferuloylmethane)(1,7 bis(4 hydroxy-3-methoxyphenyl)1,6 hepadiene 3,5-dione), **(Fig. 1.3)** a form of liposoluble polyphenol pigment isolated from the rhizome of curcuma, has a long history as a dietary supplement and is noted for a range of biological action including anti-inflammatory, anticoagulant, hypolipidemic, antioxidant, free radical scavenging, anti-atherosclerosis, etc [4]. It is the main active compound in the Chinese medicine turmeric which has significant pharmacological effect [14]. Curcumin, an anti-inflammatory compound has the ability to inhibit cyclooxygenase-2 (COX-2), lipoxygenase (LOX), and inducible nitric oxide synthase (iNOS), which are essential for inflammatory processes [15]. Moreover, inhibition of NF-Kβ signaling pathway supports anti-inflammatory effect of curcumin in cancer cells. Curcumin upregulates the heat shock family genes in addition to genes involved in cell cycle [16].

Curcumin has recently been found in clinical trials to be healthy and well tolerated in humans. Curcumin at a concentration of 40 μm inhibits the growth of multiple cancer lines *in vitro*, including lung cancer [18]. These results strongly indicate that it has a possible therapeutic use in cancer prevention especially in lung cancer.

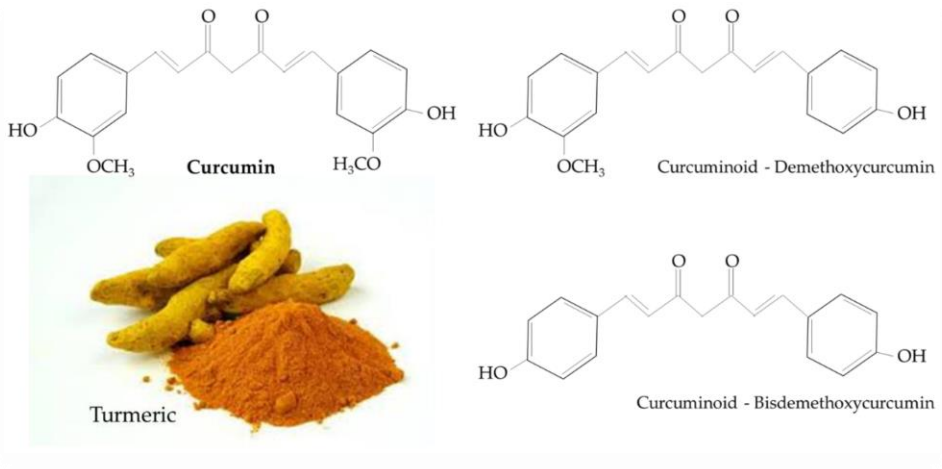


Figure 1.3: Chemical structure of curcumin and curcuminoids found in turmeric [18].

Curcumin has been suggested to induce cell cycle arrest and activate apoptosis-mediated cell death in several cancer cell types, including lung cancer [23]. While it is widely assumed that apoptosis is the primary toxic function of curcumin in tumor cells, it is unclear if autophagy is triggered in curcumin-induced cancer cell death [4]. Simultaneously, in recent years, there has been a lot of concern over the fact that curcumin can upregulate autophagy to achieve its antitumor function. It was found that curcumin can induce autophagy, a catabolic process for the degradation and recycling of macromolecules and organelles which can be activated during stress conditions was identified as an important part of the tumor control procedure [15].

1.8 Resveratrol

Resveratrol (3,5,40-trihydroxy-trans-stilbene) is a member of the stilbenoids group of polyphenols, with two phenol rings bound by an ethylene bridge **(Fig.1.4)**. This natural polyphenol has been found in over 70 plant species, most commonly the skin and seeds of grapes, and has been found in trace quantities in red wines and a number of human foods. It is a phytoalexin that inhibits the growth of pathogens such as bacteria and fungi. Numerous tests have shown that resveratrol has a very high antioxidant capacity as a natural food product [41]. Resveratrol also has antitumor activity and is being treated as a possible candidate for the prevention and treatment of multiple cancers. It induces promyelocytic leukemia cell differentiation, and exhibits anticancer properties by mediating apoptosis, arresting cell cycle progression, antiproliferation and inhibiting ribonucleotide reductase, ornithine decarboxylase, and cyclooxygenase through modulation of prostaglandin production [29].

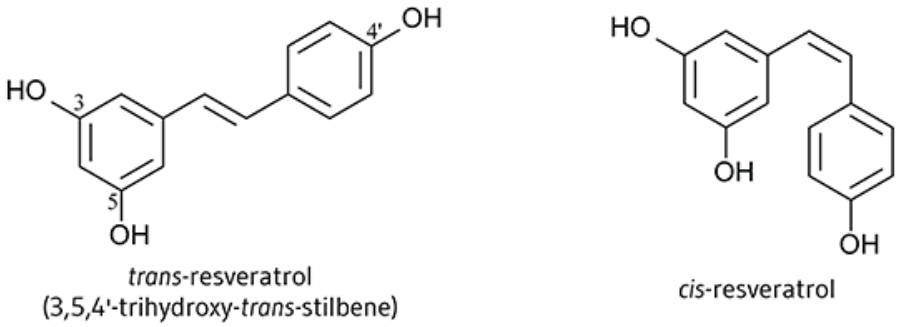


Figure 1.4: Chemical structure of resveratrol and resveratrol glucoside [41]

Many *in vitro* and *in vivo* experiments have confirmed resveratrol's anticancer properties, demonstrating that it can suppress all stages of carcinogenesis. Furthermore, anti-inflammatory and anticarcinogenic effects have been identified. Resveratrol has received great attention as an upcoming preventive and therapeutic agent against breast cancer. Indeed, resveratrol is believed to target intracellular signaling pathway components such as regulators of cell survival and apoptosis, pro-inflammatory mediators, and tumor angiogenic and metastatic switches by modulating a distinct set of transcription factors, upstream kinases, and their regulators [2].

Resveratrol has also shown promise as part of combination therapy, particularly in breast cancer. A resveratrol concentration of 10 μM/L was able to keep estrogen metabolites from interacting with DNA [22]. This compound has been shown to reverse drug resistance in a wide variety of *in vitro* cell systems by sensitizing tumor cells to drug-mediated effects in combination with other chemotherapeutic agents. Resveratrol is also a histone deacetylase inhibitor that display its antiproliferative action by activating cell cycle arrest, inducing apoptosis and autophagy, angiogenesis inhibition, increasing reactive oxygen species generation causing oxidative stress, and mitotic cell death in cancer cells [3]. Resveratrol-loaded nanoparticles have also demonstrated antioxidant potential in cancer cells [48]. Nonetheless, resveratrol delivery is still a major challenge for the pharmaceutical industry, due to its poor solubility and bioavailability.

