POLYPHENOLICS FROM MANGO (*MANGIFERA INDICA* L.) SUPPRESS BREAST CANCER DUCTAL CARCINOMA *IN SITU* PROLIFERATION BOTH *IN VITRO* AND *IN VIVO*: POTENTIAL ROLE OF THE IGFR-1-AKT-AMPK-MTOR-SIGNALING AXIS

A Dissertation

by

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ABSTRACT

More than 25% of all newly diagnosed breast cancer cases are ductal carcinoma *in situ* (DCIS), the most commonly diagnosed form of non-invasive breast cancer. This incidence coupled with the detrimental side effects associate with traditional cancer treatment makes it critical to investigate the efficacy of alternative treatment regimens that are not as toxic to non-cancer cells. The tumor-cytotoxic activities of secondary plant compounds including polyphenolics from mango (*Mangifera indica* L.) have previously been reported; however, the underlying mechanism, especially with DCIS breast cancer, has not been elucidated.

In both an *in vitro* and an *in vivo* xenograft assessment of mango extract and pyrogallol (PG), a tannin-metabolite formed by bacteria in the gastrointestinal tract, had anti-proliferative activities in the DCIS cell line MCF10DCIS.com. Reduced proliferation *in vitro* was attributed to a down-regulation of multiple constituents along the AKT/mTOR signaling axis. Within a low concentration of 10 mg/L both treatments significantly decreased total protein for mTOR and p-AKT and p-P70S6. Neither treatment had significant interaction with 5-fluoro-uracil.

In a xenograft model, the mango extract (0.8mg/day) and PG (0.2mg/day) significantly reduced tumor volumes by 50% over a 4 week exposure window where similar downregulation of the AKT/mTOR signaling axis was observed as *in vitro*, however, AMPK and p-AMPK at Thr172 were also upregulated. Sestrin, Becklin, and p-ULK were all significantly elevated in tumor tissue by treatments. Both treatments were

shown to cause elevated ROS production, which might have initiated the activation of AMPK. *In silico* modeling demonstrated PG ability to directly bind with the allosteric site of AMPKα resulting in its activation. PG treatment following siRNA knockdown to AMPK resulted in the rescue of total and p-AMPK levels *in vitro*. This indicates that PG is not only involved in the activation of AMPK but also in the upregulation of its constituent expression, possibly by the downregulation of HDAC1, a suppressor of AMPK expression. An assessment of mouse plasma indicated that PG is rapidly metabolized into pyrogallol sulfate, likely by intestinal and hepatic sulfotransferases. Findings indicate that tannin-containing foods or PG may delay the development of DCIS breast cancer.

DEDICATION

I dedicate this body of work to my mother Cathy, my father Mark, and my two brothers Marcus and Jordan.

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There are many people that I need to acknowledge and that have been beneficial contributors to my life long goal of obtaining a PhD. My mother Cathy and my father Mark have been as supportive and motivating as any child could hope for. My mother has always had my best interest at heart and helped me make the best decisions, even if at the time I may have thought differently. She is someone who always puts others before herself, and ultimately only wanted one thing for me, and that was to give my best no matter the task or the final outcome. She has contributed in numerous ways, but it is her giving nature and her ability to make personal connections that have contributed the most to my advancement. Luckily for me I come from a lineage of scientists. My father has been a great mentor and resource throughout my life, and without a doubt my biggest motivator. He has been a great sounding board for me to bounce different scientific ideas off of and supply both support and expertise when I needed them. My two brothers Marcus and Jordan have always been supportive and a lot of fun and good company in down times. I come from a close knit family, and everyone in my immediate family has inspired me in different ways.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Incidence of Cancer

It is estimated that 11 million new cases of cancer are diagnosed annually worldwide, with 7 million deaths being attributed to this disease [1, 2]. Therefore, cancer is estimated to cause mortality in more than 50% of those diagnosed; and therefore even small advancements in treatment could result in significantly increased survival and/or benefits to the quality of life for those suffering from it. Asia has the greatest incidence, mortality, and 5-year prevalence of cancer (Figure 1).

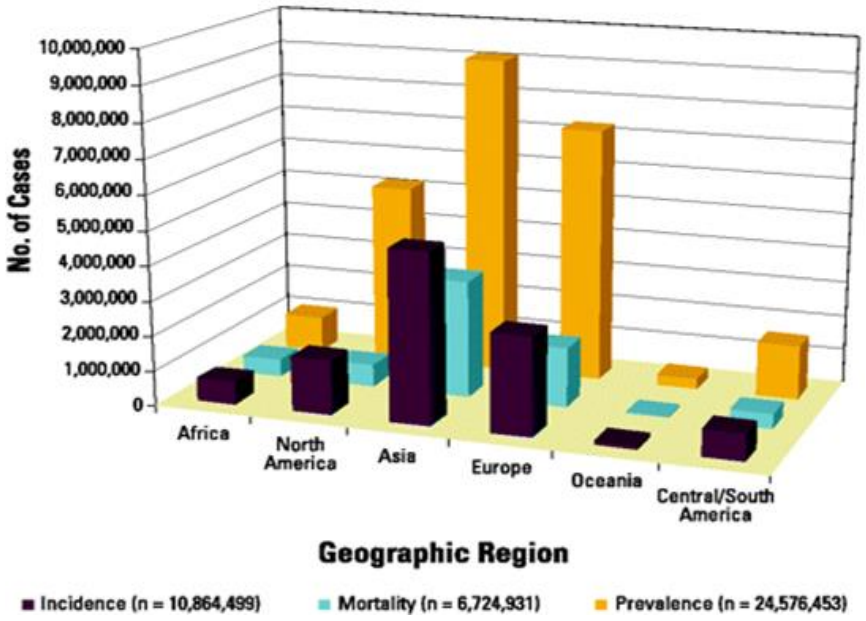

Figure 1. Breast cancer incidence, mortality, and prevalence for Africa, North America, Asia, Europe, Central/South America and Oceania [1].

Cancers that are gender-specific, for example, female breast and male prostate cancer, have the greatest incidence rates. If gender-specific cancers are eliminated from analysis, lung, stomach, colon, rectum, liver, in descending order, followed by cancer of the esophagus have the highest incidence worldwide.

Table 1. Demographic of women diagnosed with DCIS [5].

Variable	n	Percent
Age (years)		
<35	612	6.0
35-44	5,219	10.9
45-54	10.400	11.6
55-64	9,366	9.5
65-74	9,499	9.6
75-84	5,188	7.7
≥85	939	4.3
SEER site		
San Francisco/Oakland	7,763	10.4
Connecticut	6,381	9.0
Detroit	7,341	9.6
Hawaii	2,131	12.5
Iowa	4,150	7.4
New Mexico	1,663	0.8
Seattle	6,040	9.9
Utah	1,769	0.8
Atlanta	3,796	11.6
Race category		
White	34,490	9.2
Black	3,306	10.2
American Indian	82	7.3
Asian/Pacific Islander	2,967	15.0
Other	117	11.5
Surgery		
BCS	21,394	54.5
Mastectomy	17,687	45.5
RT	11,630	26.2

Of all newly diagnosed cancers, 1 out of 10 will be female breast cancer [3]. There is a relatively large variation of breast cancer incidence depending on geographical location, which is estimated to be greater than 10-fold. This range results from to multiple etiology including differences in hormonal, reproductive and nutritional factors [4]. Another contributing factor is soco-economic status; and societies that practice self-examinations and are able to diagnose breast cancer sooner. Self-

examination techniques and mammography are associated with an increase in early diagnoses of breast cancer at the ductal carcinoma *in situ* stage, and this examination occurs with the greatest frequency among women of ages 35-54 (Table 1) [5]. Asian and Pacific Islanders are two of the least likely populations to be diagnosed due to limited access to medical resources.

Hallmarks of Cancer

Cancer development is a multi-step cascade of events made up of 6 distinct stages (Figure 2) that include ongoing proliferation, desensitization to growth suppressors, invasion and metastasis, immortality and continuous replication, angiogenesis, and cell death resistance [2]. As of 2011, two additional stages have been proposed to be added to these traditional hallmarks of cancer, and these are reprogramming energy metabolism and evading immune destruction [6].

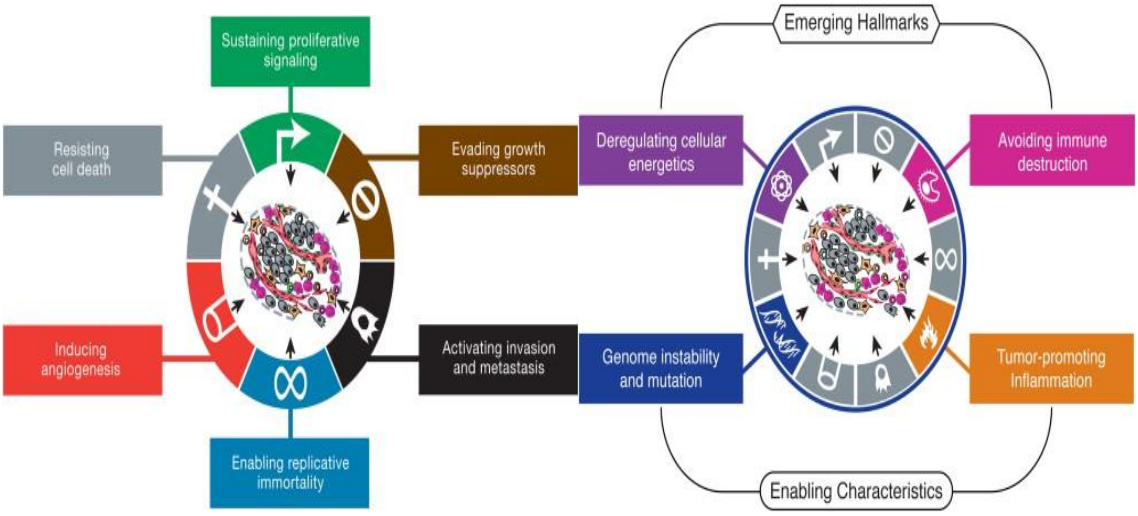

Figure 2. Schematic for the hallmarks of cancer [6].

Continuous Proliferation

In non-cancerous cells, cellular proliferation is a highly choreographed process controlled by multiple checkpoints that allow the cell to either move into mitosis or inhibit the cell cycle depending on the conditions. Cell division is generally classified into two different processes, 1) DNA duplication, and 2) the telophase portion of mitosis. These processes are regulated by specific proteins referred to as cyclin-dependent kinases, mostly classified as serine/threonine protein kinases [7]. Upon activation, these proteins cause a phosphorylation cascade specific to proteins responsible for cell cycle progression (Table 2) with nine major cyclins that navigate the mitotic cell cycle.

Table 2. Cyclin-dependent kinases and their function related to cell cycle progression

CDK	Cyclin	Cell cycle phase activity
CDK4	Cyclin D1, D2, D3	G_1 phase
CDK6	Cyclin D1, D2, D3	G_1 phase
CDK2	Cyclin E	G_1/S phase transition
CDK2	Cyclin A	S phase
CDK1	Cyclin A	G_2/M phase transition
CDK1	Cyclin B	Mitosis
CDK7	Cyclin H	CAK, all cell cycle phases

In cancer, this checks and balance system of cyclins and kinases is dysregulated due to genetic mutation or epigenetic modifications to either oncogenes or to tumor-suppressor genes. Without functional checkpoints, cells continuously replicate resulting in hyperplasia in the absence of normal phases of cellular quiescence. In this process, cancer cells may become less dependent on exogenous growth factors and continue to

proliferate in their absence. Along with this, cancer cells are able to produce their own peptide growth factors and express an increased number of surface receptors that causes them to be more sensitive to lower concentrations of growth factors [8].

Desensitization to Growth Suppressors

The cell cycle is regulated by anti-growth signaling pathways which maintain cells in a quiescence (G_0) stage [9]. Surrounding cells can secrete factors that would normally cause a cell to halt proliferation, and cancer cells loose sensitivity to these factors. One of the key tumor suppressor proteins associated with antiproliferative signaling is the retinoblastoma protein (Rb) [10]. This protein elicits its effect within the G_1 phase. This phase is a critical point in the cell cycle and drives cells to either advance towards duplication or quiescence. Hyperphosphorylation of Rb (pRb) results in the loss of its antiproliferative activities. Upon hyperphosphorylation, pRb dissociates from the transcription factor E2F, which then allows E2F to enter the nucleus and begin gene transcription resulting in cell cycle progression and growth [11]. Common mutations in multiple types of cancer include the Rb gene [12].

Bypassing Apoptosis

Not only are cancer cells able to desensitize themselves to growth-inhibitory signaling, but they are also able to avoid programmed cell death (apoptosis). Many different stimuli initiate apoptosis, including activation of the death receptor FAS (CD95 or APO-1) with a FAS ligand [13]. This process causes a cascade of signaling that normally causes an increase in mitochondrial ion permeability, resulting in the release of multiple constituents. DNA damage causes the upregulation of the tumor

suppressor protein p53 (P53) and the apoptotic promotor BAX [14]. When cytosolic levels of unphosphoralated BAX are increased, it interacts with Bcl-2 at the mitochondrial membrane and causes the release of cytochrome C, a critical apoptotic catalyst. P53 is commonly mutated in multiple cancer types, causing a loss of function either by genetic mutation or silencing via gene methylation. This results in phosphorylated BAX staying sequestered in the cytosol where it is unable to interact with Bcl-2 and prevent apoptosis [15].

Continuous Replication

Non-cancerous cells have a proliferative limit allowing only a certain number of mitotic events to occur. Cancer cells lack this limitation [16]. Telomeres are repetitive nucleotide regions at the end of chromatids, that prevent degradation and typically shorten by 50-100 base pairs during the process of DNA replication [17]. The shorting is contributed to DNA polymerase not being able to fully replicate the 3 prime ends of the chromosome resulting in progressive shortening that will leave the chromosome ends unprotected, causing genomic instability and terminal fusions triggering cell death [18]. Telomerase-elongating enzyme is upregulated in cancer cells, preventing telomere shortening [19].

Prolonged Angiogenesis

Tissues require blood flow to supply nutrients and promote gas exchange; and developing cancers trigger growth of new blood vessels. Vascular endothelial growth factor (VEGF) stimulates the proliferation of blood vessels in a process called angiogenesis. One main player in this process is thrombospodin 1 (TSP-1) an inhibitor

of tumorigenesis in healthy tissue [20]. TSP-1 is found in greater quantities in cells with wild-type p53. With certain mutations in p53, levels of TSP-1 drop significantly [21]. It is hypothesized that this downregulation of TSP-1 is responsible for an angiogenic phenotype rather then up-regulation pro-angiogenic genes. In studies where p53 was knocked out initially and reintroduced later, TSP-1 increased following the reintroduction of p53 and the angiogenic phenotype was lost [20].

Invasion and Metastasis

The metastatic stage of cancer is responsible for over 90% of deaths associated with this disease [22]. In this stage, tumor cells may separate and invade new locations in the body. The ability of cancer to metastasize is associated with several factors including adhesion proteins, like E-cadherin (E-cad). E-cad has many critical functions, including interacting with catenins which link E-cad to the internal actin cytoskeleton (Figure 3) [23]. In this configuration, E-cad is not only responsible for intercellular

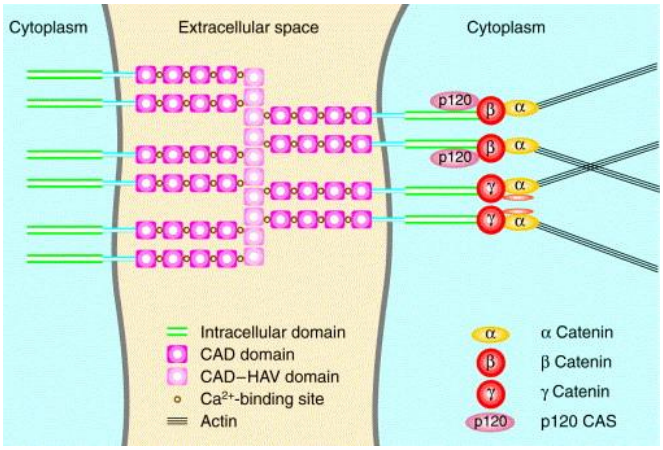

Figure 3. Intra and extracellular E-cadherin cell adhesion [23].

adhesion, but is also able to transduce transmembrane signaling regulating gene expression [24]. E-cad is lost in a majority of epithelium-derived cancers associated with genetic mutation, deletion, or hypermethylation along the promoter region. In general, downregulation of E-cad is associated with poor prognosis [25-27]. Mutation of other complex constituents, like α, β, and γ catenin also results in a loss of adhesion [28]. Also, upregulation of extracellular proteases cause degradation of stromal, blood vessels, and epithelial layers [29].

Breast Cancer

Breast cancer includes a broad class of carcinomas affecting the mammary glands and tissue, and occurs in both female and male breast tissue. The breast is filled with 15-20 lobules, and these radiate around the areola (Figure 4).

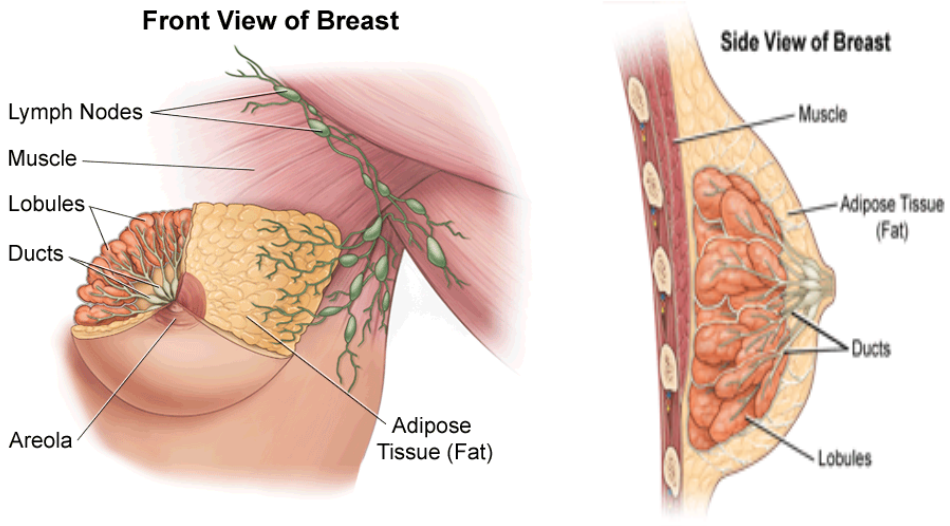

Figure 4. Anatomic features of the female breast [30].

The lobules are the functional part of the breast responsible for lactation, which is triggered by hormonal signaling. The lobules are connected to ducts that lead to the areola, which is where milk is ejected from the breast. This internal structure is surrounded by adipose tissue [31].

There are multiple classifications for breast cancer, and the American Cancer Society categorizes them in the following way:

Ductal Carcinoma In Situ (DCIS): This is a form of pre-invasive breast cancer in which hyperplastic cells surround the ducts and are morphologically similar to cancer cells. It is believed that nearly all invasive breast cancers begin as DCIS [32]. DCIS progression is shown in Figure 5 along with critical genetic and epigenetic shifts which are believed to chaperone disease progression.

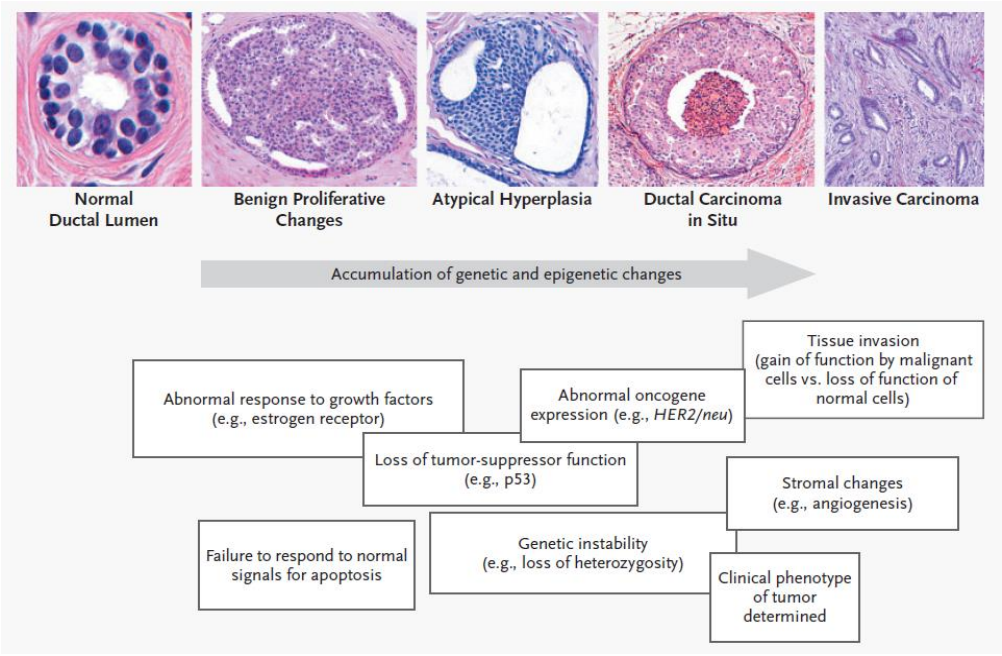

Figure 5. Histological and genetic changes in the progression of DCIS invasive ductal carcinoma [32].

There are multiple pathological features that differentiate DCIS from normal breast tissue including chromosomal imbalances. It is estimated that as much as 70% of DCIS patients experience a loss of heterozygosity. Furthermore, HER2 is overexpressed in almost half of DCIS cases while p53 is mutated 25% of the time in affected tissue [33, 34]. DCIS shows similar molecular characteristics of invasive cancer without being malignant. It is thought that a final set of events and alterations to both the DCIS and surrounding tissue causes the transformation into invasive cancer [35]. A high treatment success rate is associated with successful surgical removal of the tumor tissue. It is estimated that 25% of newly reported breast cancer cases are classified as DCIS [36].

Clinically, this form of breast cancer has the highest occurrence and is estimated to represent 8 out of every 10 cases. The site of origin is the milk duct. This tissue goes through a proliferative remodeling process that is estrogen-dependent making this tissue vulnerable to excessive proliferation [37]. Invasion beyond the confinement of the duct into surrounding adipose tissue is the initiation of metastasis [38].

Invasive Lobular Carcinoma: This form is very similar to that of invasive ductal carcinoma, except the origin of the pathology is the lobules instead of the milk duct [38]. It too has the ability to metastasize and is estimated to be associated with 1 in 10 cases of breast cancer [39, 40].

There are additional, less common types of breast cancer such as inflammatory breast cancer, triple-negative breast cancer, Paget's disease of the nipple, and phyllodes tumors [41-44].

MCF10DCIS.COM Cell Line

The model used in this study, is a MCF10DCIS.com cell line that is unique for its ability to form ductal carcinoma *in situ*-like tumors when xenographed into a nude mouse model. This stage of cancer, DCIS, is common in 25%-30% of newly diagnosed breast cancer cases [45]. This cell line was more recently established as a model for early stage breast cancer [46]. The carcinoma *in situ* stage is a critical point in cancer development, because it is the last stage prior to metastasis. At this stage, the growth may be surgically removed without the need for chemotherapy or radiation but beyond this stage requires a more aggressive treatment regimen.