1.9 Rutin

Rutin is a glycoside consisting of the flavonol quercetin and the disaccharide rutinose **(Fig.1.5)**. It is also known as rutoside, quercetin-3-O-rutinoside, and sophorin. It is a citrus flavonoid present in many species, including citrus. Many common plants contain rutin, including buckwheat, apples, black tea, and vegetables. It has been used in a variety of traditional Chinese medicines and has been shown to have many positive effects, inhibiting platelet aggregation, reduction of inflammation, antioxidant activity, and a lowering of blood fat and cholesterol [1]. In breast cancer MDA-MB-231 cells, rutin has bioactivity and anticancer activity [7]. Furthermore, rutin protects DNA damage from pro-carcinogens in HTC hepatic cells. Rutin has also been shown to have *in vitro* cytotoxic effects on cancer cell lines, including human colon cancer cells [19].

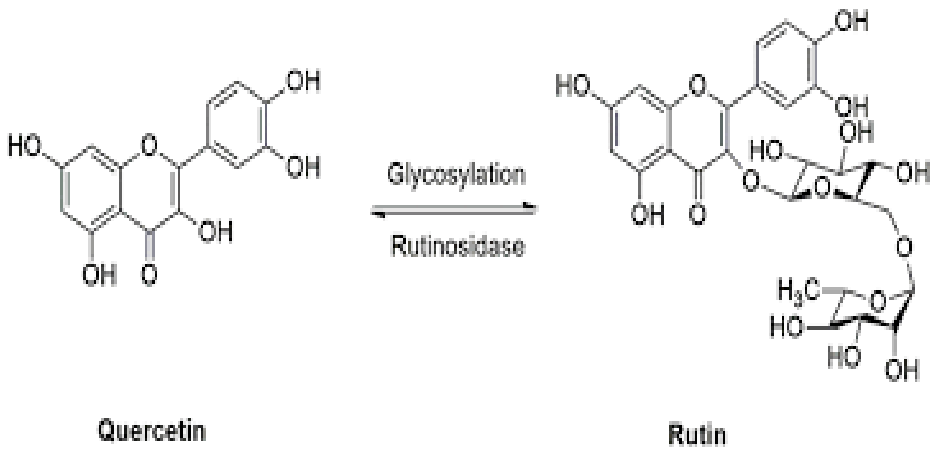


Figure 1.5: Rutin, a glycoside from quercetin flavonoid [21]

Rutin and its analogues, such as EGCG and quercetin, are effective radical inhibitors and have been shown to have chemopreventive efficacy in a number of

colonic cancer cell lines and murine models [29]. In some *in vivo* models, such as NK/Ly ascites and B16F10 cells, rutin has antitumor effects [19]. As a result, it was shown that rutin had a major beneficial effect on reducing the number of precancerous lesions and inducing apoptosis in large intestine cancer and human neuroblastoma LAN-5 cells. Anticancer effects of rutin were studied by Yang et.al., in Human leukemia HL-60 cells, and it was found that rutin inhibited tumor growth in a xenograft animal model. It was demonstrated that treatment with 120 mg/kg of rutin resulted in a reduction of tumor weight and volume when compared with the control groups. Rutin decreased tumour weight by 62.99% when compared to control and tumour size was also found to be significantly smaller [24].

Curcumin, resveratrol and rutin have a strong ability to modulate the immune system and have shown vast anticancer activities against most varieties of cancers, including adenocarcinoma cells (A549 cell lines), a NSCLC that has become highly prevalent among the population [34]. Though many studies have been carried out showing these polyphenols can have anti-tumor activity, a detailed study has not been done on the effects of minimal dosage of polyphenols on cell migration or on A549 cells viability. In this study I perform a time and concentration dependent analysis on the effects of curcumin, rutin and resveratrol on A549 cell's migration, viability and cytomorphology.

CHAPTER II

MATERIALS AND METHODS

2.1 Culture medium preparation

- 450 mL of RPMI complete medium (pH 7.3) was mixed with 100mg/mL antibiotics (penicillin and streptomycin with a final concentration of 75 µg/mL) and 12% fetal bovine serum (FBS).
- Cytiva Hyclone RPMI 1640 medium and Gibco FBS was purchased from Fisher Scientific.
- The solution was then filtered using vacuum suction having membrane of pore size 0.2 µm. The media was then stored at 4 ˚C.

2.2 Preparation of stock solutions of compounds

10ml of stock solutions were made for the below mentioned compounds.

- Curcumin: 3.68 mg of curcumin (1 mM) was dissolved in 1 mL DMSO and was made 10 mL by adding PBS.
- Rutin: 6.29 mg of rutin (1 mM) was first dissolved in 1 mL DMSO by vortex. It was made 10 mL by adding 9 mL PBS.

- Resveratrol: 2.28 mg of resveratrol (1 mM) was dissolved in 10 ml PBS and vortexed.
- The above stock solutions were filter sterilized using 0.2 µm membrane filters.

2.3 Cell culture

- A549 cells were thawed and grown in T-75 flask with RPMI medium for 24 h. They were kept at 37 °C, 5% CO_2 in humidified air in an incubator.
- The cells were then rinsed with 20 ml of pre-warmed PBS and trypsinized with 0.25% trypsin/0.53 mM EDTA for 15 min.
- When the cells have been detached the trypsin/EDTA was neutralized by addition of 10 mL growth medium.
- The cell suspension was centrifuged for 6 min at 1200 rpm.
- The supernatant was discarded, and pellet was suspended in 4 mL growth medium.
- 1 mL of cell suspension was plated in two new T-75 flasks and let grow for 24 h. Remaining cell suspension (2 ml) was frozen in liquid Nitrogen.
- The logarithmic growth phase for A549 cells were determined by changing the media every day and passaging the cells once every 2 to 3 days.

2.4 Morphological observation

- $4x10^4$ /mL suspension of A549 cells were seeded in 12-well plates at 1 mL per well cultured overnight until the cells adhered to the wall.

- The cells were exposed to curcumin, rutin and resveratrol (40 µM) and the same volume of the consumption of DMSO for solubilizing the compounds was considered a control group.
- After cultured for 24 h, cell morphological changes were observed and photographed under inverted phase contrast microscope.

2.5 Cell counting

- Cell counting was performed by The Neubauer counting chamber (hemocytometer)
- 10 µL of the cell suspension was taken and pipetted underneath the coverslip of the hemocytometer.
- All cells in areas A, B, C and D (each area made up of 16 squares as detailed for area A in fig. 2.1) were counted.

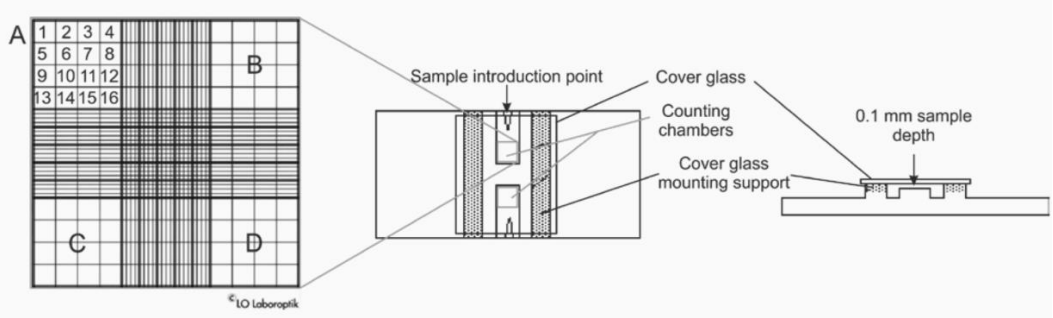


Figure 2.1: Schematic illustration of a hemocytometer

Picture modified from:

http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html.

- The average cell count per area [(A+B+C+D)/4] was calculated.
- The number of cells per unit volume (cells/mL) was calculated using the following equation.

  Number of cells/mL = average cell count x 10^4 x dilution factor
- Required number of cells (taken directly from the cell suspension) in the required volume was resuspended in the complete cell culture medium and seeded into appropriate cell culture flasks.

2.6 Cell viability assay

The MTT assay is a colorimetric assay for measuring cell metabolic activity. It is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye MTT to its insoluble formazan, which has a purple color. This assay therefore measures cell viability in terms of reductive activity as enzymatic conversion of the tetrazolium compound to water insoluble formazan crystals by dehydrogenases occurring in the mitochondria of living cells although reducing agents and enzymes located in other organelles, such as the endoplasmic reticulum are also involved [43].

- After trypsinization cells were seeded in 96 well plate at a density of 5000 cells per well in RPMI media.
- 24 h after culture cells were treated with curcumin, rutin and resveratrol at different concentrations (0 mM, 2 mM, 4 mM, 6 mM and 10 mM)

- Cell viability was assessed by MTT assay after 24 h treatment with curcumin, rutin and resveratrol.
- The cells were exposed to 0.5 mg/ml of MTT for 6 h at 37 ˚C in the RPMI medium.
- Medium and MTT were removed and after solubilization in 100% isopropanol, the amount of insoluble formazan crystals was evaluated by measuring the optical density at 570 nm.
- Each condition was performed in triplicate.

2.7 Scratch Assay

A wound healing assay is a lab technique for studying cell migration and cell–cell interactions. This is also known as a scratch assay because it involves scratching a cell monolayer and using a time lapse microscope to capture images at regular intervals. It's a 2D cell migration method for measuring cell migration in a sheet of cells semi-quantitatively [20].

- Cells were detached from T-75 flask by trypsinization and seeded in a 12 well plate such that each well had 200k cells.
- The cells were grown until they reached 70 – 80% confluency (monolayer). The confluency was constantly monitored under microscope.
- The cells were then treated with curcumin, rutin and resveratrol at 2 mM, 4 mM and 8 mM concentrations.
- The cell layer was scrapped in a straight line using a 1mm pipette tip. The tip should be kept perpendicular to the bottom of the well.

- Another line was scratched perpendicular to the first line to create a cross in each well. This removed the cell layers.
- After scratch the cell monolayer was gently washed with PBS to remove detached cells and replenished with new growth medium.
- It was then incubated and imaged with phase contrast microscopy for every 6 h till 24 h (6h, 12h and 24h) until cells migrated to meet in the middle.

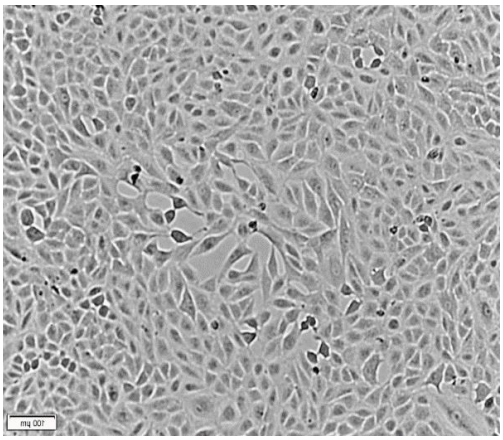
CHAPTER III

RESULTS

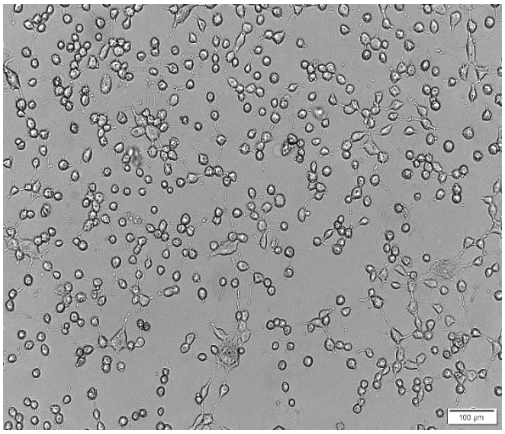
3.1 Cell morphology

The inverted microscope was used to examine A549 cell morphology alterations. As expected, untreated lung adenocarcinoma A549 cells showed a long fusiform shape, small size, clear cell boundaries with consistent pebble-like growth **(Fig. 3.1A)**. Cells were treated with 40 µM curcumin, rutin and resveratrol and pictures were taken after 24 h incubation. All three compounds inhibited growth of A549 cells to some extent with curcumin showing maximum inhibition.

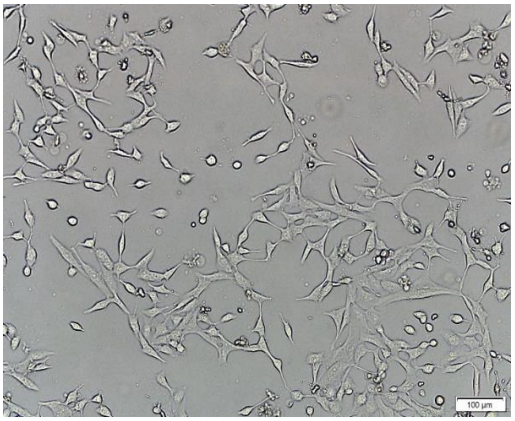
Curcumin caused A549 cells to become round with a disappearance of the normal spindle shape. Compared with the corresponding control group, bright-circular dead cells floating increased at 24 h incubation **(Fig. 3.1B)**. Rutin did not affect long fusiform shape of A549 cells but affected cellular contact, making them round after disrupting contacts resulting in the increase of circular dead floating cells **(Fig. 3.1C)**. Resveratrol caused the cells to decrease in size with a disappearance of cell boundaries. Resveratrol did not affect the contacts between cells. Bright field appeared around shrunk cells increasing the number of floating dead cells **(Fig. 3.1D)**.