This cell line was established using premalignant immortalized breast epithelia variants from the MCF10AT cell line. MCF10AT cells were xenographed into mice and resulted in lesions that were then used to start a cell culture colony, resulting in the MCF10DCIS.com cell line, characterized by its ability to form rapidly growing lesions that are predominately DCIS [46] (Figure 6).

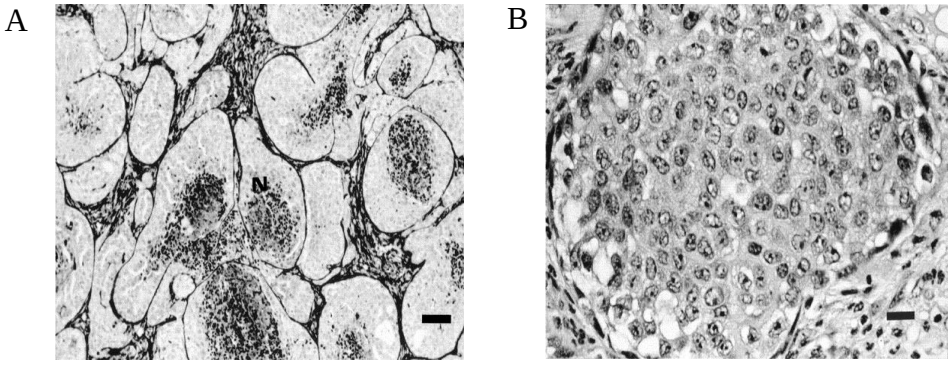

Figure 6. Histologic appearance of MCF10DCIS xenograft in nude mice.
A. is at day 36 of xenograph and passage 17, and B. is day 22 at passage 22 [46].

DCIS.com cells have similar physical characteristics of naturally formed tumors. The dark areas in Figure 6A represent areas of necrosis which is common in a growing tumor when nutrients can no longer diffuse to those areas because of the increasing mass. Tightly packed tubuli make up a majority of the tumor mass. In Figure 6B, cells maintain both their nuclei and cell membranes. Use of this cell line allows researchers to study breast cancer progression and provides a useful model to test novel chemotherapies and determine their efficacy in halting cell growth in this stage *in vitro*.

Conventional Cancer Treatments

The treatment of breast cancer is contingent on many factors, including the stage and the type of breast cancer. Generally treatment regimens are divided into 6 different classes and include: targeted therapy, hormone therapy, radiation therapy, chemotherapy, sentinel lymph node biopsy, and surgery [47].

Targeted Therapy

This treatment utilizes drugs or other compounds to specifically target cancer cells while minimizing effects to regular noncancerous cells. Common treatments include the utilization of monoclonal antibodies, PARP inhibitors, anti-angiogenesis compounds, and tyrosine kinase inhibitors.

Hormone Therapy

Many types of breast cancer are hormone sensitive, therefore blocking or removing hormones, results in decreased cancer growth. Both aromatase inhibitors and

tamoxifen are commonly used to treat estrogen-dependent neoplasia for early stage breast cancer.

Radiation Therapy

This therapy uses high-energy radiation to decrease cancer cell proliferation or decrease cancer cell viability. There are two main ways to administer this treatment including external radiation with uses an external machine to emit radiation toward the cancer site. The other method is internal radiation involving a radioactive compound that is directly administered via needles or catheters into or near the cancer site. The high energy radiation damages the DNA of rapidly dividing cancer cells, causing mutations resulting in cell death. The downside of radiation therapy is that normal cells are not excluded from the effects, resulting in side effects such as skin irritation, fatigue, and gastrointestinal irritation.

Chemotherapy

In this treatment, cancer-targeting drugs are either administered systemically or regionally in order to decrease cancer growth. Administration is dependent on type and stage of cancer. The main goal of this treatment is to either kill the cancer cells directly or halt their proliferation.

Sentinel Lymph Node Biopsy

In the progression of cancer, metastasis initiates at the lymph node responsible for lymphatic drainage from the tumor. A screening utilizing a radioactive substrate or dye injected into the tumor determines if the lymph node was affected or not. If the lymph node is found to have the dye or radioactive substrate present, then the lymph

node is removed. Following this, a biopsy is done on the removed node to determine if cancer cells are present, and if cancer is present, additional nodes maybe removed.

Surgery

This is the most invasive treatment option which results in the removal of the tumor. Two common forms of surgery include breast-conserving surgery and total mastectomy. In breast-conserving surgery a lumpectomy can be performed, and consists of removing just the tumor itself, or a partial mastectomy, which removes some healthy tissue along with the tumor mass. In a full mastectomy, the entire affected breast is removed, along with other tissue assumed to be affected including lymph nodes under the arm and chest muscle.

Alternative and Adjunct Cancer Treatments

Alternative cancer treatment methods are now becoming commonplace as adjunct treatment with traditional treatment regiments. Both nutritional and naturopathic medicines are aiding in minimizing side effects and strengthening the immune system.

Nutrition Therapy

During cancer treatment, such as chemotherapy or radiation, a common side-affect is gastrointestinal agitation resulting in weight loss and lack of nutritional uptake. In order for the body to have energy to help fight the cancer and increase treatment compliance among patients, altering the diet to maintain proper nutrition is critical. Maintaining ones weight during treatment with a well-rounded diet consisting of protein,

carbohydrates, fat, water, vitamins, and minerals also lowers the risk of infection, and expedites healing and recovery times.

Naturopathic Therapy and Treatment

This approach of treatment utilizes non-toxic and natural therapies to promote healing and increased quality of life. The main goals are to reduce the side effects experienced from chemotherapy, to boost the immune system, and to mitigate pain. Following is a list of recommended treatments by the Cancer Treatment Centers of America: herbal and botanical preparations, acupuncture, hydrotherapy, physical therapy, dietary supplements, and homeopathic remedies.

Polyphenol Production

A secondary plant compound is conventionally defined as an organic compound that is produced by the plant that is not required for growth, survival or nutrition. These compounds aid as defense mechanisms for the plants that produce them [48]. Polyphenols are the largest subset of secondary plant compounds and are produced in plants via the shikimate-phenylpropanoids-flavanoids pathway (Figure 7) with the main purpose of protecting the plant from multiple stresses including, heat, bacteria, viruses and mechanical damage [49]. Additionally, phenolic compounds give color and flavor to many fruits and vegetables [50]. Mangos are rich in mangiferin, gallotanins, the gallotanin penta-*O*-galloyl-glucoside, and methyl gallate [51-53].

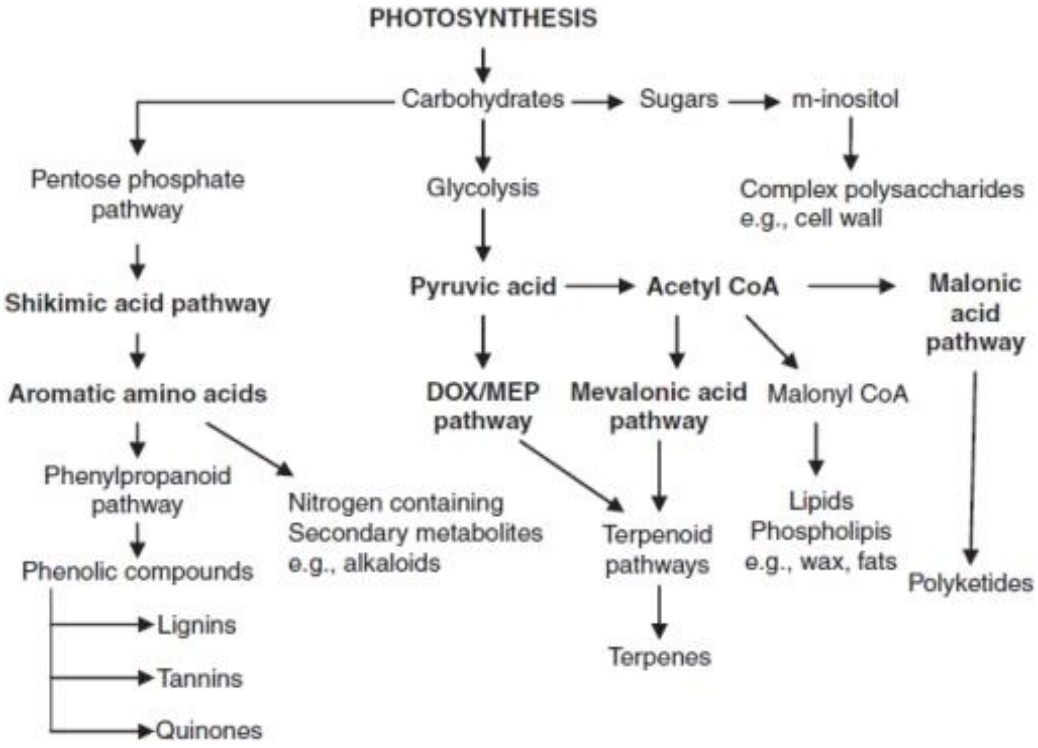

Figure 7. The shikimate-phenylpropanoids-flavanoids pathway

*Mango Fruit (*Mangifera indica *L.)*

Mango is one of the most cultivated tropical fruit crops in the world and ranks number 5 in production with the other four being bananas, pineapple, papaya, and avocado [54]. While the pulp is the most readily consumed part of the mango, beneficial health properties have also been attributed to both their peel and seed. Mangos are known for their high levels of vitamin C, β-carotene, and minerals (Table 3). It is believed that this fruit was first grown in Asia over 5,000 years ago. At one time this fruit was considered exotic, but now it is commonly grown in both North and South America. Over half of the world production of all mangos takes place in India and make

Table 3. Nutrient values in 100g Mango Pulp

Proximate	Quantity
Calories	62.1-63.7 Cal
Moisture	78.9-82.8 g
Protein	0.36-0.40 g
Fat	0.30-0.53 g
Carbohydrates	16.20-17.17 g
Fiber	0.85-1.06 g
Ash	0.34-0.52 g
Calcium	6.1-12.8 mg
β-carotene	1 mg
Phosphorus	5.5-17.9 mg
Iron	0.20-0.63 mg
Vitamin A (carotene)	0.135-1.872 mg
Thiamine	0.020-0.073 mg
Riboflavin	0.025-0.068 mg
Niacin	0.025-0.707 mg
Ascorbic Acid	7.8-172.0 mg
Tryptophan	3-6 mg
Methionine	4 mg
Lysine	32-37 mg

up its primary fruit crop. The four other large mango producing countries are China, Thailand, Mexico, and Indonesia [55, 56]. Due to strict USDA import restrictions in the US, the US market predominantly carries Haden, Tommy Atkins, Kent, and Keith varieties [57].

Every part of the mango including the skin, seed and bark of the tree have been used in ancient medicines for a variety of different ailments [58]. Currently hypothesized medicinal uses for mango fruit include reduction or prevention of macular degeneration, multiple gastrointestinal (GI) disorders including Crohn's Disease,

ulcerative colitis and colon cancer, improving general GI health including digestion, and even improving bone health [59].

Anti-proliferative and Preventive Activities of Polyphenols in Cancer

Several studies report the anti-proliferative activities of polyphenols. Resveratrol is a heavily studied polyphenol found in grapes and has shown to have antiproliferative effects in colon, breast, and prostate cancer [60-62]. Another highly published polyphenol EGCG, found in green tea, has been implicated to impede melanoma, hepatocellular carcinoma, and colon cancer proliferation as just some of its beneficial health properties [63, 64].

Pyrogallol

A unique characteristic of mango is the level of gallic acid it contains. Unfortunately, gallic acid has poor bioavailability systemically, which could be due to the fact it is quickly metabolized or because of its polar characteristics [65, 66]. Following gallic acid consumption, gut microbiota metabolize gallic acid into pyrogallol (Figure 8) [67]. It is this conversion by tannase producing bacteria which is hypothesized to potentially increase the bioavailability of gallic acid and pyrogallol [68]. Pyrogallol has been examined for its health benefits in multiple disease models, and has been done so as a single compound, or as a pyrogallol-type structure attached to other compounds. One study that focuses on tea catechins concluded that catechins that had a pyrogallol in a β-ring had a structural activity relationship with causing apoptosis in cancer cell lines [69]. When histiocytic lymphoma cells were exposed to pyrogallol containing catechins there was a significant induction in caspases, which are enzymes

with an essential role in apoptosis, while catechins that did not contain pyrogallol did not induce apoptosis. Another study that focused on free radical scavenging ability concluded that flavonoids containing pyrogallol were more efficient in scavenging toxic superoxide anion radicals than flavonoids that did not have the pyrogallol moiety [55]. These radicals are toxic in many biological systems and contribute to pathogenesis in many diseases.

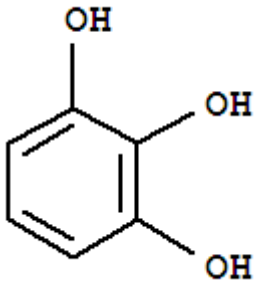

Figure 8. Chemical structure of pyrogallol

Analysis of the binding affinity of pyrogallol-based molecules, demonstrated pyrogallol's affinity for Bcl-2 protein [70]. In that experiment, different pyrogallol-based compounds were modeled with Bcl-2, and results indicated that the hydroxyl groups form hydrogen bounds with R146 and N143 in the BH3 binding grove of Bcl-2 [71]. This binding promoted apoptosis by acting as an antagonist and decreasing the ability of Bcl-2 to bind with pro-apoptotic constituents like Bad, Bax, Bim, and Bid.

Some conflicting evidence describes pyrogallol as an $O_{2}^{\bullet -}$ generating compound which results in cell death [72]. It has been a concern that antioxidants in high concentrations may become pro-oxidants [73].

While the body has its own defense mechanisms against reactive oxygen species (ROS) that include superoxide dismutase, glutathione, catalase, glutathione peroxidase, ingested exogenous antioxidants such as polyphenols, vitamin C & E, and carotenoids are also able to quench ROS and prevent their negative effects [74]. The problem arises at high concentrations of exogenous antioxidants. Firstly, a pro-oxidative effect can occur at elevated concentrations, and secondly a certain level of ROS is required for some cell signaling pathways, and therefore eliminating all ROS is not a suitable solution for disease prevention [75]. As with most compounds, a proper dose is required to obtain the desired effect without enduring toxic effect associated with elevated concentrations.

Pyrogallol also has health benefits as a mono-therapy. In one study, pyrogallol was able to halt cell cycle progression of lung cancer cells in G2-M phase while showing less toxic effects in non-cancerous bronchial epithelial cells *in vitro* [76]. These effects were attributed to decreased B1 and Cdc25c, both of which are cell cycle proteins, and increased phosphorylated levels of Cdc2 in as little as 4 h post treatment. This study also demonstrated that pyrogallol was able to upregulate key constituents along the apoptotic pathway, including up-regulating protein levels of poly (ADP)-ribose polymerase (PARP) and BAX, along with decreased levels of Bcl-2. These results were also demonstrated in a nude mouse xenograph model, and animals that were treated with 75 μg/kg/day pyrogallol for 5 weeks had decreased tumor sizes compared to PBS treated control animals [77].

While pyrogallol has been implicated to have therapeutic benefits in certain disease states, the data base for toxicological assessment is less robust. Pyrogallol has been used in a variety of commercial applications which includes being used in the development of photography and holography, along with being an ingredient in both hair and wood dyes [78-80]. The industrial application of pyrogallol causes concern for potential occupational exposure. Besides the occupational route of exposure, pyrogallol exposure could occur following the consumption of tea and smoked meats [81, 82]. Along with this, inhalation exposure to pyrogallol occurs during the smoking of tobacco products [83].

Table 4. Acute oral LD50 for pyrogallol in four different species [77].

Species	LD_{50}
Rats	738-1800 mg/kg
Rabbits	1600 mg/kg
Dog	25 mg/kg
Redwing Blackbirds	75mg/kg

Reports have previously shown that acute oral consumption of elevated levels of pyrogallol can result in renal injury, gastrointestinal tract irritation, methemoglobinemia which decreases the binding affinity of oxygen to hemoglobin, and even death in extreme circumstances [77]. Table 4 lists the acute oral LD_{50} for various species.

Gallic Acid

Gallic acid or 3,4,5-trihydroxylbenzoic acid, is naturally found in fruits, red wine, and green tea (Figure 9). A unique characteristic about mangos is that gallic acid is the major polyphenol present [59] and one reason this compound is commonly examined when discussing the beneficial health properties associate with mango consumption. Gallic acid has been implicated as having beneficial anti-inflammatory, cancer preventing, and anti-microbial properties. When examining effects of these compounds on breast cancer, it was demonstrated that gallic acid is able to affect multiple factors associated with cancer progression. Gallic acid was tested in MCF-7 breast cancer cells, and significantly reduced cellular proliferation by 42% compared to controls at concentrations as low as 5 μg/mL [84]. This compound also caused

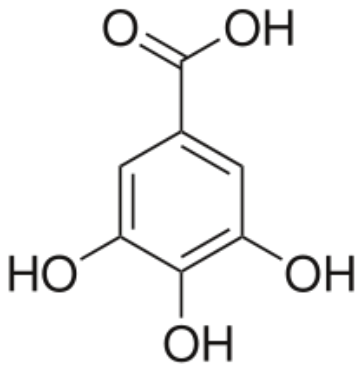

Figure 9. Chemical structure of gallic acid.

significantly induced G2/M phase arrest in this cell line at concentrations ranging from 2-12 μg/mL. Western blots were done in order to determine which constituents of the cell cycle were being altered, and protein levels of cyclin A, CDK2, cyclin B1 and

CDC2/CDK1 were all decreased in a dose-dependent manner. Further investigation revealed diminished levels of Skp2, which is a member of the SCF-type E3 ligase. Skp2 has many functions, which include the ability to ubiquitinate various cancer proteins, including $p27^{Kip1}$, $p21^{Cip1}$, $p57^{Kip2}$, and c-Myc.

Gallic acid, besides being present in numerous food stuffs, is also present in paint, color developers, ink, and pharmaceuticals [85]. With multiple routes of exposure to humans, it is important to examine some of the adverse effects that could results from gallic acid exposure at elevated levels. One study that used F344 rats that were exposed to 0, 0.2, 0.6, 1.7 and 5% gallic acid for 13 weeks revealed that animals that received 5% gallic acid had reduced body weights throughout the duration of the study when food consumption was equal across all groups [86]. It was concluded the gallic acid, like tannins, may bind with essential proteins causing them to precipitate, therefore reducing their ability to be absorbed along the gastrointestinal tract [87]. It was also concluded that gallic acid may inhibit some essential digestive enzymes such as trypsin, α-amylase, and lipase [88].

Underlying Mechanisms of Action of Polyphenols

Antioxidant and Free Radical Scavenging Activities

A majority of the health related effects associated with polyphenols are attributed to their antioxidant activities associated with their phenolic ring structures. All polyphenols contain aromatic rings with hydroxyl groups. The hydroxyl group is able to donate a hydrogen atom to quench a free radical. The remaining positive charge is then displaced amongst the aromatic ring [89]. Many health conditions are associated with

damaged caused from free radicals, therefore reducing radicals is advantageous. In various *in vitro* studies, it has been shown that mango polyphenols are able to prevent DNA damage caused by oxidative stress in various cell lines [90]. Along with their antioxidant activity, multiple polyphenols have demonstrated the ability to form complexes with multiple metal ions, such as iron, vanadium, manganese, aluminum, and calcium [91]. Iron for example binds polyphenols which could prevent it from forming additional radicals by interrupting the Fenton reaction [92].

Protein Binding

Polyphenols have an affinity for proteins, and form soluble complexes. It is believed that each polyphenol has a fixed number of binding ends that are able to bind with a limited number of protein binding sites [93]. When there is a greater quantity of protein present compared to polyphenols, the interaction between the two causes bridging between two different proteins molecules resulting in protein dimers and small aggregates. When this ratio is reversed and there are more polyphenols present than protein, binding between two proteins via a polyphenol is less likely. The binding potential of the two is also both temperature and pH dependent. It has also been concluded that proline-rich proteins had the highest affinity for polyphenols [94]. The affinity of these compounds for proteins could impact multiple biological processes including enzyme function.

PI3K Signaling and Activation of AKT

Phosphoinositide 3-kinase (PI3Ks) are a group of lipid kinases responsible for signaling that promote growth, cell survival, glucose homeostasis, and metabolism that

are dysregulated in many cancer types [95]. Upon extracellular activation by FGF (fibroblast growth factor), VEGF (vascular endothelial cell growth factor), HGF (human growth factor), Ang1 (angiopoietin I), or insulin to receptor tyrosine kinase (RTK) causes autophosphoryalation of PI3K. Currently there are three different classes of PI3K; and Class 1 PI3K will be the focus here (Figure 10).

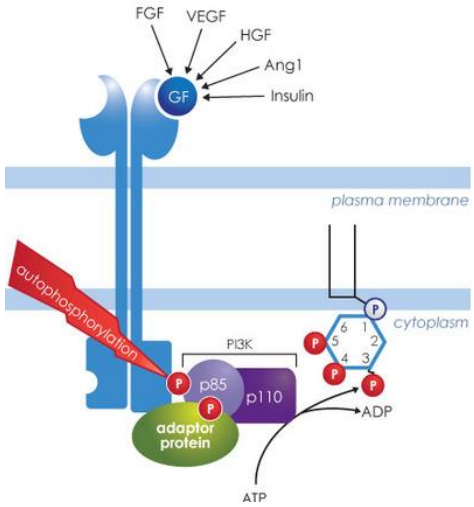

Figure 10. PI3K signaling and activation of AKT.

PI3K is a heterodimer which contains a catalytic and regulatory subunit. SH2 and SH3 domains make up the regulatory subunit and p110α, β, δ, γ are the four different catalytic subunits. When activated by insulin, insulin receptor substrate (IRS) is required for proper PI3K binding and, when activated by integrins, focal adhesion kinase (FAK) is required as an upstream regulator. Following this, PI3K phosphorylates the 3'-OH group on phosphoinositides (PI), a lipid present on the membrane. This activation of P13K by the receptor causes the phosphorylation of multiple PIs with the primary

conversion being PIP_2 to PIP_3 in cancer. This phosphorylation results in AKT mobilization to the phospholipid membrane which leads to its activation. AKT is phosphorylated on two distinct sites, Thr 308 by PDK1, and Ser 473. The Ser 473 phosphorylation has been hypothesized to occur by multiple factors, including mTOR, however, a definitive activator of this phosphorylation site has yet to be determined.

mTOR Pathway

A main phenotypical response following polyphenol exposure *in vitro* is decreased cellular proliferation. Due to this response a major cell signaling pathway of interest is the mammalian target of rapamycin (mTOR)**.** This pathway is important for multiple cell functions which include protein synthesis and lipid biogenesis, cell cycle progression, proliferation, and inhibition of autophagy. mTOR is normally subdivided into two main complexes, which include mTORC1 and mTORC2 (Figure 11), and each responds to a variety of stimuli such as oxygen, amino acids, genotoxic stress, energy

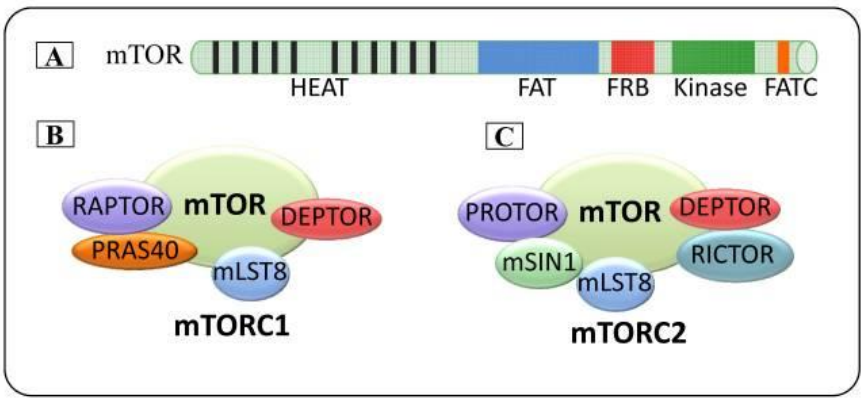

Figure 11. Protein composition for mTORC1 and mTORC2 [96].

status, growth factors and nutrients. These two complexes have two different protein compositions, and the reason they are both initiated by different factors and control different biological processes. The proteins along with their functions are summarized in Table 5.