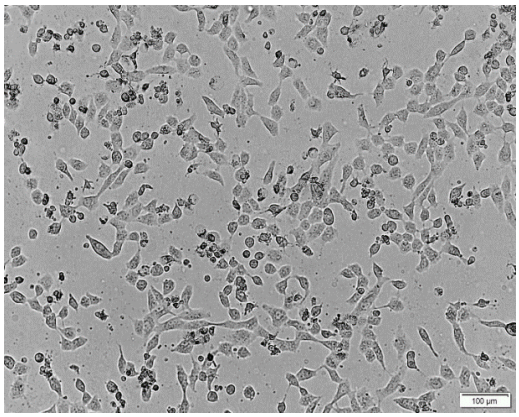
3.1A (Control)



3.1B (40 µM Curcumin)



3.1C (40 µM Rutin)



3.1D (40 µM Resveratrol)

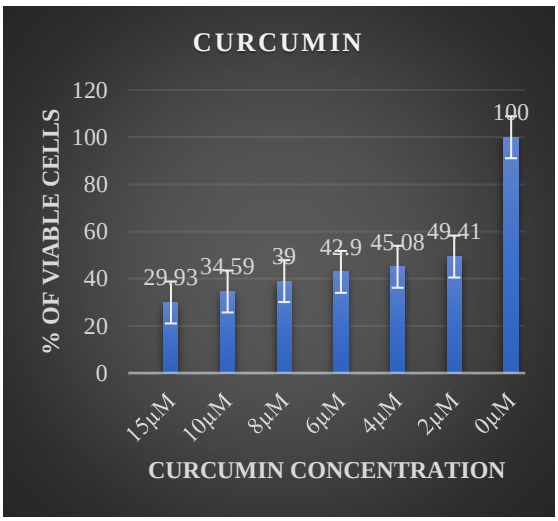
Figure 3.1: Morphology of adenocarcinoma cells under inverted phase contrast microscopy after 24 h incubation. (A) Morphology of normal A549 cells. (B) Morphology of A549 cells treated with curcumin (C) Morphology of A549 cells treated with rutin (D) Morphology of A549 cells treated with resveratrol. Scale bar = 100 µm.

3.2 Cell viability

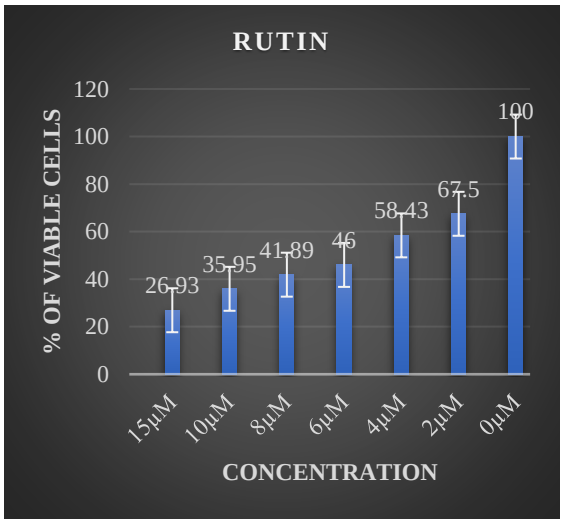
We utilised the commonly used MTT assay to quantify cell viability. Succinate dehydrogenase (SDH) in living cells can reduce exogenously applied MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble formazan, a blue-violet crystalline.The formazan in the cell is dissolved in DMSO and quantified by measuring absorbance at 570 nm using a microplate reader. The larger the value, the more live cells.

To assess the effects of the curcumin, rutin and resveratrol on cell viability, we treated A549 cells, cells with various concentrations of these compounds (0, 2, 4, 6, 8, 10 and 15 µM) for either 24 h or 48 h followed by MTT assays. We found a negative correlation betweent the viability of A549 cells and both the concentration of the compounds and the length of treatments.

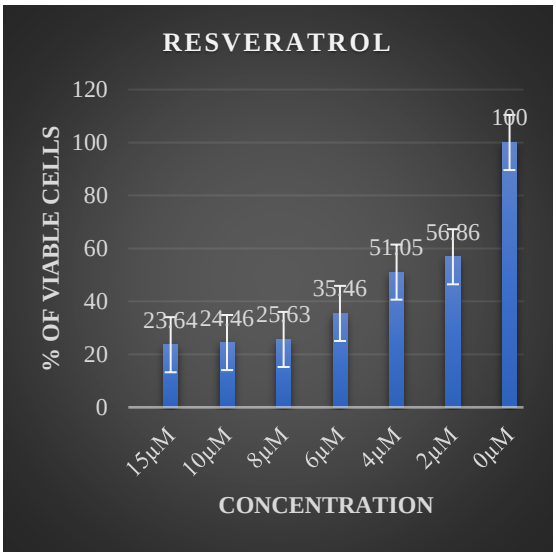
Curcumin treatment had a significant effect on A549 cells resulting in viability ranging from 49% with the 2 µM treatment to 30% with the 15 µM treatment after 24 h **(Fig. 3.2.1A)**. Rutin treatment for 24 h resulted in 68% cell viability at 2 µM with a steep decrease to 27% viable cells at 15 µM **(Fig. 3.2.1B)**. Resveratrol treatment resulted in 57% cell viability at 2 µM with a drastic decrease to 26% viability at 8 µM **(Fig. 3.2.1C)**. For the 24 h treatment with the lowest dosage of compound, curcumin showed the most inhibition followed by resveratrol and rutin **(Fig. 3.2.1D)**. When the incubation time was increased to 48 h, viability fell to 10% for curcumin **(Fig. 3.2.2A)**, 12% for resveratrol **(Fig. 3.2.2B)** and 18% for rutin at 15 µM **(Fig. 3.2.2C)**. The cell viability was significantly reduced with an incubation period of 48 h at higher concentrations irrespective of the compounds **(Fig. 3.2.2D)**.



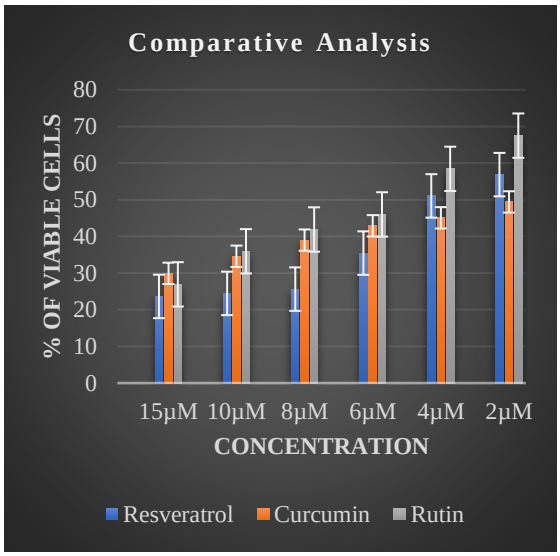
3.2.1A



3.2.1B



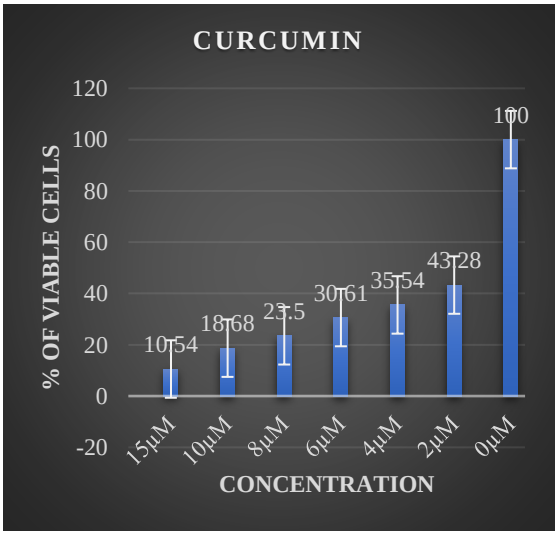
3.2.1C



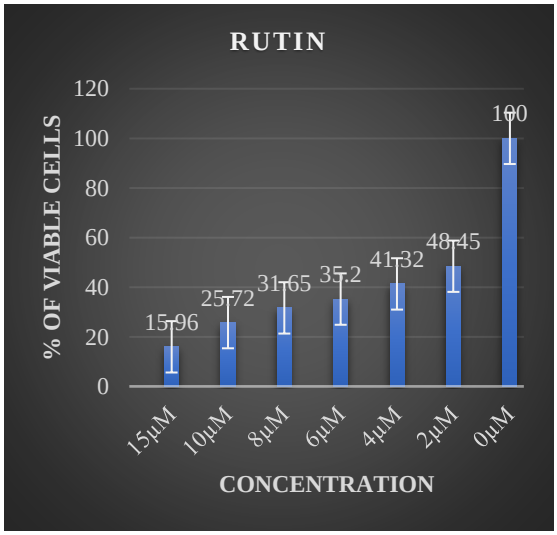
3.2.1D

Figure 3.2.1: The inhibitory effects of various compounds on lung adenocarcinoma cells (A549) after 24h incubation. **(A)** The inhibitory effect of curcumin on the proliferation of A549 cells. Cells were plated at 5 x 10^3 cells per dish in the absence and the presence of curcumin.Time-dependent decrease in the number of A549 cell at the indicated concentrations. As control, the cells were treated without curcumin. **(B)** The inhibitory effect of rutin on the proliferation of A549 cells. Cells were plated at 5 x 10^3 cells per dish

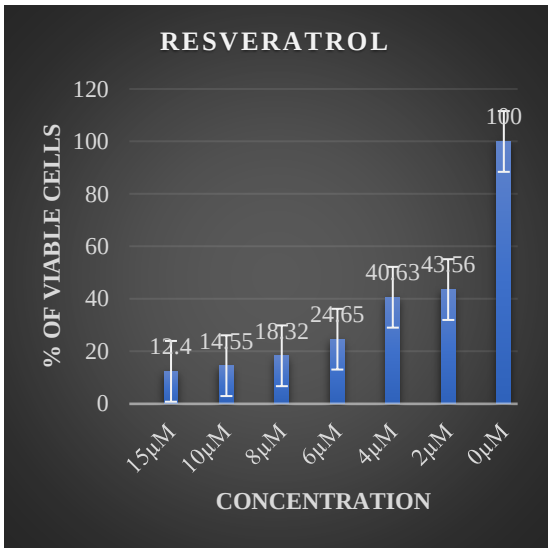
in the absence and the presence of rutin.Time-dependent decrease in the number of A549 cell at the indicated concentrations. Control cells were not treated with rutin. **(C)** The inhibitory effect of resveratrol on the proliferation of A549 cells. Cells were plated at 5 x 10^3 cells per dish in the absence and the presence of resveratrol. Time-dependent decrease in the number of A549 cell at the indicated concentrations. As control, the cells were treated without resveratrol. **(D)** Comparative analysis of curcumin, rutin and resveratrol after 24h incubation. Thebars represent mean viability across 5 replicates +/- standard error .

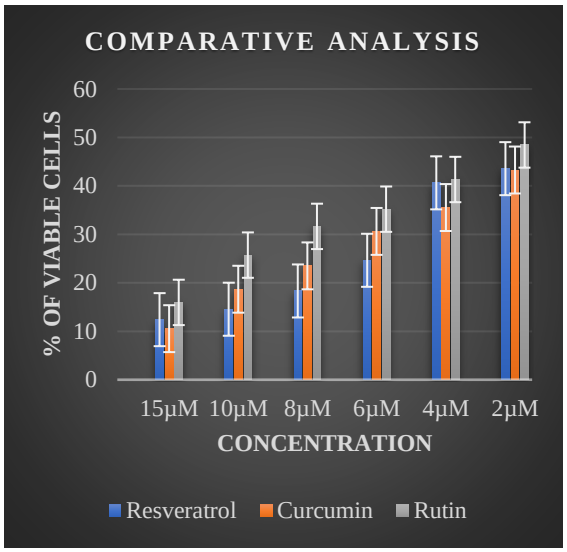


3.2.2A



3.2.2B





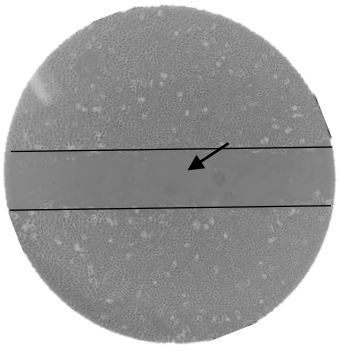
3.2.2C **3.2.2D**

Figure 3.2.2: The inhibitory effects of various compounds on lung adenocarcinoma cells (A549) after 48 h incubation. **(A)** The inhibitory effect of curcumin on the proliferation of A549 cells. Cells were plated at 5 x 10^3 cells per dish in the absence and the presence of curcumin.Time-dependent decrease in the number of A549 cell at the indicated concentrations. Control cells were not treated with curcumin. **(B)** The inhibitory effect of rutin on the proliferation of A549 cells. Cells were plated at 5 x 10^3 cells per dish in the absence and the presence of rutin.Time-dependent decrease in the number of A549 cell at the indicated concentrations. As control, the cells were treated without rutin. **(C)** The inhibitory effect of resveratrol on the proliferation of A549 cells. Cells were plated at 5 x 10^3 cells per dish in the absence and the presence of resveratrol. Time-dependent decrease in the number of A549 cell at the indicated concentrations. As control, the cells were treated without resveratrol. **(D)** Comparative analysis of curcumin, rutin and resveratrol after 48h incubation. The bars represent mean viability across 5 replicates +/- standard error.

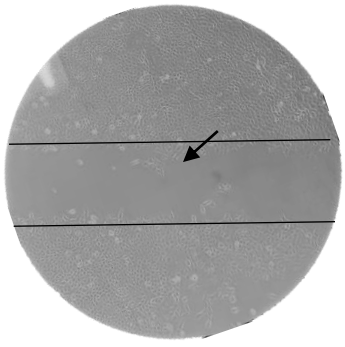
3.3 Scratch Assay

Cell migration is a finely regulated process including successive steps of cell adhesion and de-adhesion. In order to assess the ability of compounds to inhibit cancer cell motility, scrape damaged A549 monolayers were incubated in the presence of curcumin, rutin and resveratrol (15 µM) at three different time intervals (0 h, 12 h and 24 h). A cell-free area in a confluent monolayer is formed in this assay by removing the cells from the area by mechanical disruption. The cells migrate into the gap after being exposed to the cell-free environment. In this study, the monolayer was scratched with a pipette tip and the migration into the gap was imaged over several hours using a transmitted-light microscope equipped for live-cell imaging.