Autophagy and Programmed Cell Death

Autophagy, along with apoptosis and necrosis, is a type of programmed cell death (PCD) [97]. PCD is a critical cellular function that balances cell survival with death; and an imbalance in this process can result in either cells becoming resistant to death stimuli or decreased viability. Apoptosis is characterized by specific biochemical processes along with alterations in morphology. A cell undergoing apoptosis will have a unique phenotype including condensed and fragmented nucleus, blebbing of the cellular membrane, and an overall reduction in cell size [98]. Necrosis is another type of

Table 5. Individual proteins with their function that make up mTORC1 and mTORC2

mTOR Complex	Proteins	Function
mTORC1	Raptor	Linker to mTOR, responsible for regulators and substrates
	Deptor	Negative Regulator
	PRAS40	Negative regulator by inhibiting substrate binding
	mLST8	Mediates protein-protein interaction
mTORC2	Protor	Assists with complex assemble
	Deptor	Negative Regulator
	Rictor	Critical for the phosphorylation of AKT
	mLST8	Essential for function and stability of complex
	mSIN1	Mediates SGK1 interaction with complex and activates epithelial sodium channels

programmed cell death which exhibits organelle dysfunction, cellular enlargement, and cell lysis. There are many key biochemical signaling components that are associated with autophagy and these are outlined in Figure (12).

Autophagy is a catabolic process that utilizes autophagosomes which are double membrane vesicles that engulf unwanted cytoplasmic contents. Following the encapsulation of material the autophagosome binds with a lysosome/vacuole which leads to the degradation of the unwanted material [99]. Autophagy occurs at housekeeping levels under normal conditions, but upregulated during times of stress which include nutrient deprivation [100]. Dysregulation of this process has been associate with cancer progression, cardiovascular disease, and both neurodegenerative and metabolic disorders [101]. In humans Atg1 (also known as ULK1 and ULK2), Atg13, and FIP200 form a complex which results in autophagy [102]. This complex formation can be inhibited by hyper-phosphorylation of Atg13 from mTORC1 [103]. When this occurs Atg13 is no longer able to bind with Atg1.

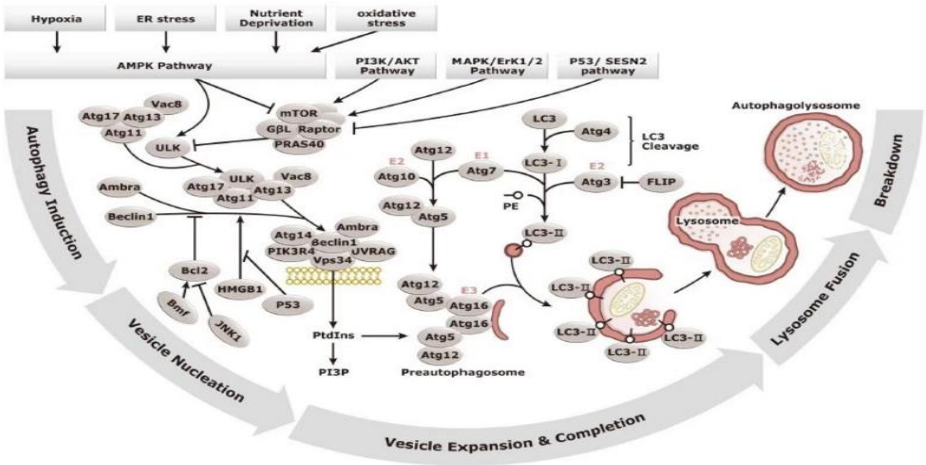

Figure 12**. Summary of AMPK Signaling [104]**

5-Fluorouracil (5-FU)

The widely used cancer drug, 5-FU, has been used for over 40 years in multiple cancer types, including breast cancer, all gastrointestinal cancers, bladder cancer, and both head and neck cancers [105]. This compound is considered an antimetabolite which causes dysregulation of various essential biosynthetic processes including inhibition of thymidylate synthase (TS) [106]. TS is an enzyme responsible for the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate, which is a constituent required for thymine formation. Thymine is one of the four nucleobases constituents that comprise DNA [107]. When the TS enzyme is inhibited it causes increased levels of deoxyuridine monophosphate and decreased levels of deoxythymidine monophosphate, resulting in DNA damage. 5-FU is also able to incorporate itself into both DNA and RNA, which causes dyregulation with protein translation. 5-FU enters the cell via facilitate diffusion and readily incorporates into these macromolecules as a uracil [108]. Figures 13 depicts the metabolism and mechanism of action for 5-FU.

5-FU treatment has multiple drawbacks including developing drug resistance and a plethora of side effects including cardiotoxicity, alopecia, maculopapular eruption, nausea, diarrhea, vomiting, mood disorders, and neurological damage [109]. Due to the multiple toxicological effects of 5-FU treatment, additional research needs to be conducted in order to maximize the benefits of 5-FU with mitigating the deleterious side effects. It is hypothesized that co-administration of 5-FU with natural compounds, such as polyphenols may reduce the efficacious dose of 5-FU therefore reducing the side

effects or preventing drug resistance. One example of this is with curcuminoids which were found to reduced 5-FU resistance in colon cancer cells by suppressing the multidrug resistance gene [110].

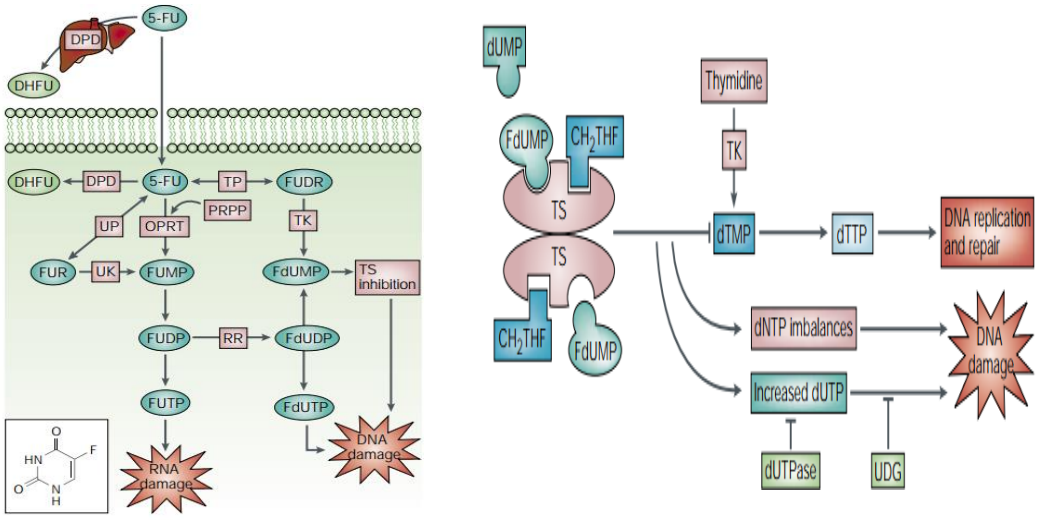

Figure 13. 5-FU metabolism and mechanism of action [122].

Polyphenols are estimated to be ingested at gram levels in the western diet and have been shown to affect (CYP) cytochrome P450, and therefore it is rational to conclude that they could have drug interactions [111]. CYP3A4, an abundant liver enzyme believed to be responsible for metabolizing more than 50% of prescribed drugs, and CYP2C9, another major drug metabolizing liver enzyme, activities were examined following exposure to a panel of over 60 different polyphenols [112]. The polyphenols tested included flavanones, chalcones, isoflavones, coumarins, flavan-3-ols, flavones, and other closely related compounds. They were tested at a concentration of 100 µM. A summary of the results can be seen in Figure 14.

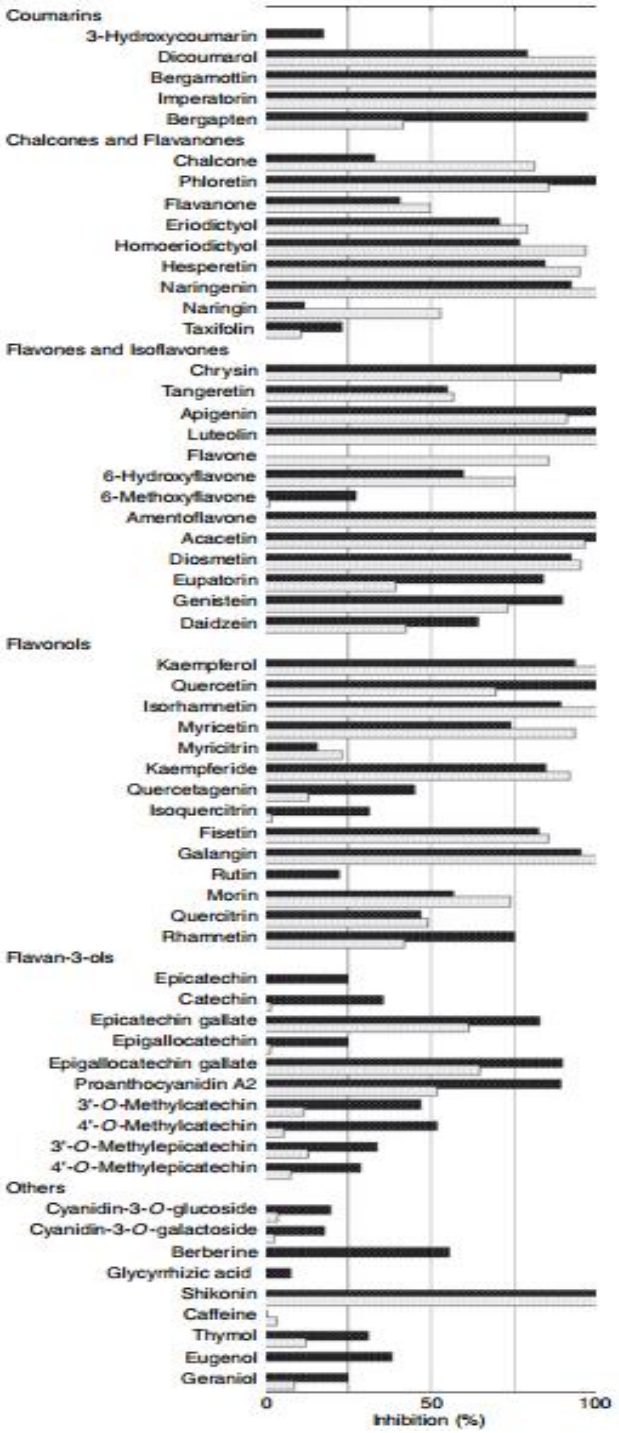

Figure 14. Inhibitory effects of polyphenols on selected p450 enzymes.
CYP3A4-catalyzed TST 6β-hydroxylation (black background) and CYP2C-catalyzed DIC 4'-hydroxylation (white background) [112].

In summary, most of the flavones, flavonols and coumarins investigated caused greater than an 80% inhibition of either CYP3A4 or CYP2C, while flavones, isoflavones, flavan-3-ols, and chalcones exhibited weaker inhibition that was less than 50%. Conclusively, these compounds could alter a drug's metabolism and could also affect the mode in which a drug is absorbed, distributed, and eliminated.

Besides affecting CYP activity, polyphenols also affect transporters. These transporters are responsible for multiple biological processes including the efflux of chemicals from intestinal cells. These transporters include multidrug resistance proteins, including P-gycoprotein among others, and are a principal factor for drug absorption along the gastrointestinal (GI) tract. Table 6 lists intestinal ATP binding cassette transporters (ABC transporters) and their physiological function.

As can be seen in the table, polyphenols have the ability to alter the absorption of many different compounds, and therefore the concern for food-drug interaction needs to be examined further in both *in vitro* and *in vivo* models. One objective in this research is to examine if mango polyphenols modulate the effects of 5-FU. 5-FU also has utility as a positive control allowing for comparisons to be made between polyphenol treatment and a widely used conventional cancer therapy.

Table 6. Intestinal ABC transporters and their physiological function.

Transporter	Tissue	Physiological substrates
Pgp (MDR1) (ABCB1)	Adrenal gland, blood-tissue barrier, brain, epithelia, heart, intestine, kidney, liver, lung, ovary, prostate, skeletal muscle, spleen, stomach	Amphipathic drugs, neutral and positively charged hydrophobic compounds
MRP1 (ABCC1)	Intestine, kidney, liver, lung, testis, blood-cerebral spinal fluid barrier	Anionic drug conjugates, GSH, oxidized GSH, GSH glucoronate and sulfate conjugated organic anions
MRP2 (ABBC2)	Brain, intestine, liver, placenta, lung kidney	Acidic bile salts, amphipathic organic anions and xenobiotics Bilirubin glucuronides, GSH, sulfate conjugates of endogenous and exogenous compounds
MRP3 (ABCC3)	Adrenal cortex, intestine, kidney, liver, lung, prostate, placenta, spleen, pancreas	Bile salts, endogenous organic anions
MRP4 (ABCC4)	Adrenal gland, brain, bladder, kidney, liver, ovary, lung, skeletal muscle, spleen, testis, thymus	cGMP, cAMP, conjugated steroids, bile acid, folic acid
MRP5	Brain, erythrocyte, heart, kidney, testis, Intestine, liver, lung, skeletal muscle	cAMP, cGMP, folate, GSH, organic anions

Hypothesis

The overall hypothesis of this research is mango polyphenols and mango derived metabolites may have anti-cancer effects on *in situ* breast cancer through inhibition of proliferation. Polyphenols may delay breast cancer progression through suppressing proliferation by modulating the IGFR-1-AKT-AMPK-mTOR-signaling axis. The downregulation of mTOR could be directly caused through the activation of AMPK.

Objectives

1) Investigate the cytotoxic and anti-proliferative activities of mango polyphenols and pyrogallol in cultured MCF10DSCIS.com cells with the IGFR-1-AKT-mTOR signaling axis being of primary interest. Possible food/drug interaction between polyphenols from mango and the widely used cancer drug 5-FU will also be investigated for possible additive or synergistic effects from joint treatment.

2) Investigate if the anti-proliferative effects of mango polyphenols that occur *in vitro* translate to an *in vivo* xerograph model. Mice will orally receive a mango extract or pyrogallol at physiologically relevant doses to determine their effects on tumor growth. Tumors will undergo a mechanistic evaluation to determine if the AKT/mTOR/AMPK is being modified. ROS production will also be a major focus because of its ability to initiate AMPK signaling. *In silico* modeling of AMPK will also be conducted to determine if the structure of pyrogallol could cause direct activation.

3) To further investigate the mechanism associated with the anti-proliferative effects of pyrogallol *in vivo*, and to verify results. Escalated doses will be used in order to determine if toxicity manifests. The bioavailability of pyrogallol and associated metabolites will be examined in tumor tissue and in plasma.

5) Determine if pyrogallol has the ability to cause epigenetic modification as it relates to HDAC1 inhibition.

CHAPTER II

MANGO POLYPHENOLICS REDUCE PROLIFERATIOIN OF DCIS BREAST CANCER *IN VITRO*- POTENTIAL INVOLVEMENT OF THE IGFR-1-AKT-MTOR SIGNALING AXIS AND POSSIBLE FOOD/DRUG INTERACTIONS WITH 5-FU

Summary

In situ breast cancer is a late stage cancer, and intervention during this stage of tumor progression may prevent metastasis. In this study, we elucidated the molecular mechanism underlying the anti-proliferative activities of a low molecular weight fraction of mango (*Mangifera indica L.*) polyphenols (ML) in an *in situ* breast cancer cell line, MCF10DCIS.COM. This line was selected due to its ability to form DCIS lesions *in vivo* which includes the formation of a myoepithelial layer, and is a validated model for studying DCIS therapeutics. Mangos are rich in large gallotannin moieties but also contain low molecular weight gallic acid derivatives. In contrast to gallotannins, low molecular size polyphenols are hypothesized to have a greater prospect of crossing phospholipid membranes and being bioactive intracellularly. Gallotannins themselves are not intestinally absorbed through the intestinal lumen but yield metabolites upon digestion by the intestinal microflora. Pyrogallol (PG), a central microbial gallotannin-metabolite, was therefore included in this investigation because of its bioavailability and relevance as a physiological metabolite. The objective of this study was to investigate the anti-proliferative activities of ML and PG and to identify pathways involved including the PI3k/AKT/mTOR signaling axis in MCF10DCIS.COM. Fluorouracil (5-

FU), a widely used genotoxic cancer therapeutic, was used a positive control and in combination with ML and PG to assess potential interactions. Non-cancer mammary fibroblasts (MCF-12F) were treated with ML and PG and non-cytotoxic dietary relevant concentrations were selected for the investigation in MCF10DCIS.COM cancer cells. In addition to proliferation and viability, mRNA and expression of total and phosphorylated protein were investigated. Both, ML and PG significantly reduced proliferation in MCF10DCIS.COM, but did not significantly reduce viability following a 48h exposure. ML significantly reduced mRNA expression of AKT and mTOR while PG significantly reduced mRNA of HIFα, AKT, mTOR and IGF1-R. LF and PG reduced protein expression of IGF1-R, IRS1, IF, AKT, and P70S6. In addition, PG reduced mTOR protein. Both treatments also had an effect on phosphorylated protein levels, with PG significantly reducing IGF1-R, AKT, and P70S6 levels. ML had a similar effect and significantly decreased IR, AKT, and P70S6 phosphorylation levels. Within the low concentration-range, ML and PG did not interact with the cytotoxic activities of 5-FU. Overall, the PI3k/AKT/mTOR signaling axis appears to be implicated as causal in decreased proliferation induced by diet-relevant concentrations of MG and PG.

Introduction

It is estimated that 1 in 8 women will be diagnosed with breast cancer in their lifetime, and this incidence is the highest among cancers affecting women [113]. Breast cancer is a multi-factorial disease with several stages, and ductal carcinoma *in situ* represents stage 0. This stage is pre-invasive and characterized by clonal proliferation of

cells that accumulate within the lumina of the mammary duct. Essentially, all invasive breast cancers arise from this stage [32]. Therefore, preventing the progression of this stage or decreasing proliferation of these cells may prevent metastasis. The development of this cancer can be dependent on multiple risk factors, which include genetic susceptibility, family history, age, lifestyles, and breast tissue composition [114-116].

Considering the increased safety profiles of naturally derived polyphenol compounds compared to synthetic small molecule chemotherapeutics, natural compounds may have utility as a standalone treatment or as adjuvant therapy [90]. Multiple health benefits including cancer-cytotoxic properties have been ascribed to polyphenolics, but their intestinal microbial metabolites have not frequently been investigated [117-121].

Mangos (*Mangifera indica L.)* are rich in gallotannins and lower molecular gallates including gallic acid[122]. Gallic acid is known for its cancer-cytotoxic activities that has been investigated *in vitro* and *in vivo* [90, 123]. Pyrogallol, which is produced as a metabolite by the gut microflora following consumption of mango, also has been found to be anti-carcinogenic, although the molecular mechanism has not been intensively investigated [68].

The PI3K/mTOR/AKT axis plays a critical role in multiple cellular functions, including proliferation, metabolism, survival, migration and angiogenesis. This signaling axis is frequently dysfunctional in cancer cells resulting in ongoing proliferation [124]. The mTOR pathway can be regulated through intracellular or extracellular stimuli [96, 125]. Polyphenols have previously been demonstrated to downregulate the mTOR

pathway and therefore it is hypothesized that this pathway is involved in the mechanism [126-129].

The objective of this study was to examine the cancer cytotoxic activities of mango gallates and pyrogallol on *in situ* breast cancer cells *in vitro*. Additionally, the interactions of these natural compounds with 5-fluorouracil (5-FU), a common cancer drug that has been used clinically for more than 40 years, was investigated. 5-FU causes dysregulation of various essential biosynthetic processes, including thymidylate synthase [106]. Also, 5FU is able to incorporate into macromolecules such as DNA and RNA and cause downstream deficiencies in protein translation. In the current study, 5-FU was included as a positive control when administered independently and to determine if there were any interactions with the gallates or pyrogallol when co-administered.

LF mango extract was investigated as a source of multiple structures of gallates and pyrogallol was of interest as the major microbial metabolite of gallotannins and in previous preliminary studies it had the highest anti-proliferative activities in cancer cells compared to other gallate compounds.

Materials and Methods

Extractions and Chemical Analysis

ML was fractionated from a complete polyphenolic extract as previously reported with slight modifications [130]. In brief, the mesocarp of mangoes was homogenized and polyphenols were extracted with 1:1 Methanol: Acetone for 30 minutes. Insoluble solids underwent three serial extractions. Solvents were evaporated with a Buchi RII

Rotavap (Waltham, MA) at 45°C. Polyphenols were concentrated using C18 solid-phase extraction. The C18 column removed all polar reducing agents including reducing sugars and ascorbic acid. To retain water-soluble phenolics that did not bind to C18, the unbound fraction was extracted in 1:1 ethyl actate:water. After evaporation of solvents, tannins were removed by filtering the crude extract through Sephadex LH-20. After solvent evaporation, total phenolics were measured spectrophotometrically using the Folin-Ciocalteu assay with values expressed as mg gallic acid equivalents (GAE)/L. Individual polyphenolics were identified using C18 reverse phase chromatography on a Waters Alliance 2690 HPLC-PDA system (Milford, MA). Characterization and quantification of individual polyphenolics was accomplished by HPLC-MS Thermo Finnigan Surveyor HPLC-PDA coupled to a Thermo Finnigan LCQ Deca XP Max MSn ion trap mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA). Briefly, gradient separations were performed using a Phenomenex Kinetex C18 column (150 × 4.6 mm, 2.6 μm) at room temperature. Injections were made into the column by use of a 25 μL sample loop. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in methanol run at 0.45 mL/min. A gradient was run of 0% phase B for 2 min and changed to 10% phase B at 4 min and held to 10 min, from 10 to 40% phase B in 25 min, from 40 to 65% phase B in 35 min, and from 65 to 85% phase B in 41 min and held to 50 min before returning to initial conditions. The electrospray interface worked in negative ionization mode. Source and capillary temperatures were set at 275°C, source voltage was 4.00 kV, capillary voltage was set at −42 V, and collision energy for MS/MS analysis was set at 35 eV. The instrument

operated with sheath gas and auxiliary gas (N2) flow rates set at 20 and 10units/min, respectively.