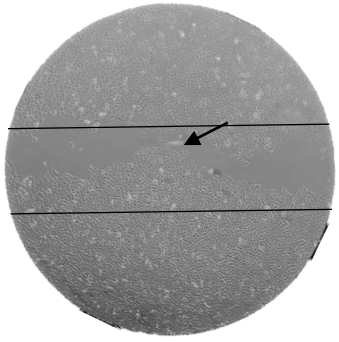
Control cells completely covered the wounded area after 24 h of incubation at 37 ^{o}C **(Fig. 3.3c)**. In contrast, treatment with curcumin, rutin and resveratrol strongly reduced wound repair and significantly inhibited the migration of both A549 at 5 µM concentration **(Fig. 3.3d – 3.3l)**.



3.3a (0h)



3.3b (12h)



3.3c (24h)

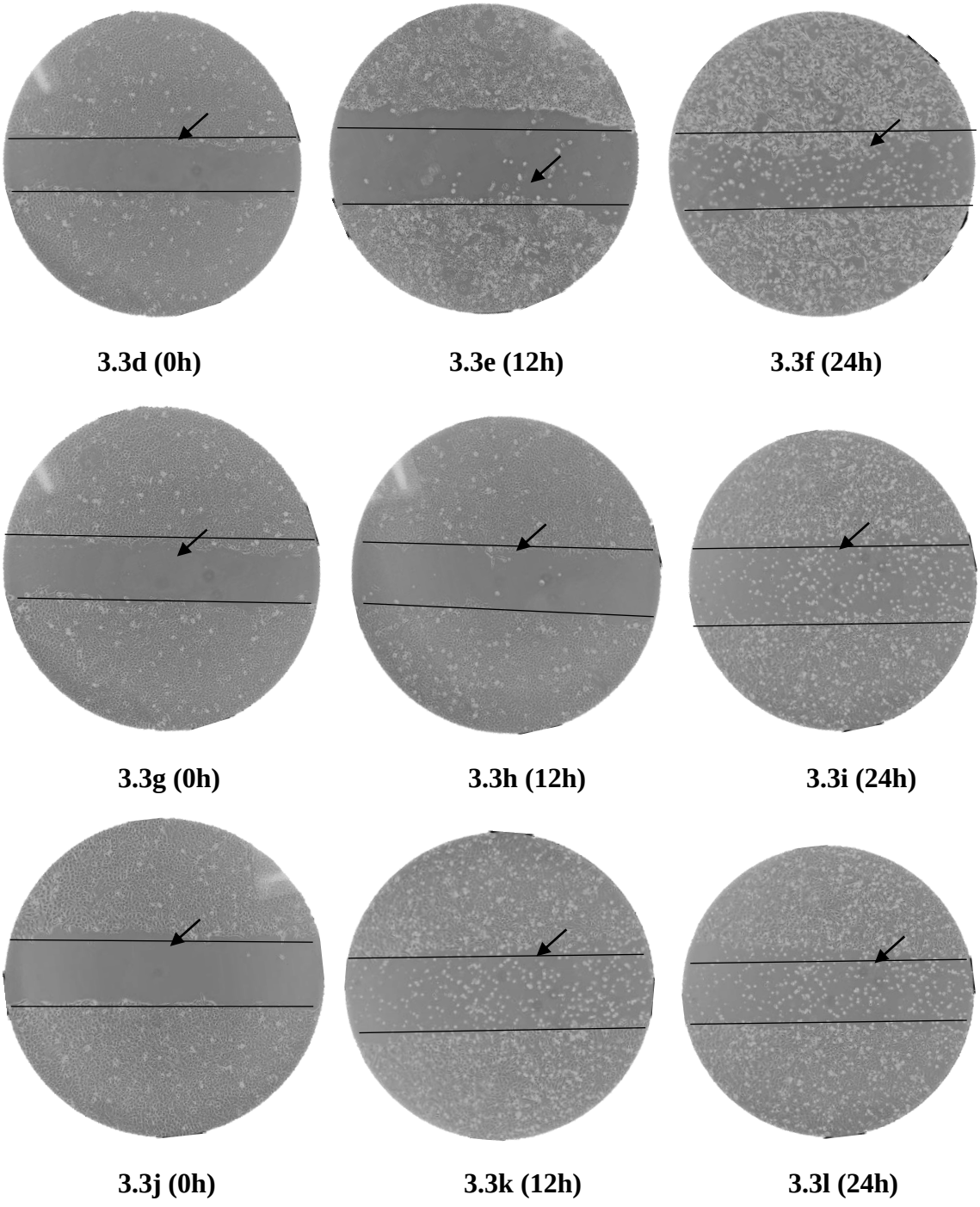


Figure 3.3: The inhibitory effects of various compounds on the migration of human adenocarcinoma cells photographed at 0^{th} hour, 12^{th} hour and 24^{th} hour. The zone between the two lines (represented by arrows) indicates the area occupied by the initial wound.

(3.3a, 3.3b, 3.3c) Scratch assay of control groups without treatment with compounds at 0^{th} hour, 12^{th} hour and 24^{th} hour, respectively. **(3.3d, 3.3e, 3.3f)** Scratch assay of A549 cells treated with 5 µM curcumin at 0^{th} hour, 12^{th} hour and 24^{th} hour respectively. **(3.3g, 3.3h, 3.3i)** Scratch assay of A549 cells treated with 5 µM rutin at 0^{th} hour, 12^{th} hour and 24^{th} hour respectively. **(3.3j, 3.3k, 3.3l)** Scratch assay of A549 cells treated with 5 µM resveratrol at 0^{th} hour, 12^{th} hour and 24^{th} hour, respectively.

CHAPTER IV

DISCUSSION

Polyphenols have broad applications and are considered to be a safe and effective antitumor drug. However, the mechanism of the antitumor activity of polyphenols like curcumin, rutin and resveratrol is still not fully elucidated. In this study, the human lung adenocarcinoma A549 cell line was selected as experimental cells. The anti-proliferation mechanism of curcumin, rutin and resveratrol in A549 cells was examined. Morphologically, curcumin (40 µM) administration caused the cells to lose their normal long shuttle shape and resulted in the increase of floating dead cells. This effect was time dependent (Fig. 3.1B). Rutin did not affect long fusiform shape of A549 cells but affected cellular contact, making them round after disrupting contacts resulting in the increase of circular dead floating cells (Fig. 3.1C). Resveratrol caused the cells to decrease in size with a disappearance of cell boundaries. Resveratrol did not affect the contacts between cells. Bright fields appeared around shrunken cells with an increase in the number of floating dead cells (Fig. 3.1D).

Curcumin could significantly inhibit the proliferation of human lung adenocarcinoma A549 cells at various concentrations and time points. After 24 hours, curcumin therapy had a major effect on A549 cells, with viability ranging from 49 percent with the 2µM treatment to 30 percent with the 15µM treatment (Fig. 3.2.1A). Rutin therapy for 24 hours resulted

in 68 percent cell viability at 2µM, decreasing to 27 percent viable cells at 15 µM (Fig. 3.2.1B). Resveratrol therapy resulted in 57 percent cell viability at 2µM, but just 26 percent viability at 8µM (Fig. 3.2.1C). Curcumin inhibited the most in the 24h treatment with the lowest compound dose, followed by resveratrol and rutin. When the incubation period was raised to 48 hours, viability dropped to 10% for curcumin (Fig. 3.2.2A), 12% for resveratrol (Fig. 3.2.2B), and 18% for rutin at 15µM. (Fig. 3.2.2C). With a 48-hour incubation time at higher concentrations, cell viability was greatly decreased.