Cell Culture

Human breast cancer cell line MCF10DCIS.COM were obtained from Asterand, Inc. (Detroit, MI) and cultured in DMEM-F12 media from (Invitrogen. Carlsbad, CA) supplemented with 10% horse serum from (Atlanta Biologicals, Lawrenceville, GA). The non-cancerous breast epithelial line MCF-12F was purchased from ATCC (Manassas, VA) and maintained per the vendor's recommendations. Cells for the proliferation and viability assay were seeded at a density of 15,000 cells/well in a 24-well plate and allowed to attach overnight. Following attachment, cells were treated in 2% FBS media for 24 h with ML or PG at 1-10 mg GAE/L (7.93-79.3μM) for cancer cells and 1-100 mg GAE/L (7.93-793μM) for non-cancer cells. The stock solutions were prepared as 5000 mg/L polyphenols in 25% DMSO. The final DMSO concentration was 0.1% (1μl in 1 ml), and the uniform concentration was treated to control and treatment groups. 5-FU was used as a positive control at 0.5 mg/L (3.85 μM), and the same concentration was used for co-incubations with ML or PG for 24 h. After 24 h, cells were detached using trypsin EDTA at 0.25%. Proliferation and viability were assessed using a Muse Cytometer from Millipore (Billerica, MA). We performed cell proliferation assay with the objective of selecting the relevant dose-range that does not induce cytotoxic activities in normal cells but cytotoxic effects in the breast cancer cells. ML was investigated as a source of multiple structures of gallates, and PG was of interest as the major microbial metabolite of gallotannins. The selected 5FU-

concentration caused low cytotoxicity in cancer cells in order to detect any possible synergistic activities of ML and PG.

Analysis of mRNA Expression by RT-PCR

mRNA analysis was performed as previously described [131], using the Qiagen RNA isolation kit (Valencia, CA) according to the manufacturer's protocol after 12 h of treatment with ML or PG. Cells were seeded at 100,000 cells/well in a 12 well plate for 24 h, and treated in either ML or PG at 1-10 mg GAE/L for 24 h. mRNA was extracted from treated cells, and cDNA was synthesized from the isolated RNA with the use of a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY). Primers were designed using the Primer Express Software Version 3. (Applied Biosystems, Foster City, CA) and purchased from Integrated DNA Technologies Inc. (Coralville, IA). qRT-PCR was conducted on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA).

Multiplex Bead Assay

MCF10DCIS.COM cells were seeded at 200,000 cells/well in a six well plate and allowed to attach overnight. Cells were treated with ML or PG at 1-10 mg GAE/L for 24 h. Protein was extracted (25 µg) from tumor cells and prepared to determine total and phosphorylated protein levels of key components of the mTOR pathway utilizing multiplex kits and protocols (Millipore, Billerica, MA). Analysis was performed using a Luminex L200 (Luminex, Austin, TX). Data was analyzed using Luminex xPONENT Version 3.1.

Statistical Analyses

Quantitative data represent the means ± SE. Data was analyzed by one-way analysis of variance using JMP 10 (SAS Institute Inc., Cary, NC). Tukey's HSD was used ($p<0.05$) to determine statistical differences between treatment groups.

Results

Chemical Analysis

Utilizing HPLC-MS analysis, polyphenols were identified in the mango extract (Fig. 15A). Four major compounds were identified (Fig. 15B) including, mono-galloyl glucoside (Peak 1 and 3), gallic acid (Peak 2), and hydroxybenzoic acid hexoside (Peak 4) (Fig. 15B). Fig 15C is the chemical structure of pyrogallol. The concentration of total phenolics was quantified using the Folins Ciocalteu assay as 1174 mg/L GAE.

Cell Cycle Kinetics

Previous studies have indicated that anti-proliferative polyphenols may arrest cell cycle in different stages [132-134]. In this study, PG did not significantly alter cell cycle kinetics following 12h exposure. ML caused a minor arrest in the S-phase (data not shown). The absence of effect in the PG treatment may be attributed to the low concentration used or the treatment requiring a longer exposure window.

Anti-proliferative Effects of Mango Polyphenols and Pyrogallol in MCF10DCIS.COM

In a preliminary screen, the anti-proliferative activities of selected polyphenolic compounds and their metabolites were evaluated in MCF10DCIS.COM cells at 1 mg/L for 48 h (Fig. 16A). The greatest anti-proliferative activities were observed in cells

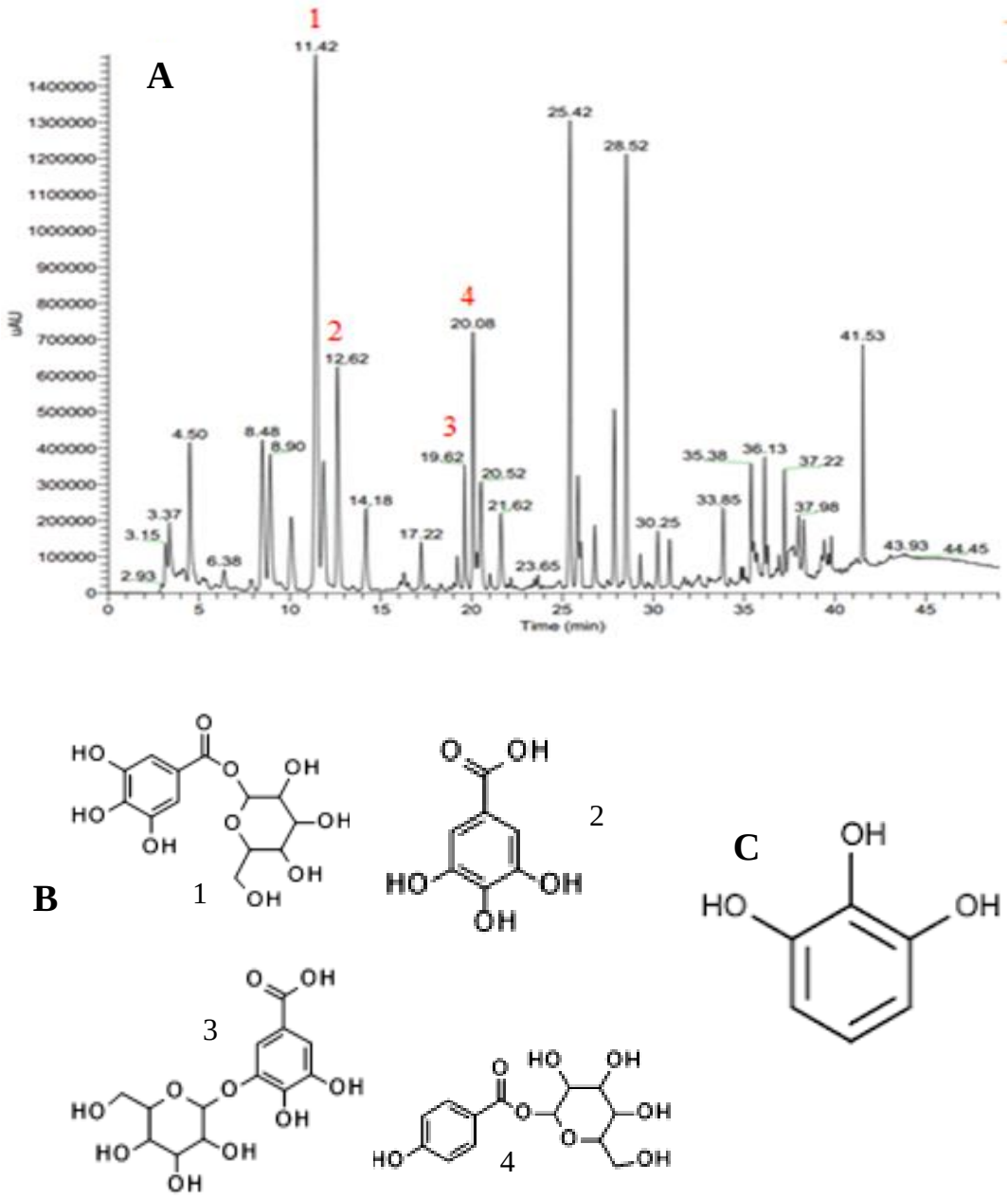

Figure 15. Characterization of compounds in mango polyphenols.
A) Representative chromatogram at 280 nm of the low molecular weight mango polyphenol fraction, B) tentative structures for compounds 1-4: Peak 1 and 3 monogalloyl glucoside, Peak 2 gallic acid, Peak 4 p-hydroxybenzoic acid hexoside,21 C) chemical structure for pyrogallol.

treated with ML and PG decreasing proliferation greater than 55% and 50%, respectively. For this reason, these two treatments, ML and PG, were selected for additional analyses.

In previous studies, synergistic interactions between polyphenols and conventional cancer treatment drugs were observed, including 5-FU [110, 135]. 5-FU treatment was included to act as a positive control, and to evaluate if a combinational treatment with both polyphenol treatments could produce additive or synergistic effects. These results are presented as paired bars to compare the effect of ML or PG treatment individually and in combination with 5-FU. In this study, cells were pretreated with PG and ML at 10 mg/L for 24 h followed by a treatment with 5-FU at 0.5 mg/L for an additional 24 h (Fig. 16B). Both, ML and PG at a relevant dietary dose of 10 mg/L, significantly reduced cellular proliferation by 34% and 25%, respectively, compared to the control. These treatments did not reduce proliferation to the levels seen in a treatment of 48 h at 1 mg/ml because the exposure window was reduced by half. A longer exposure window in the initial screen was done in order to determine if the compounds have an anti-proliferative effect even at lower concentrations. The 10 mg/L concentration was selected because it is relevant to dietary intake from foods and dietary supplements. Viability was assessed in the non-cancer cell line MCF-12F for 48 h, where ML and PG decreased viability at concentrations of 25 mg/L or greater (Fig. 16C). For this reason, a concentration-range of 10 mg/L was selected for its cytotoxic effects in cancer cells while producing to cytotoxic effects in normal cells.

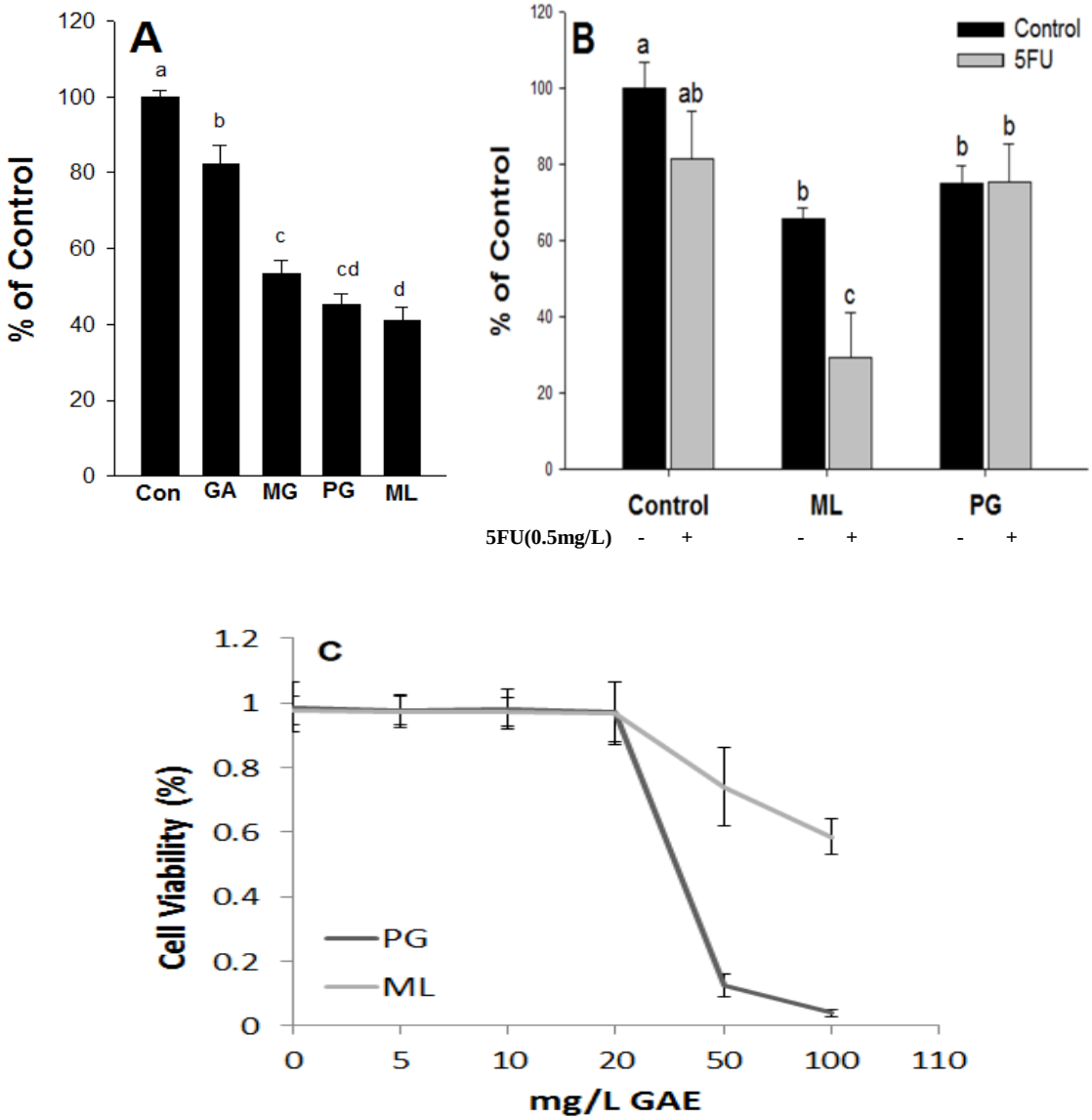

Figure 16. Effects of mango polyphenols and their metabolites on cell proliferation. A) Anti-proliferative activities of non-treated controls (Con), gallic acid (GA), methylgallate (MG), pyrogallol (PG) and low molecular fraction of mango polyphenols (ML) each at 1 mg/L for 48 h of exposure, B) Subsequent treatment of 10 mg/L ML or PG, followed by a 24 h exposure of 5-FU at 0.5 mg/L for 24 h, C) Anti-proliferative activities of ML and PG in non-cancer breast-fibroblast cells MCF12F. Values are the mean ± SEM (n=3). The different letters indicate significance at $p < 0.05$ (Tukey's HSD).

Effects of Mango Polyphenols and Pyrogallol on Gene Expressions

Polyphenols are known to downregulate different vital constituents of the IGF1-R/PI3K/AKT/mTOR-axis in cancer cell lines [127, 136, 137]. Based on the anti-proliferative activities of ML and PG, the gene expression of constituents of the mTOR-pathway were investigated in this study (Fig. 17A-D). After 24 h of exposure, mRNA of the extracellular protein IGF1-R was decreased by more than 40% when cells were treated with PG at 10 mg/L compared to 5-FU, which at the selected concentration did not significantly affect mRNA expression. The combination of PG + 5-FU did also not significantly decrease mRNA expression of IGF1-R compared to control cells. In contrast, ML mRNA expression of IGF1-R was increased compare to control cells, and conversely, in combination with 5-FU it decreased the gene expression of IGF1-R (Figure 17A). Neither ML nor 5-FU altered the gene expression of AKT but the combination of both significantly reduced AKT mRNA. ML may not have decreased the mRNA level of AKT, while PG alone and in combination with 5-FU decreased mRNA of AKT (Figure 17B). mTOR mRNA levels were significantly decreased by ML and PG but in combination with 5-FU, these treatments did not potentiate the effects of 5-FU alone (Figure 17C). ML and PG also decreased HIF1α mRNA but the co-treatment with 5-FU did not potentiate the effects of 5-FU alone (Figure 17D). While changes in mRNA expression allow limited conclusions about protein-activity along this pathway it indicates any potential reduction of protein production due to reduced mRNA expression [138, 139].

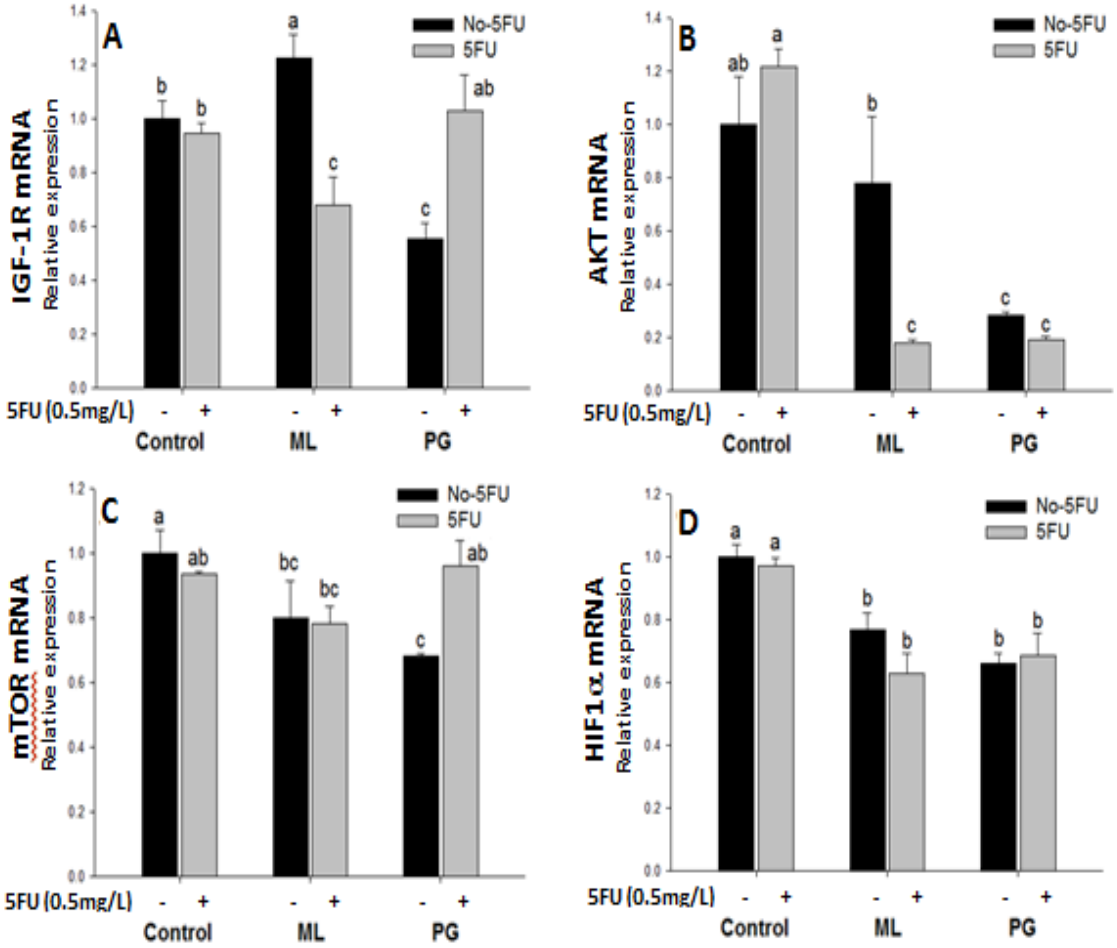

Figure 17. Effects of mango polyphenols (ML) and pyrogallol (PG) on the gene expressions involved in the mTOR signaling.
mRNA expressions following exposure to 10 mg/L ML or PG for 24 h with and without subsequent exposure to 5-FU at 0.5 mg/L for 24 h in MCF10DCIS.COM cells. (A) IGF-1R, (B) AKT, (C) mTOR, and (D) HIF1α. Values are the mean ± SEM (n=3). The different letters indicate significance at $p < 0.05$ (Tukey's HSD).

Effects of Mango Polyphenols and Pyrogallol on Protein Expression

Total and phosphorylated protein levels provide a better characterization of the anti-proliferative activities of ML and PG as it represents an indication of the activity of a certain pathway. ML and PG significantly decreased total IGF-1R, and PG also

decreased phosphorylated protein levels. In addition, PG followed by 5-FU significantly reduced total protein levels compared to either treatment individually (Fig. 18A). Insulin receptor (IR) total protein was significantly reduced by both polyphenol treatments where ML also significantly reduced phosphorylated levels. PG in conjunction with 5-FU decreased total protein levels greater than either compound individually; however, this effect was not observed at the phosphorylated level (Fig. 18B).

IRS1 was only significantly decreased at the total protein level with PG. A combination treatment with 5-FU and PG decreased total protein levels greater than each treatment individually. IRS1 phosphorylation levels were not significantly altered by either ML or PG (Fig. 18C). The polyphenol treated groups decreased both total and phosphorylated AKT protein levels. PG reduced total AKT protein levels to a greater extent than 5-FU alone (Fig. 18D). mTOR showed significantly decreased protein levels with both polyphenol treatments, and PG alone reduced mTOR total protein greater than 50% compared to the control. Neither treatment altered mTOR phosphorylation. 5-FU alone elevated mTOR protein levels, and when pretreated with ML or PG, mTOR total protein was decreased (Fig. 18E). The downstream kinase P70S6K that is directly involved in cell proliferation upon mTOR activation was downregulated at both the total and phosphorylated protein levels by both ML and PG. Furthermore, when pyrogallol and 5-FU were combined, an additive effect was apparent for total protein expression (Fig. 18F).

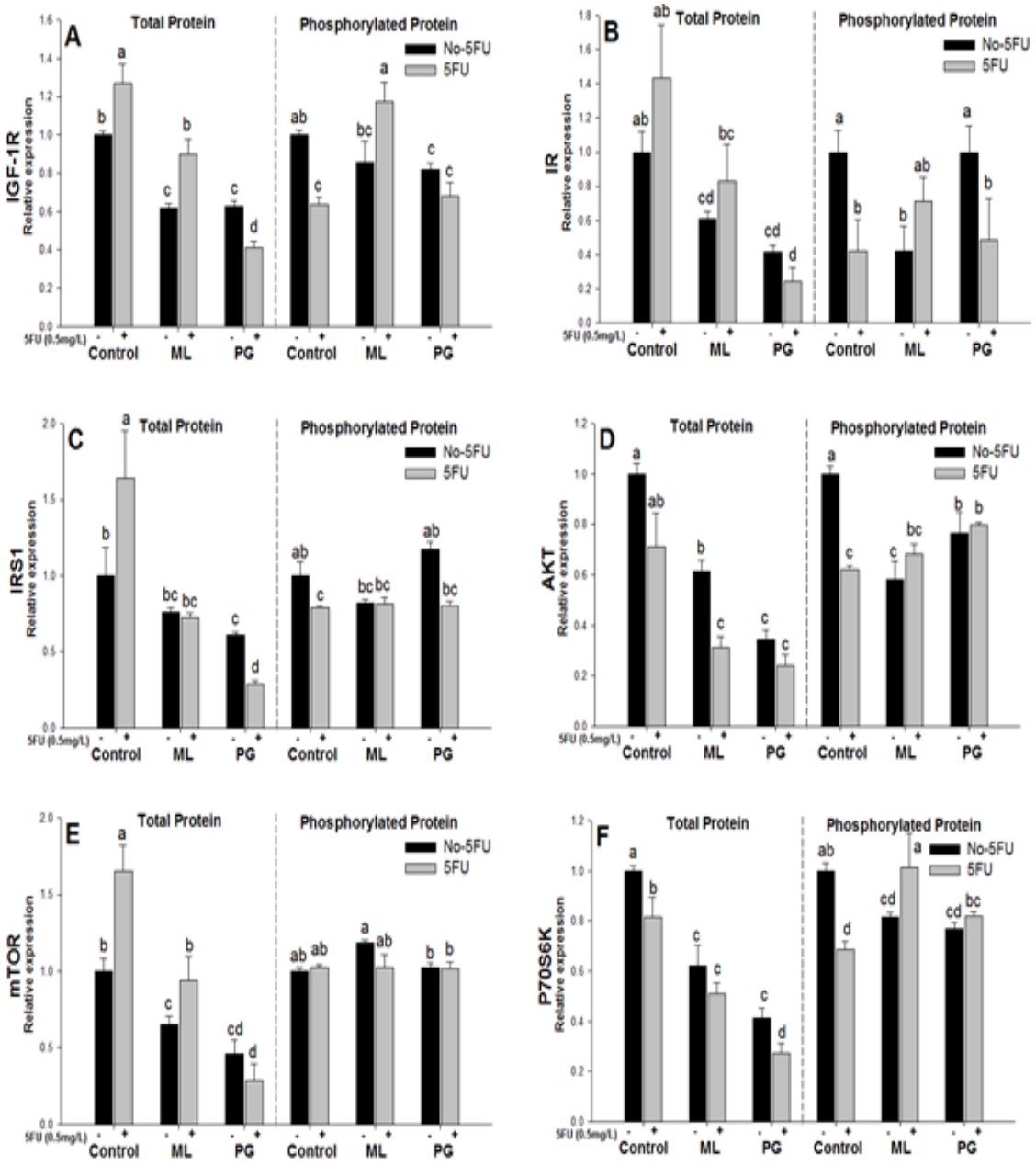

Figure 18. Effects of mango polyphenols (ML) and pyrogallol (PG) on the protein expressions involved in the mTOR signaling.
Expression of total and phosphorylated protein levels following 10 mg/L ML and PG treatment for 24 h followed by 24 h incubation with and without 5-FU at 0.5 mg/L. (A) IGF-1R, (B) IR, (C) IRS1, (D) AKT, (E) mTOR, and (F) P70S6K. Values are the mean ± SEM (n=4). The different letters indicate significance at $p < 0.05$ (Tukey's HSD).

Discussion

Previous studies have demonstrated the anti-proliferative activities of polyphenols in cancer cells, while no cytotoxicity was observed in non-cancer cells within the same treatment concentration-range [60, 140, 141]. The concentrations selected for this study are within a physiological range, which can be expected to occur *in vivo* upon consumption of mango fruit or a dietary supplement [65, 142]. Conversely, few reports include the investigation of PG, a microbial metabolite, that occurs systemically after the ingestion of gallotannins [68, 143] as they occur in mangos and many other fruits and vegetables, teas and spices [144-147]. The results of this study confirm that mango polyphenols decrease proliferation in breast cancer cells that seem to be at least in part modulated by the mTOR-signaling axis [148].

ML and PG decreased proliferation in MCF10DCIS.COM, but did not decrease viability in the non-cancerous breast cell line MCF-12F at 10 mg/L. This initial result supplied justification for the dose concentration used through the duration of the study, because 10 mg/L had a cytotoxic effect in the breast cancer cell lines while displaying no cytotoxic effects in normal cells. The low molecular weight fraction is composed of multiple compounds, which included mono-galloyl glucoside, gallic acid, and hydroxybenzoic acid hexoside. In addition, our lab reported that mango polyphenols include galloyl diglucoside, coumaric glycoside, dihydrophaseic acid glucoside, ferulic acid hexoside, eriodictyol-O-hexoside.[130] A complete extract, along with the single compound PG was selected due to their similarities in effect observed in the proliferation assay, and allowed for a comparison between an extract containing multiple polyphenols

to a single polyphenolic metabolite with physiological relevance that has yet to be investigated in a breast cancer model. Intuitively, each of these compounds can be expected to differ in anti-proliferative activities. Previous studies that investigated the independent and combinational anti-proliferative efficacy of pomegranate polyphenols, and they demonstrated that an extract containing punicalagin, ellagic acid, and pomegranate tannins, had a greater anti-proliferative effect on colon cancer cell lines (HT-29 and HCT119), compared to treatment with any of the individual polyphenols[149]. This demonstrates how an extract containing a spectrum of polyphenols could produce a synergistic or additive effect compared to single polyphenol compounds. The same conclusion was drawn in a study that evaluated a total cranberry extract versus its phytochemical constituents in oral, colon, and prostate cancer cell lines.[150]

Pyrogallol is formed in the gastrointestinal tract through tannase enzymes. The intestinal conversion rate of tannins to pyrogallol has not yet been established. However, in a previous clinical study performed in our laboratory, we quantified 87.9 mg of gallic acid metabolites with the majority being pyrogallol glucuronide, pyrogallol sulfate, and methyl-pyrogalloyl sulfate as urinary metabolites after 400 g mango fruit intake (130.13 mg GAE of gallotannins). [151] With this, the concentration-range of pyrogallol used in this study includes physiological levels after the intake of food and reaches into a concentration range that may be expected after prolonged consumption or higher intake levels of tannins, e.g. from a dietary supplement.

5-FU is a widely used genotoxic cancer drug that is frequently used in breast cancer treatment, however, this drug has severe side effects that include cardiotoxicity, mood disorders, and neurological damage.[109, 148] The concentration of 5-FU selected for this study (3.85 μM) was far below the IC50 in cancer cells to determine whether a co-treatment with polyphenols might potentiate the anti-proliferative activities of 5-FU,[152] thus indicating a potential for decreasing the required dose in cancer treatment which would reduce the severity of observed side effects.[110] A low concentration of 5-FU was selected to detect any possible synergistic activities with ML or PG. A higher concentration of 5-FU would have potentially masked these effects. Previously, a combination of 5-FU and genistein resulted in decreased cyclooxygenase-2 levels (COX-2) compared to groups only treated with 5-FU, which had significantly elevated COX-2 levels in HT-29 colon cancer cells. This effect was attributed to the reactive oxygen species produced by the genistein which activated AMPK and decreased COX-2 expression. In contrast to this previous publication, the concentration-range selected for this study was much lower and this may have caused the lack of additive, potentiating or synergistic activities. Overall, in this study, the selected low concentrations of 5-FU and the low concentrations of polyphenols did not produce overwhelming evidence of an additive, potentiating or synergistic interaction between ML, PG, and 5-FU as it was observed in previous publications.

The IGF-1R- AKT/mTOR signaling axis is a major pathway involved in the regulation of proliferation.[96, 153-155] Previously, treatments of cancer cells with polyphenols have elicited a reduction of activities in this pathway; however, the effects

of a physiological microbial metabolite of polyphenols have rarely been investigated. In this study, ML decreased mRNA levels for both mTOR, and downstream transcription factor HIF1α. At the protein level, ML downregulated the cell surface receptor IGF-IR and P70S6K, a kinase regulated by mTOR, and may exert an extracellular and intracellular effect influencing both components. Previously, treatment with polyphenols showed extracellular effects through the blockage of membrane receptors such as epidermal growth factor receptor in combination with an intracellular effect through the quenching of free radicals.[156, 157] It could be rationalized that mango polyphenols exerted an effect both intracellularly and extracellularly, where the larger molecular weight compounds exert more effect on the cell surface, and the smaller compounds are able to cross the phospholipid membrane and bind with cytosolic or nuclear proteins.[158-160]

As initially hypothesized, both polyphenol-treatments demonstrated differential activities within the AKT/mTOR signaling axis *in vitro* and a summary of the results can be seen in Table (7). Compared to the low concentration-range of 5-FU, ML exerted a greater reduction on mRNA and protein along the mTOR pathway. ML significantly reduced IGF1-R, IRS1, IR, mTOR, and P70S6 protein levels compared to 5-FU. There was no consistent interaction observed between ML and 5-FU that would justify further investigation in this co-treatment paradigm within the selected concentration-range. ML decreased mRNA levels of mTOR and HIF1α, as well as total protein for IGF-1R, IR, AKT, mTOR, and P70S6K, and phosphorylated protein levels of IR, AKT, and P70S6K compared to the control. ML may not have decreased the mRNA level of AKT,

Table 7. Overview of proposed anti-proliferative mechanism of polyphenols involving IGF-1R-AKT/mTOR signaling axis.

	Mango			Pyrogallol		
	mRNA	Total protein	Phos Protein[a]	mRNA	Total Protein	Phos Protein[a]
IGF-1R	*	Decrease	*	Decrease	Decrease	Decrease
IR	-	Decrease	Decrease	-	Decrease	*
IRS1	-	*	*	-	Decrease	*
AKT	*	Decrease	Decrease	Decrease	Decrease	Decrease
mTOR	Decrease	Decrease	*	Decrease	Decrease	*
P70S6K	-	Decrease	Decrease	-	Decrease	Decrease
HIF1α	Decrease	-	-	Decrease	-	-

[a]Phosphorylated Protein, * indicates no effect, and – indicates not measured.

however, it is common for mRNA and protein expressions not to be fully correlated [161, 162]. In this study, AKT protein expression was decreased while the mRNA concentrations were not affected at the selected time point of 24 h. In addition, it is possible that with the selected exposure window (24 h) data did not capture an effect of PG on AKT mRNA, which might be changed at earlier time points.[163] PG, a central intestinal microbial metabolite of gallotannins, was selected for this study as it systemically occurs after the consumption of gallotannins.[68] In initial screening, the anti-proliferative effect of PG exceeded those of gallic acid and other mango-derived polyphenols. PG decreased mRNA levels of IGF-1R, AKT, mTOR, and HIF1α, as well as total protein for IGF-1R, IR, IRS1, AKT, mTOR, and P70S6K, and phosphorylated protein levels of IGF-1R, AKT, and P70S6K compared to the control. When co-treated with 5-FU, an additional reduction of protein-expression and protein phosphorylation

was observed along the mTOR axis compared to the individual 5-FU treatment. It indicates that PG may have a potential to enhance the anti-proliferative activities of 5-FU, thus decreasing the required dose of 5-FU. Further research would be necessary to determine whether the enhanced anti-proliferative action from the co-treatment of mango polyphenols, PG with 5-FU *in vitro* can be reproduced in animal models.

Overall, this study demonstrates that mango polyphenols have an anti-proliferative effect on DCIS breast cancer *in vitro* at physiologically relevant concentrations, and this mechanism could be partially attributed to the ability of these polyphenols to down-regulate multiple key constituents along the AKT/mTOR signaling axis.

CHAPTER III

POLYPHENOLICS FROM MANGO (*MANGIFERA INDICA* L.) SUPPRESS BREAST CANCER DUCTAL CARCINOMA *IN SITU* PROLIFERATION *IN VIVO* THROUGH THE ACTIVATION OF AMPK PATHWAY AND SUPPRESSION OF MTOR

Summary

The objective of this study was to assess the underlying mechanisms of mango polyphenols resulting in decreased proliferation and tumor volume in ductal carcinoma *in situ* (DCIS) breast cancer. Utilizing a MCF10DCIS.com xenograft model, the hypothesis that mango polyphenols reduce signaling along the AKT/mTOR axis while upregulating AMPK was tested. Tumor volumes were significantly reduced more than 50% in both the mango (MG) and pyrogallol (PG) treated athymic mice at 0.8 mg GAE/day and 0.2 mg/day, respectively, following 4 weeks of oral administration (n=10 per group). Both treatments decreased mRNA expression involved in the mTOR pathway by up to 80%. Total protein levels were significantly decreased by greater than 25% for IRS1, 45% for AKT, and 40% for mTOR in both treatment groups compared to the control. Phosphorylated protein levels of IR, IRS1, IGF-1R, P70S6K, and ERK were significantly reduced by PG, and MG significantly reduced IR, IRS1, IGF-1R, and mTOR. The level of Sestrin2, an activator of AMPK, was significantly elevated in MG and PG treated tumors, while MG significantly elevated AMPK phosphorylation and PG elevated LKB1. The modulation of reactive oxygen species (ROS) generation and the AMPK pathway by both compounds were confirmed *in vitro*. These results suggest that

mango polyphenols and their major microbial metabolite pyrogallol may inhibit proliferation of DCIS breast cancer through upregulating of AMPK and downregulating of the AKT/mTOR pathway.

Introduction

Breast cancer was estimated to contribute almost 30% to newly diagnosed cancers with 232,670 cases diagnosed in 2014 [164], contributing to an estimated 40,000 deaths [165]. Ductal carcinoma *in situ* (DCIS) stage occurs directly before metastasis. Delaying the onset of metastasis may have significant implication in extending survival, specifically since most deaths occur because of complications arising from multiple organ system involvements rather than to primary cancer site tumors [166]. Treatment of DCIS breast cancer, most commonly with a mastectomy, has a ten year survival rate of 98-99% [32]. Given the high incidence and mortality associated with breast cancer, an ongoing effort in drug development along with the refinement of current treatment options is necessary to provide the best therapeutic strategies. Currently, available treatment options commonly cause adverse side effects contributing to both a decreased quality of life and decreased compliance amongst patients [167, 168]. Dietary polyphenols are advantageous, based on their demonstrated efficacy and lack of deleterious side effects and therefore could be a potential candidate as treatment alternatives to conventional synthetic small molecules [169-171].

Mango (*Mangifera indic*a L.), once considered an exotic fruit, is now readily consumed in the U.S [56]. Mango is rich in polyphenolic moieties including gallic acid, gallotannins, galloyl glycosides and flavonoids, which all have been shown to have

potential health benefits associated with their consumption [59, 172, 173]. Polyphenolic profile and concentrations are dependent on variety, location, harvest conditions, and processing methods [174]. Mango polyphenolics have been demonstrated to have anti-inflammatory and cancer-cytotoxic properties in multiple cancer types including malignancies of the colon and breast [175, 176]. Gallic acid is a unique trihydroxibenzoic acid that is found in high concentrations in mango [177]. Gallotannins consist of gallic acid and other molecules, and may be broken down by lactobacilli to release free gallic acid or its decarboxylated form pyrogallol within the gastrointestinal tract (GI). Pyrogallol is a unique metabolite that is formed by decarboxylation of gallic acid by intestinal microflora, and has been shown to have health promoting properties in different disease models [68, 76].

The AKT/mTOR pathway is dysregulated in breast cancer, and has been a target for breast cancer therapy [178]. Previously, we reported that the anti-inflammatory and anti-cancer effects of mango polyphenols results through modulation of the mTOR pathway [179, 180]. 5' AMP-activated protein kinase (AMPK) is responsible for maintaining energy homeostasis within the cells and is activated through the accumulation of Sestrin protein, and ultimately the activation of this pathway culminates in the inhibition of mTOR along with the activation of autophagy [181, 182]. The expression of AMPK is known to be activated by reactive oxygen species (ROS), therefore, the ROS-activated AMPK pathway could be modulated by pro-oxidants [183-185].

The objective of this research is to establish if the mango polyphenols and pyrogallol inhibit proliferation of breast cancer cells in a xenograft tumor model. Our hypothesis was that mango polyphenols and their major intestinal metabolite pyrogallol may have anti-cancer effects on ductal carcinoma *in situ* through signaling along the AKT/mTOR axis while upregulating AMPK. This is the first study to investigate the inhibition of ductal carcinoma *in situ* breast cancer by mango polyphenols and their major intestinal metabolite pyrogallol *in vivo*.

Materials and Methods

Extractions and Chemical Anaylsis

The polyphenolic extract from mango cv. Keitt was prepared as previously described with some modifications [130]. Briefly, 2 kg of pulp was homogenized and extracted with 2 L of 1:1 Methanol: Acetone for 30 min proceeded by separation of insoluble solids with cheese cloth. Remaining solids underwent two additional extractions with 1 L of solvent mixture, and was evaporated with a Buchi RII Rotavap (Waltham, MA) at 45°C under vacuum. Polyphenols were concentrated using a 10 g Waters C18 sample preparatory column that was prepared with 10 columns volumes of 0.1 M HCl MeOH and 0.01 M HCl H2O. Polyphenolics were eluted with 200 mL of 0.1 M HCl MeOH. To retain water-soluble phenolics that did not bind to C18, the unbound fraction was extracted twice by 1:1 ratio of sample: ethyl acetate. The C18 eluent and ethyl acetate fractions were collected together, evaporated under vacuum to dryness, and brought up to 50 mL in 1% Citric Acid. This process was repeated 9 additional times and extracts were pooled together to make a volume of 500 mL for the final extract. Total

soluble phenolics was calculated for the final extract spectrophotometrically using the Folin-Ciocalteu assay with values expressed as mg gallic acid equivalents (GAE)/L. Additional a detailed characterization and quantification of individual polyphenolics was performed by use of a HPLC-MS Thermo Finnigan Surveyor HPLC-PDA coupled to a Thermo Finnigan LCQ Deca XP Max MSn ion trap mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA) Injections were made into the column by use of a 25 μL sample loop followed gradient separations were performed using a Phenomenex Kinetex C18 column (150 × 4.6 mm, 2.6 μm) at room temperature. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in methanol run at 0.4 5mL/min. The gradient was run with 0% phase B for 2 min and changed to 10% phase B at 4min and held to 10min, from 10 to 40% phase B in 25min, from 40 to 65% phase B in 35min, and from 65 to 85% phase B in 41 min and held to 50 min before returning to initial conditions. The electrospray interface worked in negative ionization mode. Source and capillary temperatures were set at 275°C, source voltage was 4.00 kV, capillary voltage was set at −42 V, and collision energy for MS/MS analysis was set at 35 eV. The instrument operated with sheath and auxiliary gas (N2) flow rates set at 20 and 10units/min, respectively. Pyrogallol was order from Sigma-Aldrich (St. Louis, MO) and was brought up in 0.2 M solution of citric acid buffer at a pH of 3.5.

Cell Culture

Human breast cancer cell line MCF10DCIS.COM were obtained from Astelrand, Inc. (Detroit, MI) and cultured in DMEM-F12 media (Invitrogen, Carlsbad, CA) supplemented with 10% horse serum (Atlanta Biologicals, Lawrenceville, GA).

Xenograph Study

All animal procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University before their initiation. Female athymic BALB/c nude mice at approximately three weeks of age were ordered from Harlan Teklad (Houston, TX). MCF10DCIS.com cells (5 X 10^4 cells) were harvested and mixed with 50 µL of Matrigel (BD Bioscience, San Jose, CA) and stored on ice until implantation. Mice were implanted subcutaneously into the mammary pad (bilaterally). Tumors were allowed to proliferate one week before the initiation of dosing. Animals were orally gavaged 100 µl twice daily with either MG or PG to achieve a daily exposure of 0.8 mg GAE/day or 0.2 mg/day, respectively (n=10 per group). Control animals received the same dosing regimen with a vehicle of the 0.2 M citric acid buffer (pH 3.5). Tumors size were measured weekly with calipers. Body weights were recorded weekly and at the time of necropsy. Mice were killed 4 weeks after the initiation of dosing, and tumors and organs of interest were either preserved via fixation in 10% neutral buffered formalin or flash-frozen in liquid nitrogen [186].

Analysis of mRNA Expression by Real-time PCR

Total RNA was isolated from tumor samples using a mirVana miRNA Isolation kit (Applied Biosystems, Foster City, CA). cDNA was synthesized from the isolated

RNA using of a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY). Primers were designed using the Primer Express Software Version 3. (Applied Biosystems, Foster City, CA) and purchased from Integrated DNA Technologies Inc. (Coralville, IA). qRT-PCR was conducted on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) [186].

Multiplex Bead Assay

Xenographic tumor samples collected at the time of necropsy were homogenized in tissue protein extraction reagent (Invitrogen Corp., Grand Island, NY). Protein (50 μg) samples was utilized to determine the levels of pro-inflammatory cytokines including CRP, TNF-α, and IL-1β, and the total and phosphorylated protein levels of key components of the mTOR pathway utilizing multiplex kits and protocols (Millipore, Billerica, MA). The analysis was performed using a Luminex L200 (Luminex, Austin, TX). Data was analyzed using Luminex xPONENT Version 3.1 [175].

Western Blot

Protein samples were diluted appropriately to achieve 60 ug total protein, which was loaded and run on a 4-12% sodium dodecyl-polyacrylamide gel. The gel was transferred for 1 h at 350 mA onto a PVDF membrane. The membrane was probed with the primary antibodies against Sestrin 2, Becklin, ULK, p-ULK, AMPK, p-AMPK, mTOR, p-mTOR, and LKB1 (Cell Signaling Technology, Danvers, MA). Beta-actin was used as the endogenous control (Sigma, St Louis, MO).

ROS Generation

MCF10DCIS.com cells were seeded at a density of 5,000 cells/well in a 96-well plate and allowed to attach overnight. Following attachment, Cells were treated with either MG or PG treatment for 24 h at a concentration range from 1-20 mg GAE/L followed by treatment with 5 mM dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 30 min at 37°C. Following the 30 min incubation, the medium was removed, and 100 µl Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) containing no phenol red was added. The fluorescent signal was measured at 480 nm excitation and 520 nm emission with a FLUOstar Omega plate reader (BMG Labtech Inc., Durham, NC) [187].

Cell Proliferation and Cell Cycle Kinetics

MCF10DCIS.com cells were seeded at a density of 15,000 cells/well in a 24-well plate and allowed to attach overnight. The glutathione pretreatment groups received 5 mM glutathione for 3 h and then either MG or PG treatment for 24 h at 10 mg/L. After 24 h cells were detached using trypsin. Proliferation and viability were assessed using a Muse Cell Analyzer (Millipore, Billerica, MA). For the cell cycle kinetics, MCF10DCIS.com cells were treated with either MG or PG at 10 or 20mg GAE/L for 24 h and then harvested for analysis. The MUSE ® Cell Cycle Kit and protocol were used (EMD Millipore, Darmstadt, Germany). Briefly, cells were fixed in 70% ethyl alcohol at -20°C overnight. Cells were then resuspended in 200 µL of Muse Cell Cycle Reagent and incubated at room temperature for 30 min protected from light. Samples were then run in the Muse Cell Analyzer (EMD Millipore, Darmstadt, Germany).

Mitochondrial Membrane Potential

MCF10DCIS.com cells were seeded overnight and then treated with either 10 or 20 mg GAE/L MG or PG for 24 h. Cells were suspended in assay buffer and then added to MitoPotenital working solution, which consisted of Muse MitoPotential Dye and assay buffer (EMD Millipore, Darmstadt, Germany). Cells were incubated for 20 min at 37°C in a CO_2 incubator. Following incubation 5 µL Muse MitoPotential 7-ADD reagent was added to each sample and allowed to sit at room temperature for 5 min. Samples were run in the Muse Cytometer (EMD Millipore, Darmstadt, Germany).

Molecular Modeling and Docking

The 3D coordinates of human α2β1γ1 AMPK complex was retrieved by the Protein data bank (Structure ID= 4CFF) [188]. The model was energetically refined in the internal coordinate space with Molsoft ICM software (MolSoft LLC, San Diego, CA) as previously described [189]. In addition, molecular docking was run as previously reported [175, 189].

Small Interfering RNA (siRNA) Transfection of MCF10DCIS.COM Cells

MCF10DCIS.com cells were transfected for 6 h with siRNA targeting AMPK or with a control siRNA with Lipofectamine RNAi MAX (Invitrogen Corp., Grand Island, NY). Following transfection media was replaced with treatment media containing either 10 or 20 mg/L of PG or 10 mg GAE/L of MG for 24 h. After treatment mRNA and protein were isolated following the same procedures previously mentioned.

Statistical Analysis

The sample size was determined using a priori statistical power analyses (p =

0.05, power = 0.85) [186]. Quantitative data represent the means ± SE. Data was analyzed by one-way analysis of variance using JMP 10 (SAS Institute Inc., Cary, NC). Dunnett post hoc multiple comparisons or Tukey's HSD were used ($p < 0.05$) to determine statistical difference between treatment groups.