Previous studies have shown that polyphenols suppressed the growth of multiple cancer lines. Here, evidence has been obtained that curcumin, rutin and resveratrol even at very low concentrations, could effectively inhibit the proliferation of lung cancer A549 cells, but the specific mechanism needs to be further explored. Cancer cells develop a variety of distinguishing characteristics as the tumor progresses. These abilities include the ability to proliferate in the absence of exogenous growth-promoting or growth-inhibitory signals, to invade neighboring tissues and spread to distant sites, to induce an angiogenic response, and to evade cell proliferation-limiting mechanisms including apoptosis. These characteristics are due to changes in the cellular signaling pathways that regulate cell proliferation, motility, and survival in normal cells. Many of the proteins currently being researched as potential cancer therapy targets are signaling proteins that are part of these pathways [8]. Although the mechanism of polyphenols studied is not known, they may act by interfereing with the activity of these signalling pathways.

The main biological characteristics of tumor cells are uncontrolled proliferation and higher migration ability. Cell adhesion is an important mechanism in many biological processes, including growth, tissue structure maintenance, angiogenesis, and tumor metastasis. Cells

undergo programmed cell death in the absence of sufficient extra-cellular matrix (ECM) interactions, which are primarily mediated by integrins [38]. Therefore, the characterization of new anti-integrin agents is of considerable utility for the development of therapies. Cancer cell metastasis involves multistep cellular processes, commencing with cell migration and invasion. Cell migration is tightly regulated by coordinated remodeling of membrane and actin cytoskeleton. Although the detailed mechanisms are not yet understood, it is clear that dynamic and reciprocal interactions between cell adhesion molecules, ECM and soluble factors are essential. This study showed that curcumin, rutin and resveratrol treated A549 cells have a decreased migratory capacity in scratch wound assay at very low concentration of 5μM (Fig. 3.3a – 3.3l). The inhibitory effect of curcumin, rutin and resveratrol against lung cancer A549 cells on cell migration is likely due to the reduced attachment to ECM proteins observed in the presence of these polyphenols. Future studies could investigate the effects of polyphenols on integrin expression patterns. Their effects on structural modifications of actin microfilament and its dynamics can possibly be analyzed.

CHAPTER V

CONCLUSION

Epidemiological and dietary intervention research in a variety of animal models and cancer cell lines have recently suggested that diet-derived natural compounds like flavonoids that are polyphenols may be useful as cancer chemopreventative agents [13]. Flavonoids of the flavonol type, such as rutin, resveratrol, and curcumin, have been confirmed to have a broad range of pharmacological properties, including antioxidant, antimicrobial, antifungal, and antiallergic activity, which have been used in human medicine and nutrition [34]. In addition, polyphenols have a wide range of pharmacological benefits for the treatment of chronic diseases including diabetes, hypertension, and cancer [46].

In this study I present data that supports the growing evidence that polyphenols, especially rutin, curcumin and resveratrol, could significantly inhibit the proliferation of A549 cells as well as alter cytomorphology. I found that these compounds negatively affected A549 cell viability over 48h incubation and also reduced cancer cell migration at a very low concentration (2µM) in scratch assays.

Due to the wide range of effects of natural compounds and the complexity of cell death, elucidating the mechanism of action of these compounds will require further study. We can, however, conclude that the beneficial activity of curcumin, rutin and resveratrol

against A549 lung cancer cells at extremely low doses make them attractive potential anti-tumor agents that can easily be delivered through dietary changes and/or supplementation.

It is also worth noting that the effects of curcumin, rutin and resveratrol may differ depending on cell type. Further studies with additional cell types will provide more information regarding the potential utility of these compounds against multiple types of cancer. The future study of non-apoptotic cell death mechanisms induced by polyphenols may provide targets of plant-derived compounds for future cancer therapeutics and become a powerful new direction for antitumor research and therapy.

REFERENCES

1. Al-Dhabi NA, Arasu MV, Park CH, Park SU, An up-to-date review of rutin and its biological and pharmacological activities, EXCLI Journal, 2015.
2. Beekwilder, J.Wolswinkel, R.Jonker, H.Hall, R de Vos, C.H. Bovy, A, Production of resveratrol in recombinant microorganisms, Appl. Environ. Microbiol, 2006.
3. Burns, J.Yokota, T.Ashihara, H.Lean, M.E.J Crozier, A, Plant foods and herbal sources of resveratrol. J. Agric. Food Chem. 2002.
4. Chang CC, Fu CF, Yang WT, Chen TY and Hsu Y, The cellular uptake and cytotoxic effect of curcuminoids on breast cancer cells, Taiwan J Obstet Gynecol 51: 368-374, 2012.
5. Deretic V, Saitoh T, Akira S. Autophagy in infection, inflammation and immunity. Nat Rev Immunol. 2013.
6. Deretic V. Autophagy: An Emerging Immunological Paradigm. J Immunol 2012.
7. Fabjan N, Rode J, Kosir IJ, Wang Z, Zhang Z, Kreft I. Tartary buckwheat (Fagopyrum tataricum Gaertn.) as a source of dietary rutin and quercitrin. J Agric Food Chem 2003.
8. G. Steven Martin, Cell Signaling and Cancer, Cancer Cell Book Vol. 4, Issue 3, 159-238, 2009.

9. Garcia-Mediavilla V, Crespo I, Collado PS, et al. The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappa B pathway in Chang Liver cells. Eur J Pharmacol 2007.
10. Gary D. Stoner and Hasan Mukhtar, Polyphenols as cancer chemopreventive agents, Journal of Cellular Biochemistry, 1995.
11. Giusi Briguglio, Polyphenols in cancer prevention: New insights, International Journal of Functional Nutrition, 2020.
12. Gomes LC, Dikic I. Autophagy in antimicrobial immunity. Mol Cell. 2014.
13. Gonza´lez-Gallego J, Sa´nchez-Campos S, Tun˜o´n MJ. Anti-inflammatory properties of dietary flavonoids. Nutr Hosp 2007.
14. Guan F, Ding Y, Zhang Y, Zhou Y, Li M and Wang C: Curcumin suppresses proliferation and migration of MDA-MB-231 breast cancer cells through autophagy dependent Akt degradation. PLoS One 11: e0146553, 2016.
15. Gupta SC, Kismali G and Aggarwal BB: Curcumin, a component of turmeric: From farm to pharmacy. Biofactors 39: 2-13, 2013.
16. Gupta SC, Patchva S and Aggarwal BB: Therapeutic roles of curcumin: Lessons learned from clinical trials. AAPS J 15: 195-218, 2013.
17. H. Wang, F. Xing, H. Su, A. Stromberg, and L. Yang, “Novel image markers for non-small cell lung cancer classification and survival prediction,” BMC Bioinformatics, vol. 15, no. 1, p. 310, 2014.