Results

Polyphenol Composition of Mango Extract by HPLC-MS

The polyphenol profile and concentration of the mango extract was determined using HPLC-MS analysis (Figure 19) and were as following: gallic acid at 16.89 mg/L, ester-monogalloyl glucoside (2 different isomers) at 174.75 mg/L and 22.74 mg/L, and p-hydroxybenzoic acid glycoside at 81.94 mg/L. Additionally, unresolved gallotannins were identified.

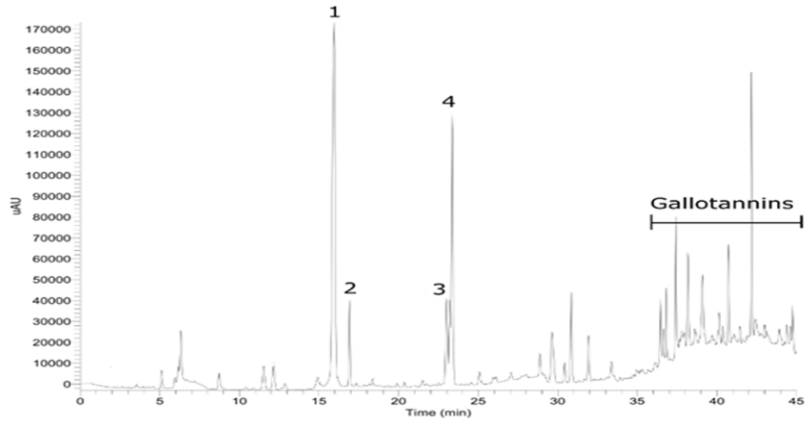

Figure 19. Representative HPLC chromatograph of mango polyphenols (280 nm). Tentative peak assignments showing the presence of: 1 Ester monogalloyl glucoside (149 mg/g of total phenolics), 2 Gallic acid (14 mg/g), 3 Ester-monogallol glucoside (19 mg/g), 4 p-Hydroxybenzoic acid glycoside (69 mg/g), and gallotannins.

Effects of Mango Polyphenols (MG) and Pyrogallol (PG) in Athymic Nude Mice Xenographed with MCF10DCIS.COM Cells

Based on the previous studies of the effects of MG and PG that resulted in decreased proliferation of MCF10DCIS.com *in vitro* primarily through the modulation of the AKT/mTOR signaling axis, we followed up with an assessment of these treatments in an *in vivo* xenograft model. Animals that were orally treated with 0.8 mg GAE/day MG or 0.2 mg/day PG had significantly reduced tumor volumes compared to the control group at weeks 3 and 4. As can be seen in Figure 20A, at week 3, tumor volumes of PG and MG treated animals were reduced approximately 75% and 45%, respectively, compared to the control animals. At 4 weeks, MG and PG had a similar effect on tumor volumes, which was greater than a 70% reduction compared to the control. These results indicate significant retardation in tumor growth by both treatments following 4 weeks of oral exposure.

Tumors collected at the time of necropsy were processed and assayed for cytokine levels based on previous reports suggesting mango polyphenols reduce inflammation in various disease models, and these results are present in Figure 20B [130]. Previous reports provided ample justification for screening a few key inflammatory markers to determine if these treatments were able to alter expression in this *in vivo* model for breast cancer. MG significantly decreased CRP and TNF-α levels by 20% and 35% respectively compared to the control. Tumors from PG treated animals had significantly reduced IF-1β, approximately 40%, compared to the control.

Genes along the mTOR signaling pathway were quantified to determine if these compounds had an effect at the mRNA level, which could impact total and phosphorylated protein levels. MG and PG decreased the mRNA expression of multiple genes along the AKT/mTOR signaling axis in xenographed tumors (Figure 21). Upstream of mTOR, IGF1R and IRS1 mRNA levels were significantly reduced by both treatments with IGF-1R and IRS1 being reduced greater than 60% compared to control tumors. mRNA levels were also reduced downstream of IGF-1R and IRS1

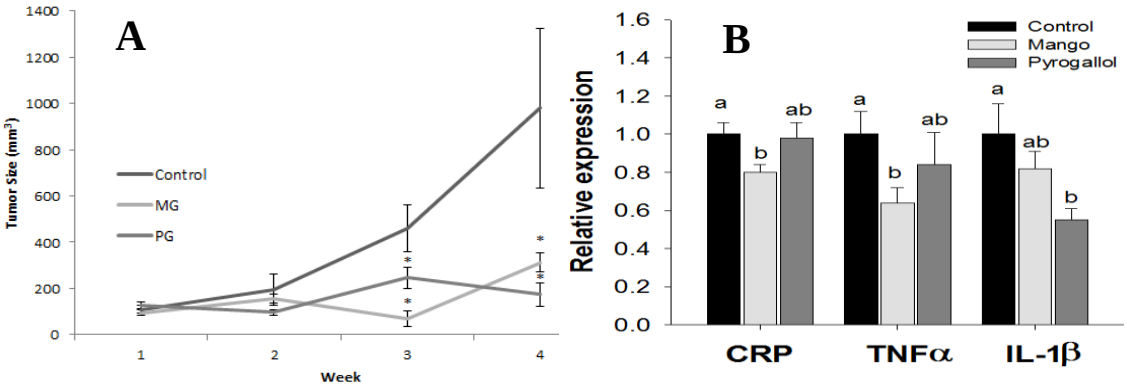

Figure 20. Effects of mango polyphenols (MG) and pyrogallol (PG) in athymic nude mice xenografted with MCF10DCIS.COM cells.
(A) Tumor volumes over four weeks were decreased by MG or PG in athymic mice compared to vehicle treated controls (n=10 per group). *$p < 0.05$ compared to the control (Dunnett's test). (B) Pro-inflammatory cytokine levels in tumor following four week exposure to 0.8 mg GAE/day MG or 0.2 mg/day PG.

and included decreased mRNA levels for PI3K and AKT. Both of these were reduced to a similar percentage with MG treatment reducing either level by nearly 80% while PG treatment reduced PI3K and AKT by 60%. The mRNA levels of mTOR were reduced

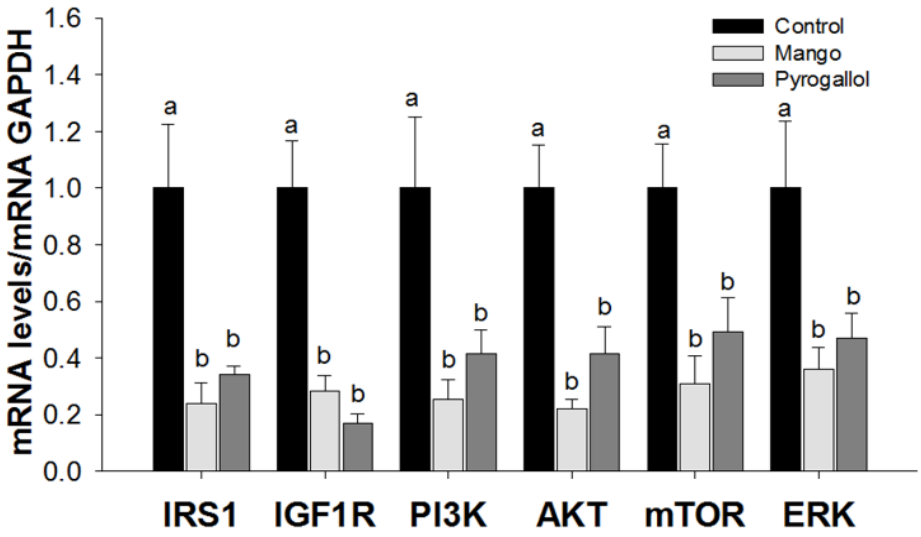

Figure 21. Effects of mango polyphenols (MG) and pyrogallol (PG) on mRNA levels in tumors following four week exposure to 0.8 mg GAE/day MG or 0.2 mg/day PG in athymic nude mice xenografted with MCF10DCIS.COM cells.

60% and 50% for MG and PG treatment, respectively. ERK mRNA levels were also reduced similarly with a 60% reduction with MG treatment and 50% reduction in PG treated animal tumors. Overall all mRNA investigated were reduced at least 50% with either treatment along the AKT/mTOR signaling axis. Because the mTOR signaling axis is so interconnected with numerous cellular functions, including proliferation, it is hypothesized that this genetic downregulation is a least in part responsible for the reduced tumor sizes in the treatment groups.

Following the mRNA analysis, tumor samples were run both with a multiplex bead assay and western blot to determine treatment effects on total and phosphorylated protein levels. The Luminex bead assay results focused on the effects of mTOR signaling and were conducted to allow a more direct comparison to the results we

previously published *in vitro* (Figures 22A and 22B). The MG and PG treatments altered both total and phosphorylated protein levels along the AKT & mTOR signaling axis. Total protein levels for IRS1 were reduced 25% for MG and 40% for PG while AKT and mTOR were significantly decreased approximately 40% by both treatments compared to the control. MG treatment was significantly reduced phosphorylated protein levels for the following: IR, IRS1, IGF-1R, and mTOR by 30%, 50%, 35%, and 45% respectively. PG treatment had a similar effect; however, it did not significantly reduce p-mTOR levels but did significantly decrease the downstream indicator of mTOR, p-P70-S6k by 80%. This downregulation could also be attributed to the downregulation seen in p-ERK levels, which were reduced more than 40%.

Western blots were conducted to examine further a potential mechanism of action that extended beyond the effects of mTOR signaling. AMPK was a novel pathway to investigate based on previous publications suggesting polyphenols as ROS-generating compounds [183-185]. Animal tumors that received the MG or PG treatment regimen had increased Sestrin2 and Becklin protein levels compared to the control. Sestrin2 is activated when elevated levels of ROS are present, and similarly, oxidative stress is also able to activate Becklin, which can ultimately trigger apoptosis or autophagy [181, 182]. Both treatments had no significant effects on total AMPK levels; however, p-AMPK was up-regulated in both treatment groups (Figures 23A and 23B). AMPK is a key regulator in maintaining cellular homeostasis, and its upregulation coincides with the down regulation of p-mTOR [190]. The reduction in p-mTOR in the MG treated animal tumors could also be potentially related to the elevated total ULK and p-ULK levels.

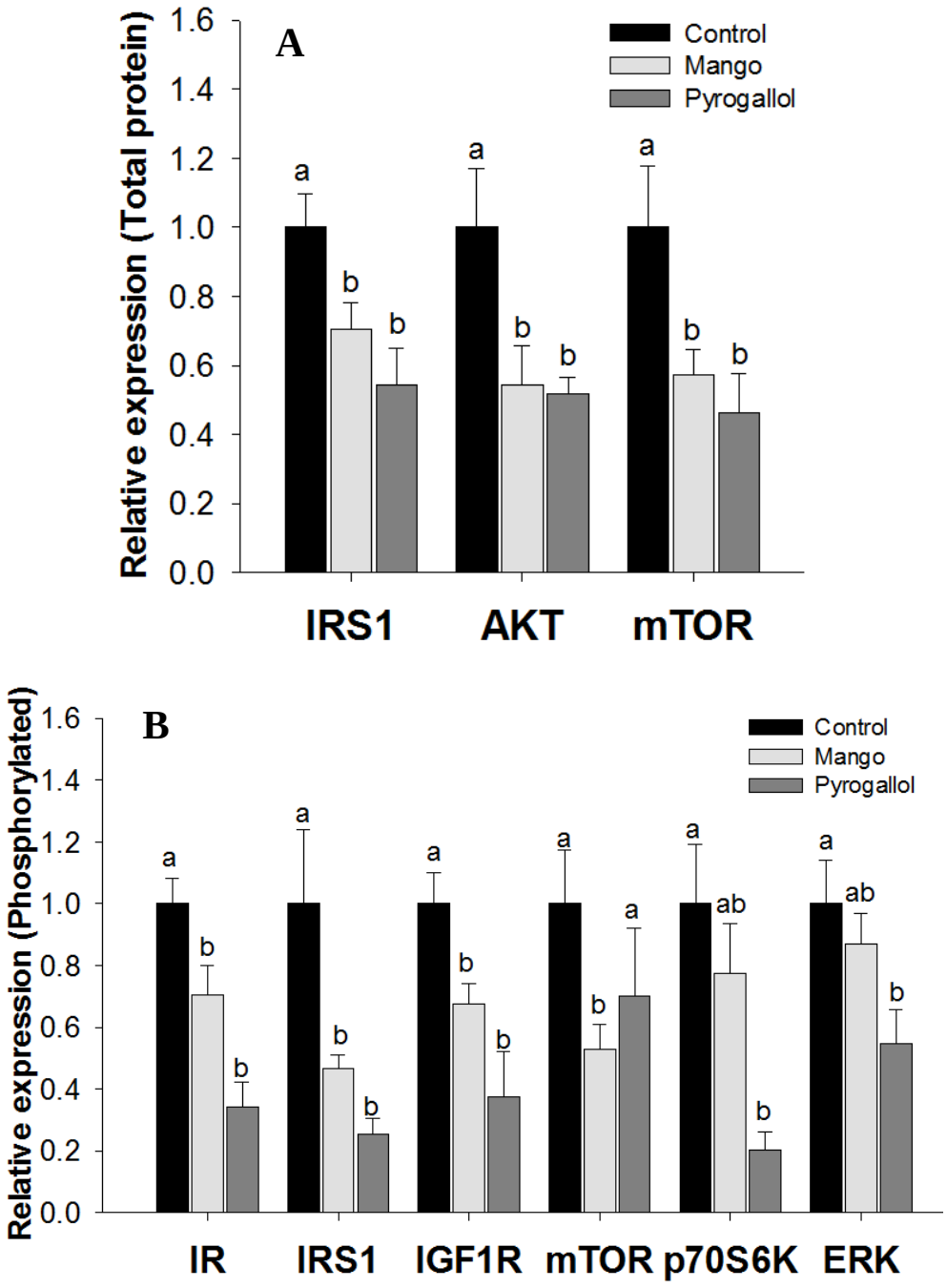

Figure 22. (A) total protein levels, and (B) phosphorylated protein levels in tumor following four week exposure to 0.8 mg GAE/day MG or 0.2 mg/day PG in athymic nude mice xenografted with MCF10DCIS.COM cells.

While the western blot data demonstrated a trend in upregulated AMPK and downregulated mTOR, further examination, possibly at higher doses, would be required to explore whether a causal relationship can be ascertained.

Effects of Mango Polyphenols (MG) and Pyrogallol (PG) in MCF10DCIS.COM Cells

Due to the results obtained in *vivo*, further investigation *in vitro* was warranted to elucidate a more definitive mechanism of action leading to the upregulation of AMPK signaling from the MG and PG treatments. A 5-20 mg/L dose range was selected based on previous work in a non-cancerous breast cell line that determined this concentration range had no effect on viability. In our previous works, both MG and PG decreased MCF10DCIS.com proliferation by 26 and 31% respectively compared to the control. At the highest level, proliferation was reduced as much as 61% for PG treated cells and 48% with MG treatment. Following this, both treatments were examined for ROS production because elevated ROS levels have shown to activate AMPK. MG and PG increased the generation of cytosolic ROS, however PG produced ROS to a greater degree and in a dose-dependent manner compared to MG. PG produced significantly more ROS, approximately 2.5 fold, at concentrations as low as 1 mg/L compared to control. At 10 mg/L MG and PG had ROS levels, 2.75 and 3.75 fold greater than controls, respectively. Doubling the treatment concentration to 20 mg/L MG did not increase ROS generation, but PG ROS generation continued to increase to 4.75 fold greater than controls (Figure 24A).

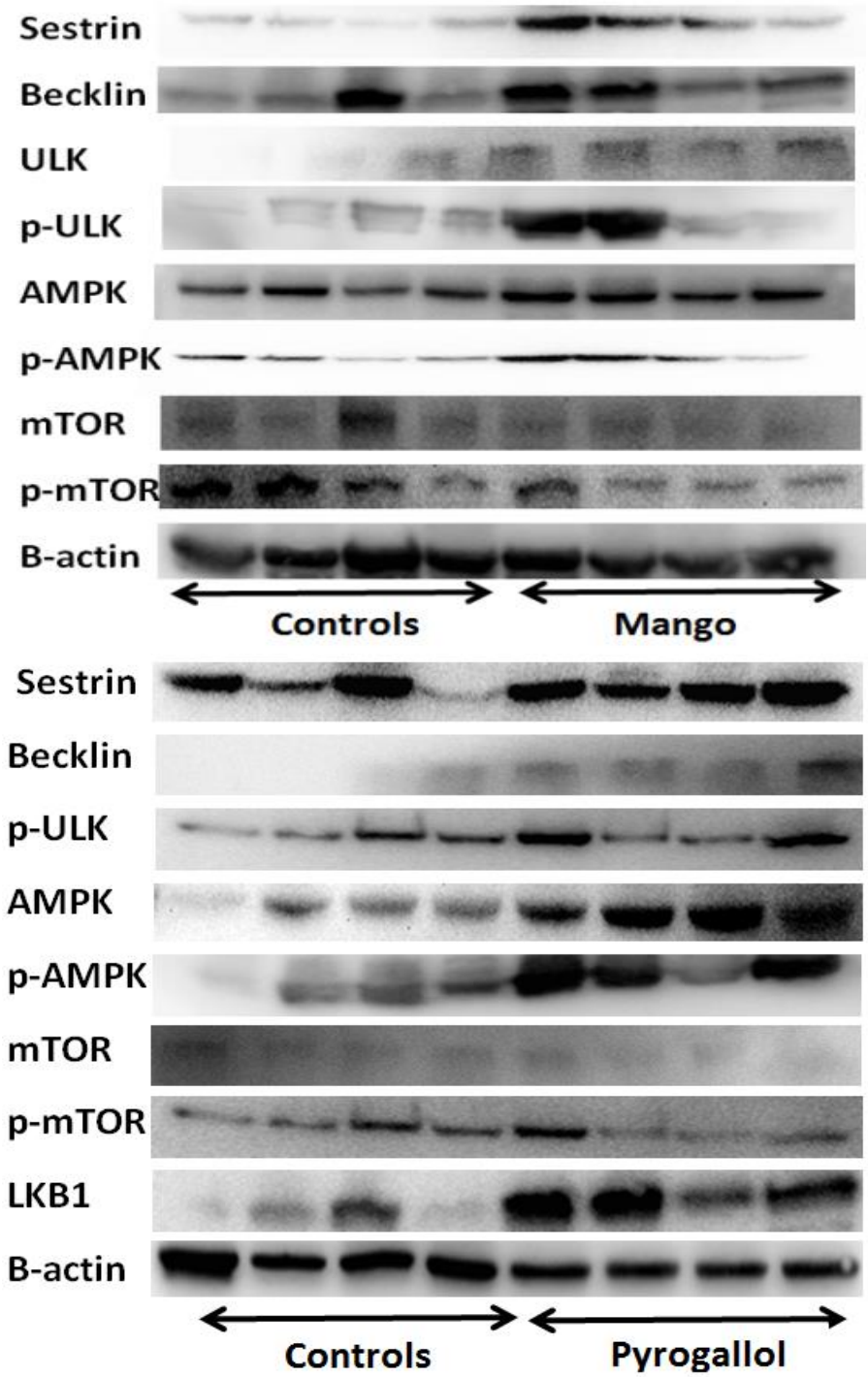

Figure 23. (A) Western blot total protein and phosphorylated protein levels in tumor following four week exposure to 0.8 mg GAE/day MG or (B) 0.2 mg/day PG in athymic nude mice xenografted with MCF10DCIS.COM cells.

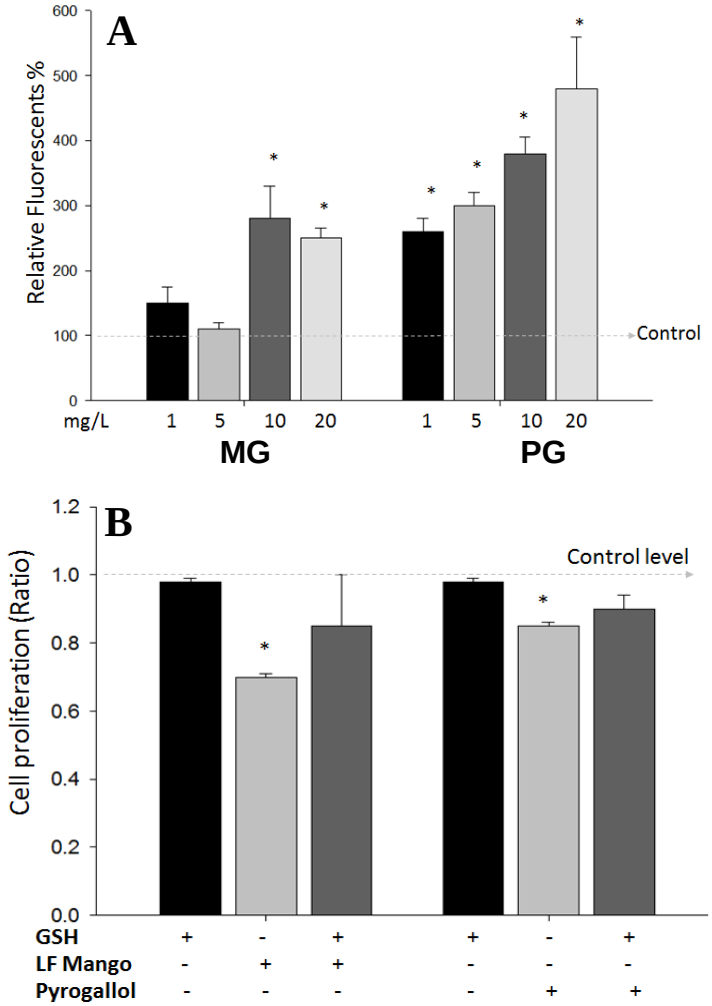

Figure 24. Effects of mango polyphenols (MG) and pyrogallol (PG) in MCF10DCIS.COM cells.
(A) MG and PG induced ROS generation following 24 h treatment in MCF10DCIS.COM cells. (B) GSH pretreatment (5mM) attenuated the anti-proliferative effects of both MG and PG at 10 mg/L for 24 h.

Based on these findings, it was hypothesized that ROS may be a contributing factor involved in the anti-proliferative mechanisms of MG and PG. To test this hypothesis, cells were pre-treated with 5 mM GSH to determine whether inclusion of an antioxidant would negate the anti-proliferative effects of either the MG or PG

treatments. Figure 24B shows either treatment at 10 mg/L significantly reduced MCF10DCIS.com proliferation compared to the control after 24 h. Cells that were co-treated with GSH for 4 h did not have a significant difference in cellular proliferation compared to the control. This data continued to support the hypothesis that the ROS producing nature of these compounds is contributing to their anti-proliferative capacities.

More experimentation was performed to determine the effects of these treatments on both cell cycle kinetics and mitochondrial function (Figures 25A & 25B). Both treatments significantly affected cell cycle kinetics where the lower concentrations mainly affected the G0/G1 phase, while the higher levels increase the amount of cells halted in the S phase. MG and PG at a concentration of 10 mg/L significantly decreased cells in the GO/G phase, 10% and 11% respectively, and did not alter the percentage of cells in the S phase compared to control cells. Both treatments significantly increased cells halted in the G2/M phase compared to the control, 3% in MG treated and 8% in PG treated. At the higher concentration of 20 mg/L, MG significantly decreased cells in the G0/G phase similarly to the lower concentration. MG and PG treatments significantly decreased cells in the G2/M phase by 9% and 8% respectively. The greatest effect seen from this higher treatment group was in the S phase with MG treatment increasing cells halted in this phase by 27% and PG treatment increased by 18%. The increase of cells in the S phase with MG treatment increasing cells halted in this phase by 27% and PG treatment increased by 18%. The increase of cells in the S phase could be a result of ROS-induced DNA damage. The only effect from the mitochondrial potential assay was

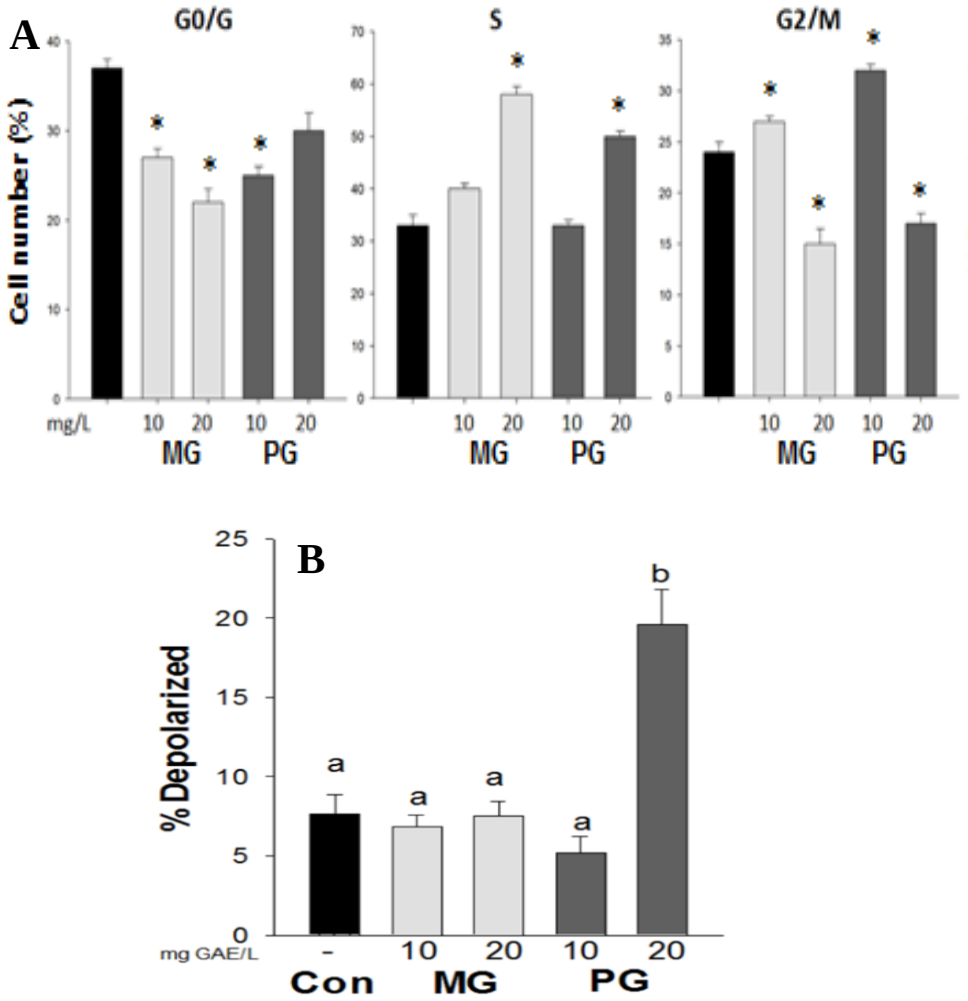

Figure 25. (A) MG and PG have varied effects on cell cycle progression determinant on concentration. (B) PG induced the mitochondrial membrane potential at 20 mg/L. All of the experiments were performed at least three times, and the results were expressed as the mean ± SEM (n=3). *p < 0.05 compared to the control (Dunnett's test).

a significant increase in dead depolarized cells at 20 mg/L PG after 24 h, increasing the number of depolarized cells to nearly 13% more than was found in control cells.

Effects of Pyrogallol (PG) on the AMPK in MCF10DCIS.COM Cells

Because of the elevated ROS production demonstrated by PG *in vitro*, molecular docking was performed against the allosteric modulatory site of the activated human α2β1γ1 AMPK in complex with A769662 (Protein Data Bank, Structure ID= 4CFF) [188]. Initially, protocols were validated by docking A769662 into the binding site of interest. The compound docked with the score of -32.61 reproducing the non-covalent binding pattern observed experimentally [191]. PG docked into the same pocket (β1 subunit) with the score of -14.43. The natural compound established hydrogen bond interactions with the side chain of Lys 31, the backbone carbonyl of Gly 19 and most importantly, with the phosphate group of pSer 108 (Figure 26A). Residues pSer 108 (carbohydrate binding module β1 subunit) and Lys 31 (β1 subunit) are part of a non-covalent network conserved in mammals [191]. This network is critical for the stability of the activated form of AMPK which subsequently protect residue Thr 172 (catalytic module α2 subunit) from dephosphorilation [192]. In addition, mutation of Ser 108 into an alanine significantly decreased binding and activation of A769662 and 991 [192]. The low molecular weight of this compound along with the ability for it to cross phospholipid membrane made it plausible that the chemical structure of PG may also be a factor in activating AMPK and supply structural integrity to the AMMPK complex.

Further investigation was conducted examining if PG could increase AMPK levels following knockdown with siRNA (Figures 26B &C). MCF10DCIS.com cells transfected with siRNA had reduced mRNA, total and phosphorylated protein levels, compared to the control. In the case of mRNA, PG treatment was able to significantly

increase AMPK mRNA levels by nearly 40%, while MG treatment had no effect on mRNA levels. Western blot analysis revealed that this pattern continued to the total and phosphorylated protein levels. Conclusively, PG treatment upregulated AMPK levels following its knockdown in MCF10DCIS.com cells.

Discussion

In this study, we demonstrated the anti-cancer effects of mango polyphenols and pyrogallol in a DCIS xenograft model. Mango polyphenols have been previously reported to have cytotoxic activities in cancer cells, however, DCIS- specific breast cancer has not been previously investigated [90]. Previously, we have reported the ability of mango polyphenols to downregulate the mTOR pathway *in vivo* by targeting upsteam regulators such as PI3K and AKT [186]. The mechanism proposed here, continues to elucidate the activities of these compounds on mTOR signaling both *in vitro* and *in vivo* with particular emphasis on the AMPK-mTOR-axis. The chromatogram from the MG extract contained four major polyphenols and residual gallotannis. It was reported that gallotanins can be broken down firstly though tannase enzymes produced by GI bacteria such as Lactobacillus Plantarum, separating the gallic acid moieties along the meta-depside bonds [193, 194]. The digestion leads to elevated free gallic acid levels, and decarboxylase enzymes produce pyrogallol [195].

In the athymic xenograft mouse experiment, tumor sizes were significantly reduced in those animals that received MG or PG treatment compared to the control. The reduction in volume was hypothesized to be a result of mTOR downregulation due to the fact that mTOR is a key regulator of cellular proliferation and has previously been

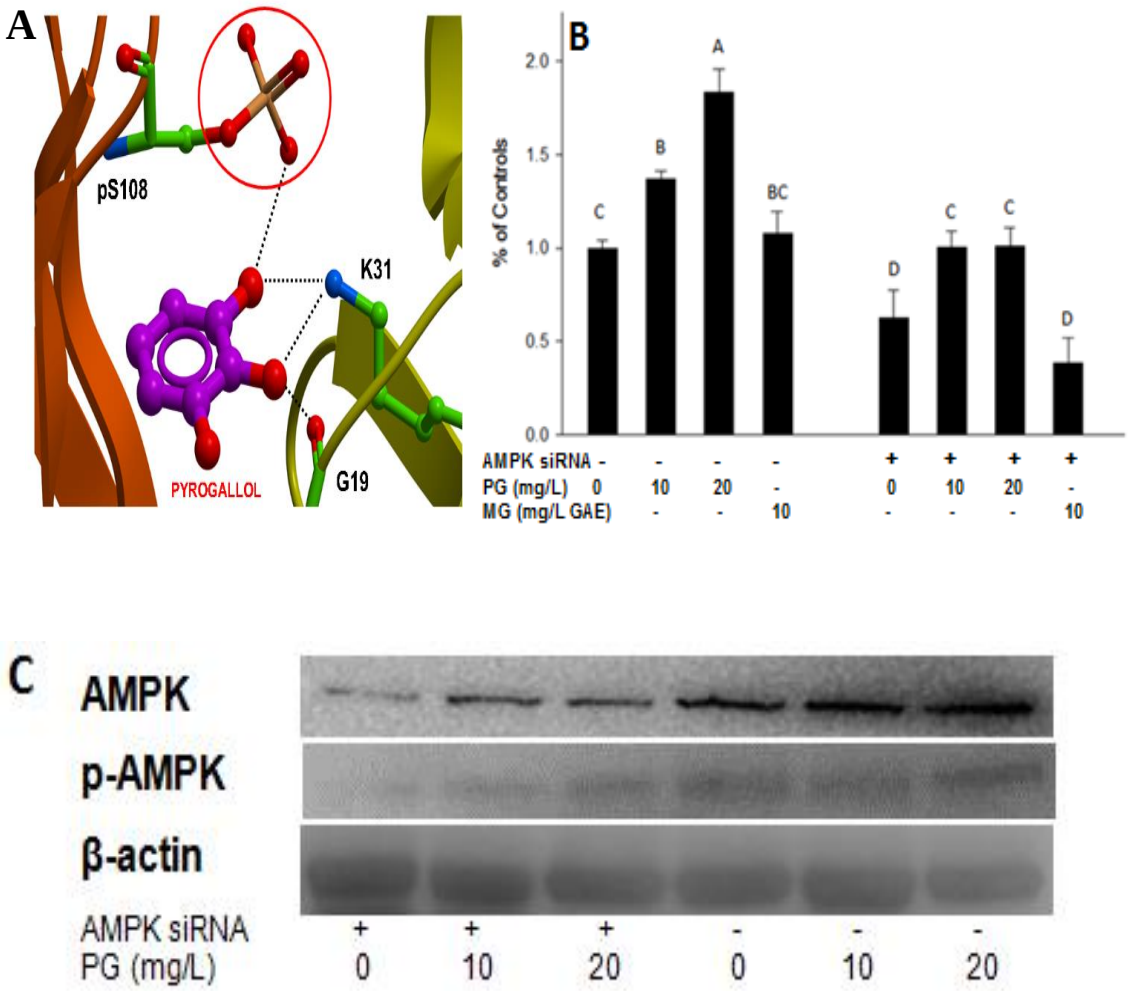

Figure 26. Effects of pyrogallol (PG) on the AMPK in MCF10DCIS.COM cells. (A) Docking of PG into the allosteric modulatory site of human α2β1γ1 AMPK holo complex (Protein Data Bank, Structure ID= 4CFF). The secondary structure is displayed as ribbon colored in yellow (β1 subunit) and orange (Carbohydrate-binding module β1 subunit). Residues are displayed as sticks and colored by atom type with carbon atoms in green. The ligand is displayed as sticks and colored by atom type with carbon atoms in magenta. Hydrogen bond interactions are displayed as black dashed lines (Molsoft ICM). (B) mRNA expression levels of AMPK in MCF10DCIS.COM cells with and without siRNA followed by exposure to either PG or MG for 24 h. The results were expressed as the mean ± SEM (n=3). The different letters indicate significance at $p<0.05$ (Tukey's test). (C) Western blot results following siRNA knockdown of AMPK in MCF10DCIS.COM cells followed by exposure to PG for 24 h.

reported to be affected by polyphenol exposure [96, 154]. Animals were treated with concentrations that were approximately 1-2% and less than 1% of the LD50 levels for pyrogallol and gallic acid, respectively [77, 196], and are equivalent to a high dietary intake of mango or dietary supplement with pyrogallol in humans [151]. Animals showed no adverse effects as determined by daily observation, body weight gain, and liver weights at these doses. In this study, MG decreased CRP and TNF-α in tumor tissue while IL-1β levels were reduced in tumor tissue in animals exposed to PG. These findings support previous reports demonstrating that anti-inflammatory activities of gallic acid derivatives where at least some of the beneficial effects of gallic acid and related polyphenols consumption contributed to their ability to reduce inflammation [131, 158, 197].

In this study, genes investigated along the mTOR pathway were downregulated by treatment with MG or PG by at least 40%. The downregulation at mRNA-level of these critical components carried over to total and phosphorylated protein levels. Multiplex-bead analysis demonstrated a downregulation of both total and phosphorylated mTOR protein levels by MG and a downregulation of total mTOR and the downstream indicator of activity p70S6K by PG. Both treatments caused increased p-AMPK levels which could be partially attributed to elevated Sestrin2 or Beclin protein levels [175, 190, 198]. Sestrin2 can activate AMPK downstream of tumor suppressor P53 leading to the inhibition of mTOR [199]. Beclin activation leads to the recruitment of vacuolar sorting protein 34 and phosphoinositide 3-kinase, forming a complex which is the precursor to autophagosome formation [200].

To elucidate the potential mechanism of AMPK activation, further *in vitro* studies were performed. PG and MG increased ROS production, with PG causing elevated ROS levels at 1 mg/L. Significant increase of ROS with MG was achieved at 10 mg/L. Cellular proliferation of MCF10DCIS.com cells was decreased following 24 h exposure to either treatment but was reversed when cells were co-treated with glutathione. The ability of this antioxidant to attenuate the effects of the treatments provides additional support to the hypothesis that the generation of ROS caused by PG and MG is at least in part responsible for the anti-proliferative activities observed in DCIS breast cancer cells. Further investigation revealed that elevated levels of PG and MG increased cells halted in S phase which is frequently attributed to DNA strand breaks [201]. These breaks could result from oxidative base damage caused by either treatment [202]. PG at the same concentration also caused depolarization of the mitochondria, which is also associated with oxidative damage [203].

Determining a finite mechanism for a complex mixture of bioactive components is difficult due to multiple chemical structures involved, and for this reason, PG was of primary focus for the remaining mechanistic evaluation. An *in silico* modeling profile determined that pyrogallol has binding-specificity to the allosteric site of AMPK [204]. Similarly, AMP can activate AMPK in very low concentrations; as little as 2 μM caused half maximal activation of AMPK in a heart model [205]. When MCF10DCIS.com cells were transfected with AMPK siRNA, PG treatment was able to rescue both the mRNA levels along with total and phosphorylated protein levels. While these findings suggest that PG is acting through AMPK, they also demonstrate that PG increases constitutive

levels of AMPK through another mechanism (other than binding to the allosteric site) in addition to increasing the activation of AMPK.

In summary, mango polyphenols and their major microbial metabolite pyrogallol may inhibit proliferation of DCIS breast cancer by upregulating of AMPK and downregulating of the AKT/mTOR pathway. To our knowledge, this was the first study that examined the activities of mango polyphenols and its GI derived metabolite pyrogallol in DCIS breast cancer progression. Results from this study support previously published work showing a downregulation of mTOR function from polyphenol exposure [206]. It seems that the impact on the AMPK-mTOR axis was in part induced by the generation of ROS and potentially by direct binding of PG to the allosteric binding site of AMPK causing its activation. Further investigations should be performed within physiological and pharmacological concentration levels to determine whether PG might advance cancer cells into autophagy. Because, basal levels of ROS are higher in cancer cells, allows for this prevention to be selective for cancer cells over non-cancerous cells. PG can cause increased ROS in cancer cells, disrupting the cellular balance and selectively advance these cells towards autophagy, while leaving non-cancerous cells unaffected.

CHAPTER IV

ORAL EFFECTS OF PYROGALLOL IN 4 WEEK EXPOSURE IN ATHYMIC MICE WITH MCF10DCIS.COM XENOGRAPH TUMORS AND HDAC1 INHIBITION *IN VITRO*

Introduction

Pyrogallol is a metabolite formed in the GI tract following mango consumption. Certain bacteria strains, such as lactobacillus platarum, bacillus subtilis, and enterococcus faecalis, within intestine are able to produce tannase enzymes [207-209]. These enzymes are produced as a defense mechanism in order to detoxify tannins, an anti-nutritional factor that binds proteins and forms indigestible complexes, which is detrimental to bacteria and other microbes [210] . The enzymatic conversion of tannins to pyrogallol is summarized in Figure 27 below.

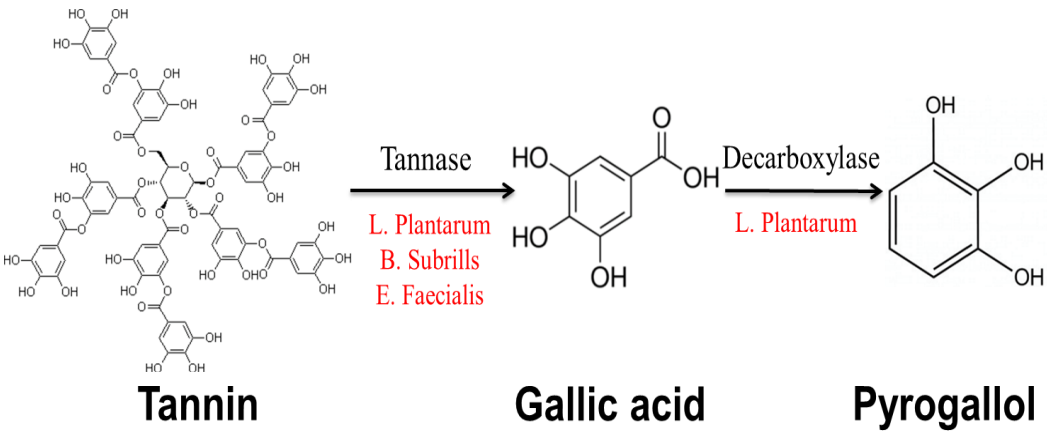

Figure 27. Conversion of tannins into pyrogallol following metabolism by L. platarum in colon.

Previous research revealed pyrogallol to have an anti-proliferative effect on DCIS proliferation in both *in vitro* and *in vivo* cancer models. This proliferative effect was attributed to dysregulation of the mTOR pathway and activation of AMPK; however, it is plausible that these compounds cause epigenetic modifications as well.

New investigations in polyphenol research have been centered on their ability to cause epigenetic modifications [211]. Epigenetics are alterations in gene expression which are not caused by changes to the DNA nucleotide sequences [212]. These modifications can occur through multiple mechanisms including DNA methylation, histone acetylation, and RNA interference [213]. Many disease conditions have been linked with epigenetic abnormalities including cancer, inflammation, and diabetes [214]. Many publications have shown the epigenome to be a better target for therapeutics than genetic disorders because they are more readily reversible [215]. Two main groups of inhibitors have emerged as promising cancer therapeutics and include histone deacethylase (HDAC) and DNA methyl transferase inhibitors (DNMT) [216].

Chromatin remodeling is a key characteristic in epigenetic regulation altering the chromosome to more open or closed conformation changes the accessibility to certain genes [217]. Histone acetyltransferases are a group of enzymes that are responsible for transferring acetyl groups to histones, more specifically their amino-terminal lysine residues, changing the chromatin architecture to a more open conformation, allowing space for regulator proteins to access DNA [218]. HDACs have the opposite effect and cause transcriptional repression by removing acetyl groups and decreasing the accessibility to DNA. HDAC1 is overexpressed in multiple cancer types including

breast, gastric, colon, and prostate [219]. The knockdown of HDAC1 *in vitro* has shown retardation in tumor growth and an increase in apoptosis [220]. Targeting HDACs with inhibitors relieves transcription repression of genes critical for apoptosis and proliferative regulation [221].

Some of the concerns with current synthetic epigenetic drugs are the lack of specificity of these compounds, potentially leading to increased cancer progression, and their high levels of toxicity [222]. Polyphenol and polyphenol metabolites have been shown to be HDAC inhibitors in both precancerous and cancerous cells [223].

The research performed here had 3 main objectives: 1) verify the reduction of MCF10DCIS.COM tumor volumes with two different pyrogallol concentrations, 2) determine if AMPK and mTOR genes are being affected by the pyrogallol treatment, and 3) evaluate if pyrogallol is able to inhibit HDAC1.

Methods

Cell Culture

Human ductal carcinoma *in situ* breast cancer cell line MCF10DCIS.COM was obtained from Asterand, Inc. (Detroit, MI). Cells were cultured in DMEM-F12 media as suggested by the vendor (Invitrogen, Carlsbad, CA) that was supplemented with 10% horse serum (Atlanta Biologicals, Lawrenceville, GA).

Xenograft Study

All animal procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University prior to their initiation. Female athymic BALB/c

nude mice at approximately 3 weeks of age were ordered from Harlan Teklad (Houston, TX). MCF10DCIS.COM cells ($5X10^4$ cells) were harvested and mixed with 50µL of Matrigel (BD Bioscience, San Jose, CA) and stored on ice until implantation. Mice were implanted subcutaneously bilaterally into the mammary pad. Tumors were allowed to grow 1 week prior to the initiation of dosing. Animals were orally gavaged 100 µl daily with either ML at 0.2 mg/L or 0.8 mg/L of PG. Control animals were dosed with a vehicle of citric acid buffer at a molarity of 0.2 and a pH of 3.5. Tumors size and body weights were taken weekly and at time of necropsy. Tumors and organs of interest were either saved via fixation in 10% formaldehyde or flash-frozen in liquid nitrogen.

Analysis of mRNA Expression by Real-time PCR

Total RNA was isolated from tumor samples using mirVana miRNA Isolation kit (Applied Biosystems, Foster City, CA). cDNA was synthesized from the isolated RNA with the use of a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY). Primers were designed using the Primer Express Software Version 3. (Applied Biosystems, Foster City, CA) and purchased from Integrated DNA Technologies Inc. (Coralville, IA). qRT-PCR was conducted on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA).

HDAC1 Inhibition Assay

HDAC1 enzyme activity was performed with the HDAC Assay Kit (Cayman Chemical Company, Ann Arbor, MI). MCF7DCIS.COM cells were seeded at a density of 5,000 per well in a 96 well black, clear bottomed plate and allowed to attach

overnight in 100 µL of media. Cells were treated for 24 h with Trichostatin A at 2 µM (PC), butyrate at 100 µM, gallic acid at 10 and 20 mg/L, and pyrogallol at 10 and 20 mg/L. The NC group was treated with at the same concentration vehicle as the treatment groups. Following incubation, treatment media was aspirated and 200 µL of assay buffer and 10 µL of diluted HDAC substrate was added (kit constituents). The plate was incubated for 3 h at 37°C. Following incubation cells were lysed in 50 µL lysis buffer and developer mixture (kit constituent) and incubated for an additional 15 min at 37°C. Fluorescence was measure at an excitation wavelength of 340-360 nm and an emission wavelength of 440-465nm.

Results

Pyrogallol Effects on Tumor Volumes and Gene Expression on mTOR and AMPK Signaling

Following the 4 weeks of PG exposure both the low and high treatment groups had significantly reduced tumor volumes compared to controls from week 1 through necropsy (Figure 28). For weeks 1-3 the tumor volumes in PG treated animals were approximately half that of the control treated animals. Interestingly, the tumor volumes between the 0.2 mg/day treatment and the 0.8 mg/day PG did not differ through the duration of the study. At the conclusion of the study all groups had similar body weights. No significant differences were observed for liver weights. Animals throughout the 4 week dosing duration never demonstrated any adverse effects to either

dose concentration. The tumor growth and final volumes were very similar to the growth pattern and tumor volumes previously reported.