18. Han X, Deng S, Wang N, Liu Y and Yang X: Inhibitor effects and molecular mechanisms of tetraphydrocurcumin against human breast cancer MCF-7 cell. Food Nutr Res 60: 30616, 2016.
19. Hongyan Chen, Qing Miao, Jing Liu, Yazhuo Hu, Lei Tian, Jingkun Pan, and Yi Yang, Anti-Tumor Effect of Rutin on Human Neuroblastoma Cell Lines through Inducing G2/M Cell Cycle Arrest and Promoting Apoptosis, The Scientific World Journal, 2013.
20. James E. N. Jonkman, Judith A. Cathcart and Pina Colarusso, An introduction to the wound healing assay using live cell microscopy, Journal of Cell adhesion and migration, 2014.
21. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
22. Kalantari, H.; Das Dipak, K. Physiological effects of resveratrol. BioFactors 2010, 36, 401–406.
23. Kang JH, Kang HS, Kim IK, Lee HY, Ha JH, Yeo CD, Kang HH, Moon HS and Lee SH: Curcumin sensitizes human lung cancer cells to apoptosis and metastasis synergistically combined with carboplatin. Exp Biol Med (Maywood) 240: 1416-1425, 2015.
24. Keylor, M.H.; Matsuura, B.S.; Stephenson, C.R.J. Chemistry and biology of resveratrol-derived natural products. Chem. Rev. 2015, 115, 8976–9027.
25. Li, M.; Kildegaard, K.R.; Chen, Y.; Rodriguez, A.; Borodina, I.; Nielsen, J. De novo production of resveratrol from glucose or ethanol by engineered Saccharomyces cerevisiae. Metab. Eng. 2015, 32, 1–11.

26. Li, M.; Schneider, K.; Kristensen, M.; Borodina, I.; Nielsen, J. Engineering yeast for high-level production of stilbenoid antioxidants. Sci. Rep. 2016, 6, 36827.
27. Liu D, You M, Xu Y, Li F, Zhang D, Li X and Hou Y: Inhibition of curcumin on myeloid-derived suppressor cells is requisite for controlling lung cancer. Int Immunopharmacol 39: 265-272, 2016.
28. Marques, F.Z.; Markus, M.A.; Morris, B.J. Resveratrol: Cellular actions of a potent natural chemical that confers a diversity of health benefits. Int. J. Biochem. Cell Biol. 2009, 41, 2125–2128.
29. Martin AR, Villegas I, La Casa C, de la Lastra CA., Resveratrol, a polyphenol found in grapes, suppresses oxidative damage and stimulates apoptosis during early colonic inflammation in rats. Biochem Pharmacol 2004; 67: 1399– 410.
30. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008; 451:1069–1075. doi: 10.1038/nature06639.
31. Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, Englert JA, Rabinovitch M, Cernadas M, Kim HP, Fitzgerald KA, Ryter SW, Choi AM. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol 2011; 12:222-230. doi: 10.1038/ni.1980.
32. Narayana KR, Reddy MS, Chaluvadi MR, et al. Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. Ind J Pharmcol 2001; 33:2-16.

33. Nawaz, W.; Zhou, Z.; Deng, S.; Ma, X.; Ma, X.; Li, C.; Shu, X. Therapeutic versatility of resveratrol derivatives. Nutrients 2017, 9, 1188.
34. Nijveldt RJ, van Nood E, van Hoorn DEC, et al. Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 2001; 74:418-25.
35. Petkov E, Nickdor N, Uzunov P. Inhibitory effects of flavonoids on xanthine oxidase. Planta Med 1981; 43:183-7.
36. Poillet-Perez L, Despouy G, Delage-Mourroux R, Boyer Guittaut M. Interplay between ROS and autophagy in cancer cells, from tumor initiation to cancer therapy. Redox Biol. 2015; 4:184-192. doi: 10.1016/j.redox.2014.12.003.
37. Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 1992, 339, 1523–1526.
38. Sabine Raab-Westphal, John F. Marshall, and Simon L. Goodman, Integrins as Therapeutic Targets: Successes and Cancers, Cancers (Basels), PMC5615325, 2017.
39. Sharma S, Ali A, Ali J, Sahni JK, Baboota S. Rutin : therapeutic potential and recent advances in drug delivery. Expert Opin Investig Drugs. 2013; 22:1063-1079. doi: 10.1517/13543784.2013.805744.
40. Shinojima N, Yokoyama T, Kondo Y and Kondo S: Roles of the Akt/mTOR/p70S6K and ERK1/2 signaling pathways in curcumin-induced autophagy. Autophagy 3: 635-637, 2007.
41. Urban Svajger, Natasa Obermajer and Matjaz Jeras, Dendritic cells treated with resveratrol during differentiation from monocytes gain substantial tolerogenic

properties upon activation, The journal of cells, molecules, systems and technologies, 2010.

42. V. K. Anagnostou, A. T. Dimou, T. Botsis, E. J. Killiam, M. D. Gustavson, R. J. Homer, D. Boffa, V. Zolota, D. Dougenis, L. Tanoue, et al., "Molecular classification of nonsmall cell lung cancer using a 4-protein quantitative assay," Cancer, vol. 118, no. 6, pp. 1607–1618, 2012.
43. V. Kuete, H. Sivas, Anticancer Activities of African Medicinal Spices and Vegetables, Medicinal Spices and Vegetables from Africa, 2017.
44. Wang, Y.; Halls, C.; Zhang, J.; Matsuno, M.; Zhang, Y.; Yu, O. Stepwise increase of resveratrol biosynthesis in yeast Saccharomyces cerevisiae by metabolic engineering. Metab. Eng. 2011, 13, 455–463.
45. Watson JL, Greenshields A, Hill R, Hilchie A, Lee PW, Giacomantonio CA and Hoskin DW: Curcumin-induced apoptosis in ovarian carcinoma cells is p53-independent and involves p38 mitogen-activated protein kinase activation and downregulation of Bcl-2 and survivin expression and Akt signaling. Mol Carcinog 49: 13-24, 2010.
46. Williams RJ, Spencer JPE, Rice-Evans C. Flavonoids: antioxidants or signalling molecules, Free Radic Biol Med 2004; 36:838-49.
47. World Cancer Report 2014, World Health Organization, 2014 pp. Chapter 5.3.
48. Xia YQ, Wei XY, Li WL, Kanchana K, Xu CC, Chen DH, Chou PH, Jin R, Wu JZ and Liang G: Curcumin analogue A501 induces G2/M arrest and apoptosis in non-small cell lung cancer cells. Asian Pac J Cancer Prev 15: 6893-6898, 2014.

49. Xiao K, Jiang J, Guan C, Dong C, Wang G, Bai L, Sun J, Hu C and Bai C: Curcumin induces autophagy via activating the AMPK signaling pathway in lung adenocarcinoma cells. J Pharmacol Sci 123: 102-109, 2013.
50. Yuk JM, Jo EK., Crosstalk between autophagy and inflammasomes. Mol Cells. 2013; 36:393-399. doi: 10.1007/ s10059-013-0298-0.