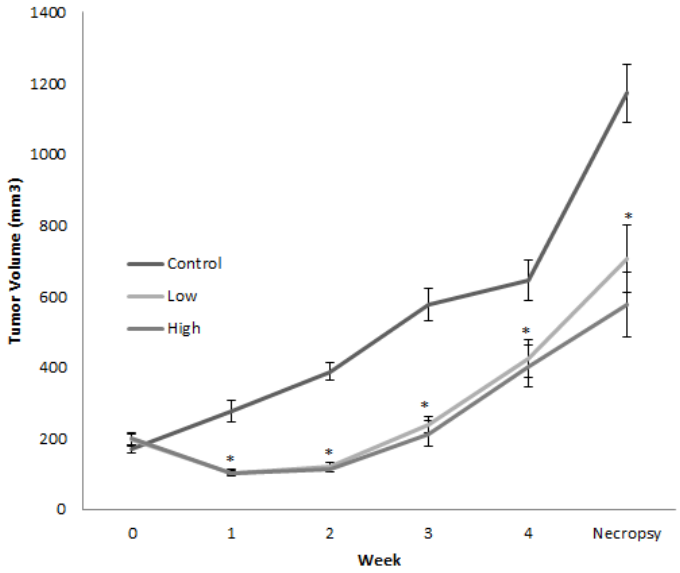

Figure 28. Effects of pyrogallol (PG) in athymic nude mice xenografted with MCF10DCIS.COM cells.
Tumor volumes over four weeks were decreased by PG 0.2 mg/day and 0.8mg/day in athymic mice compared to vehicle treated controls (n=10 per group). *p < 0.05 compared to the control (Dunnett's test).

any adverse effects to either dose concentration. The tumor growth and final volumes were very similar to the growth pattern and tumor volumes previously reported.

Gene Expression Profiles for mTOR and AMPK Pathways in Xenograph Tumor Tissue

Many genes along the mTOR and AMPK signaling axis were altered in tumor tissue in both the low and high PG treatment groups compared to (Figure 29A-B). In respect to mTOR specific genes, AKT, PI3K, IGF1-R, and mTOR were all downregulated 60%, 52%, 68%, and 60%, respectively, for the low dose treatment and

79%, 47%, 61%, and 82%, respectively, for high dose treatment. AMPK genes had an inverse response and were upregulated in the PG treatment groups. An activator of AMPK, Sestrin 2 was nearly 3 and 3.5 fold higher in the 0.2 mg/day and 0.8 mg/day treatments, respectively. The LKβ1 levels were 1.6 fold higher than control in the high treatment group. Beclin was increased 1.2 and 1.7 fold for the low and high dose treatments respectively. AMPKα, AMPKβ, and the autophagy indicator LC3 were increased 1.6, 2.0, and 3.2 fold, respectively, in the low dose treatment and 1.8, 3.5, and 4.8 fold, respectively, in the high dose treatment.

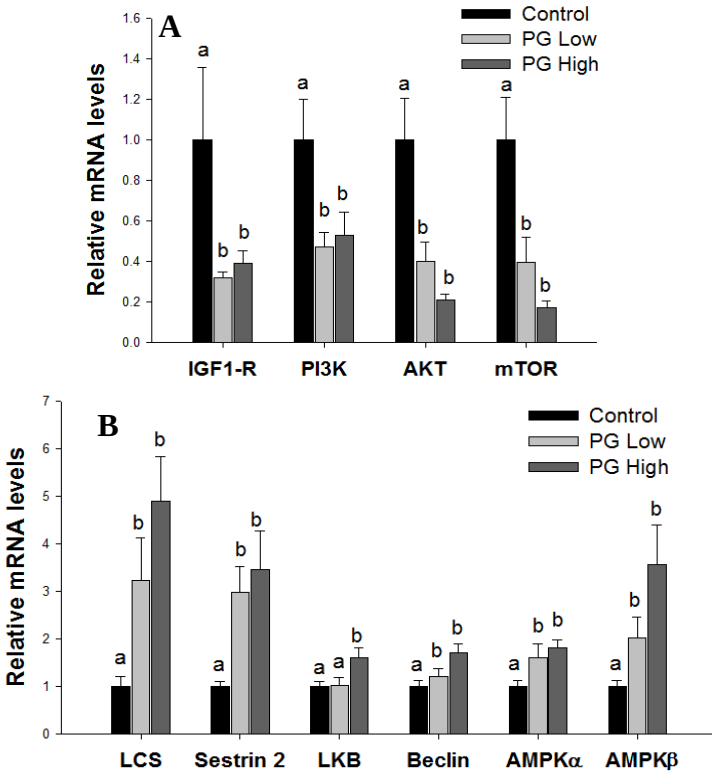

Figure 29. Effects of pyrogallol (PG) on mRNA levels in tumors following four week oral exposure to 0.2 mg GAE/day (low) or 0.8 mg/day (high) PG in athymic nude mice xenografted with MCF10DCIS.COM cells.
(A) mTOR signaling. (B) AMPK signaling.

HDAC1 Inhibition

In vitro results of HDAC1 inhibition (Figure 30) indicate the significant inhibitory effect of pyrogallol at 20 mg/L during a 24 h exposure window in the DCIS breast cancer line. The inhibition exerted by pyrogallol was about half as effective as the positive control, trichostatin A. Butyrate has been reported to be an HDAC1 inhibitor [206], and the results observed for pyrogallol had a similar effect in inhibition. Gallic acid had no effect on HDAC1 inhibition at both concentrations investigated.

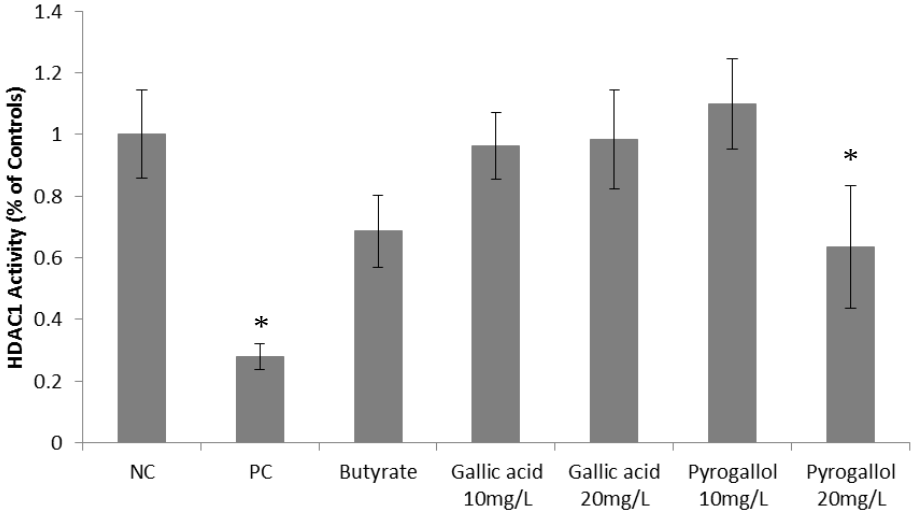

Figure 30. Effects of gallic acid and pyrogallol on HDAC1 activity compared to known inhibitors, trichostatin A (PC) and butyrate. NC is the non-treated controls.

Discussion

Oral treatment with pyrogallol in athymic mice for 4 weeks that had MCF10DCIS.com cell lines xenographed into their mammary pads produced a similar retardation in growth as our lab has previously reported. Animals that received either treatment had tumor volumes that were half the volume of the control animals. The

results here were not surprising, however, does add support for the anti-proliferative nature of the mango derived metabolite pyrogallol. Treated tumors continue to grow throughout the course of the study, showing acceleration in growth after week three. This increased rate of proliferation could be attributed to the cancer developing resistance to the treatment, which is a common occurrence in cancer development [224]. A follow up study that compares genetic and protein expression at different time intervals, such as comparing expression levels at week 1 and week 5, may supply some insight to how this cancer is seemingly able to develop resistance to the pyrogallol treatment. Previous reports have shown that polyphenols are able to induce drug metabolizing enzymes; therefore, it is plausible that continuous exposure to pyrogallol is resulting in the compound inducing its own metabolism [225].

The gene expression results also coincided with previous findings and support the hypothesis that mTOR is being downregulated [226]. All mTOR genes investigated, IGF1-R, PI3K, AKT, and mTOR itself were all down regulated by more than 40% compared to controls. A majority of the AMPK genes were upregulated by both treatments, with the exception of LKB1 in the low PG treatment group. AMPK signaling can inhibit mTOR signaling when activated, however, mTOR can also inhibit AMPK depending on cellular conditions. In the case of high nutrient levels, mTOR phosphorylates Ulk1 at Ser 757, which impedes Ulk1's ability to bind with AMPK. In low nutrient conditions AMPK activates ULK1 by phosphorylating Ser317 and Ser777 in turn promoting autophagy. AMPK is also able to directly inhibit mTORC1 through interacting directly with TSC2 and Raptor.

Pyrogallol also had an effect on HDAC1. HDAC1 is overly expressed in multiple cancer types, and leads to the repression of tumor suppressor genes. A natural compound that leads to HDAC1 inhibition would be advantageous because of their limited side effects compared to using synthetic small molecules. For the first time, a mango derived metabolite has been shown to inhibit HDAC1 in breast cancer. Interestingly, the precursor compound, gallic acid, did not have an effect. These results provide further justification that the gut microbiota can promote or hinder disease advancement based upon the population and compounds produced.

CHAPTER V

BIOAVAILABILITY OF PYROGALLOL AND ITS MAJOR METABOLITES IN PLASMA, ANIMALS TUMORS, AND INTRACELLULARLY

Introduction

Polyphenols are plentiful micronutrient present in the human diet that undergo the same fate similar to drugs once consumed, which includes absorption, distribution, metabolism and excretion (ADME) [227]. The body acknowledges polyphenols as xenobiotics, and therefore wants to eliminate them from the system through either phase 1 or phase 2 metabolism or a combination of both [159, 228]. A majority of these metabolizing enzymes can be found in the liver, small intestine, and kidney and have a primary responsibility for detoxifying harmful foreign compounds [229]. A summary of this conversion is illustrated in Figure 31 below.

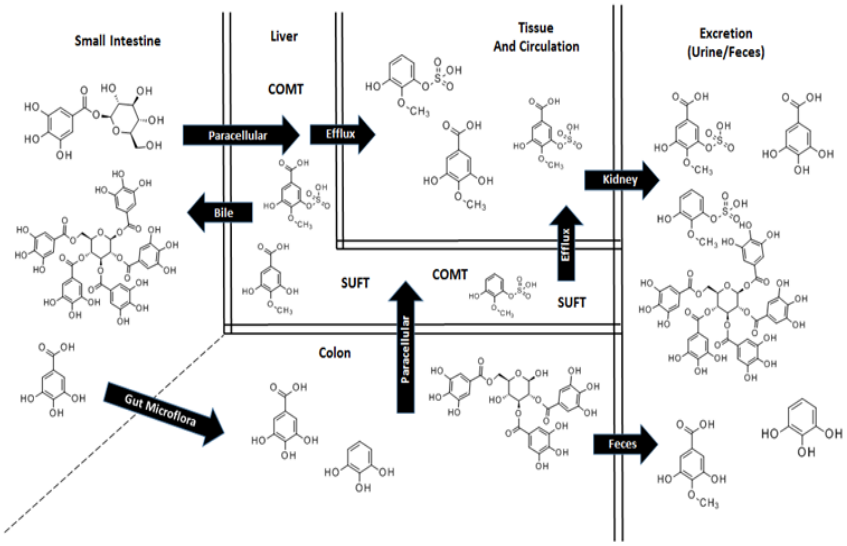

Figure 31. ADME diagram following mango consumption.

Initially, polyphenols must separate from the food matrix in order for metabolism and distribution to occur. Many factors contribute the bioaccessabilty of polyphenols and include the presence of glycosides and acylation's, solubility, and interconnectivity with the food matrix [230]. Tannins included a large group of polyphenols which are considered to have poor bioavailability because of their ability to polymerize and their protein binding affinity [231]. Another factor that limits polyphenol bioavailability is their hydrophilicity, making it difficult for them to cross the phospholipid membrane and enter cells [232]. Polyphenols also commonly undergo glycolsylation which increases their affinity for water, and studies have shown that flavonoids that undergo deglycosylation, primarily through the enzyme lactase phoridzin hydrolase (LPH), are more readily absorbed [233]. LPH resided on the luminal service of enterocytes, with cleaves aglycone and allows for flavonoids to be absorbed supposedly through passive diffusion [234]. Lower molecular weight polyphenols have been shown to be transported into cell cells via different transporters, such as monocarboxylic acid transporter and other active transporters that are responsible for the uptake of lactate, pyruvate, and ketone bodies [235, 236].

The goal of this chapter was to examine the bioavailability of pyrogallol both *in vivo* and *in vitro* in MCF710DCIS.COM breast cancer cells. Tumors collected from xenographed mice treated with pyrogallol for 4 weeks will be analyzed for pyrogallol or pyrogallol derived metabolites. Plasma collected at time of necropsy was also analyzed for these compounds. *In vitro* the same cell line will be used and treated with pyrogallol

to determine if this compound is able to enter a cell and what metabolism occurs at the cellular level. It is hypothesized that pyrogallol will not bio-accumulate in the tumor tissue due to their polarity, however, the parent compound or metabolites are expected to be in the plasma. Due to the low molecular weight, 126.1 g/mol, of pyrogallol it is expected in to be found intracellularly but is expected to be metabolized quickly.

Methods

Cell Culture

Human ductal carcinoma *in situ* breast cancer cell line MCF10DCIS.COM was obtained from Asterand, Inc. (Detroit, MI). Cells were cultured in DMEM-F12 media as suggested by the vendor (Invitrogen, Carlsbad, CA) that was supplemented with 10% horse serum (Atlanta Biologicals, Lawrenceville, GA).

Xenograft Study

All animal procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University prior to their initiation. Female athymic BALB/c nude mice at approximately 3 weeks of age were ordered from Harlan Teklad (Houston, TX). MCF10DCIS.COM cells ($5X10^4$ cells) were harvested and mixed with 50 µL of Matrigel (BD Bioscience, San Jose, CA) and stored on ice until implantation. Mice were implanted subcutaneously bilaterally into the mammary pad. Tumors were allowed to grow 1 week prior to the initiation of dosing. Animals were orally gavaged 100 µl daily with either PG at 0.2 mg/L or 0.8 mg/L. Control animals were dosed with a vehicle of citric acid buffer at a molarity of 0.2 and a pH of 3.5. Tumors size and body weights

were taken weekly and at time of necropsy. Tumors and organs of interest were either saved via fixation in 10% formaldehyde or flash-frozen in liquid nitrogen.

Bioavailability in Tumor Tissue, Plasma, and In Vitro

For analysis of pyrogallol metabolites in plasma, 500 µl of mice plasma was acidified with 25 µl of 88% formic acid. 75 µl of 10% SDS was then added followed by 325 µl of 0.1 % formic acid MeOH. After proteins were denatured, 50 µl of 3 M KCl was added to remove any residual SDS. Samples were sonicated for 10 min and centrifuged at 12,000 RPM for 5 min at 4^{o}C. Supernatants were filtered through a 0.45 um filter prior to LC-MS analysis.

Methodology for analysis of pyrogallol metabolites in tumors was based off of method by Margalef et al [202]. Briefly, mice tumor tissue was homogenized and incubated with 50 µl of 10% SDS. This was followed with two independent extractions of homogenized tissue with 400 µl of 0.1% formic acid acetonitrile. Samples were centrifuged at 12,000 RPM for 5 min at 4^{o}C, and supernatants were evaporated under vacuum using a ThermoFisher Speed Vac. Dried samples were brought up in 200 µl of 1:1 0.1% MeOH:H20, and analyzed on LC-MS.

MCF10DCIS.COM cells were grown in 10 cm cell culture dishes and allowed to grow to 100% confluency. Media was aspirated, and treatment media was added to each dish. Treatment duration was 1 or 2 h. At the end of treatment, media was aspirated out of each dish and each dish was wash twice with PBS to ensure removal on any extracellular polyphenols. Cells were scraped from their dishes with 2mL acidified

methanol. Cells were transferred to a 1.7 mL microcentrifuge tube and lysed with a syringe. Samples were stored at -80°C until analysis could be performed.

Results

Pyrogallol Characterization and Detection in Plasma and Tumor Tissue

Following the 4 week exposure window of the PG treatment both tumor tissue and plasma were analyzed via HPLC-MS in order to determine if PG and its metabolites were absorbed and metabolized. No PG or PG metabolites were found to bioaccumulate in tumor tissues. In contrast, pooled plasma extracts show the presence of the PG derived metabolite pyrogalloyl-O-sulfate with a peak at 205.2 m/z and shown in Figures 32A-B. This result demonstrates pyrogallol was absorbed and metabolized.

Pyrogallol Characterization and Detection in MCF10DCIS.COM Cells

Breast cancer cells that were incubated in a treatment media of pyrogallol at 20 and 50 mg/L had both pyrogallol and the metabolite methyl-pyrogallol-sulfate present intracellularly after 1 hour. When cells were treated at 20 mg/L approximately 0.36% of pyrogallol was absorbed and 0.74% methyl-pyrogallol-sulfate was either absorbed or formed intracellularly. When the dose was increased to 50 mg/L 0.716% of the pyrogallol was absorbed and 0.2% of the methyl-pyrogallol-sulfate was present. This data set is displayed in Figure 34.

Discussion

Pyrogalloyl-O-sulfate was found in mouse plasma in animals that received orally administer pyrogallol for 4 weeks. No parent compound was found in either plasma or in the tumor tissue itself. It can be concluded that oral administration of pyrogallol leads to rapid metabolism, presumably in the liver, following exposure. The sulfation of polyphenols has been noted with other phenolic compounds such as gallic acid, catechol,

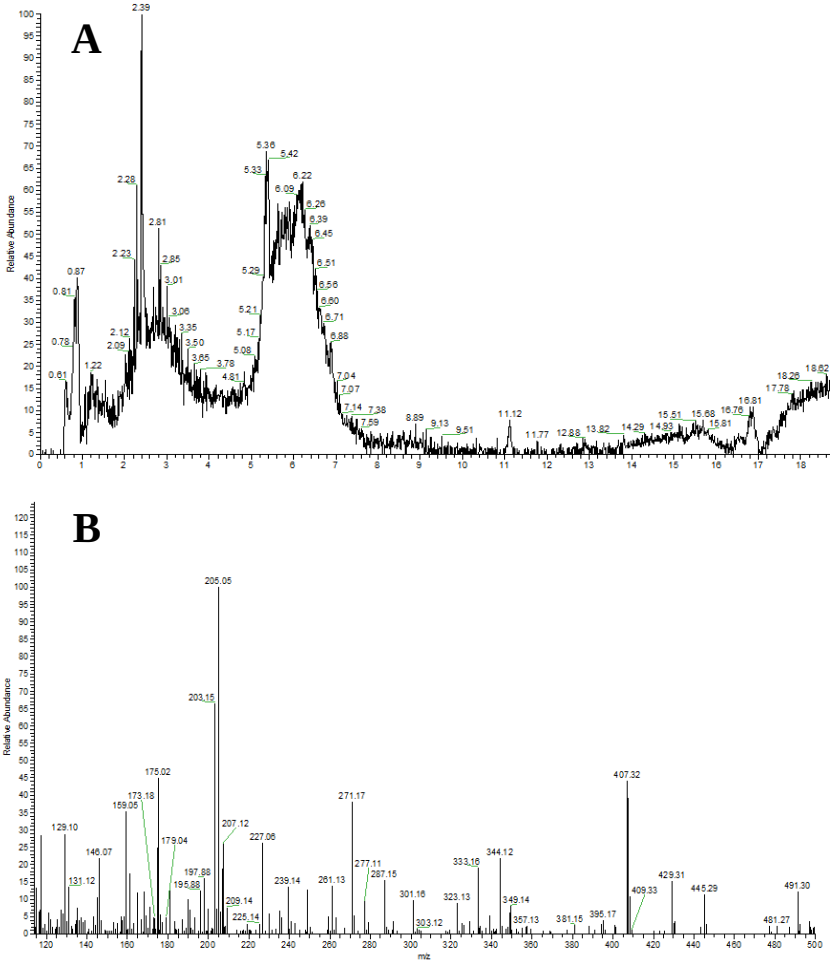

Figure 32. A. Extracted Ion Chromatogram of pyrogallol-*O*-sulfate at 205 m/z in mouse plasma following 0.8 mg dose of pyrogallol B. Mass spectrum for pyrogallol sulfate present in mouse plasma.

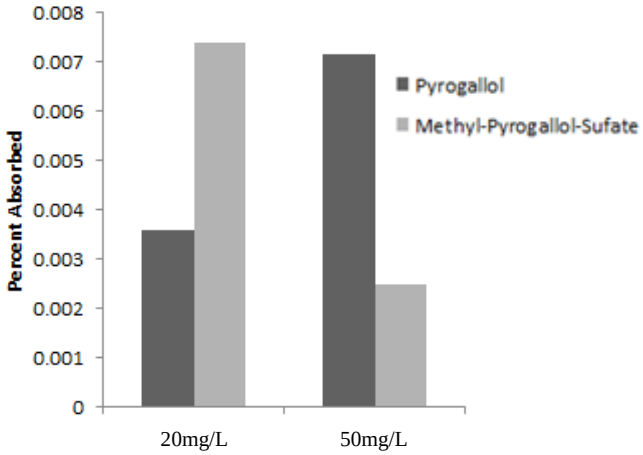

Figure 33. Percentage of pyrogallol and methyl-pyrogallol-sulfate intracellularly absorbed in MCF10DCIS.COM cells following 1 hour of exposure to 20 and 50 mg/L pyrogallol.

and resorcinol and is no surprise that pyrogallol undergoes a similar metabolism [85, 237, 238]. Sulfotransferases (SULTs) are a group of enzymes found in a variety of tissues and are responsible for the sulfonation of xenobiotics and endogenous compounds [239]. 3'phosphoadenosine 5' phosphosulfate (PAPS) is responsible for donating the $SO3^-$ group being transferred by SULTs [240]. The addition of a sulfonate moiety normally increases a compounds polarity making them more water soluble and decreases their reactivity. This is not true for all compounds, and in some instances the addition of this functional group forms reactive electrophiles [241241]. Figure 35 summarizes the sulfation and methylation of pyrogallol.

The lack of pyrogallol, or pyrogallol metabolites in tumor tissue was expected due to their polarity. This finding suggests that these compounds are exuding a systemic effect on tumor proliferation instead of eliciting an effect intracellularly. In a follow up

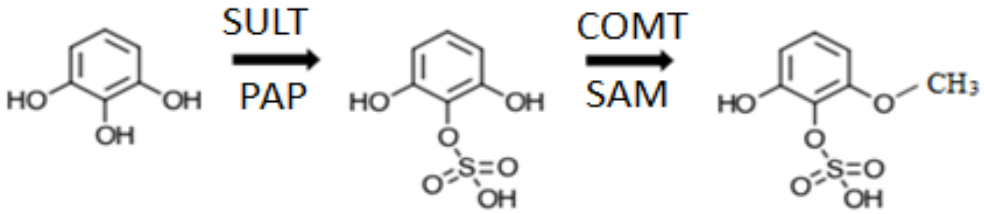

Figure 34. The sulfation and methylation of pyrogallol to produce metabolites found in plasma and intracellularly.

experiment would be interesting the monitor the sulfation of pyrogallol through the course of the study. It is possible that the mice are becoming more efficient at clearing these compounds and consequently could be the reason we saw more aggressive growth in the treatment animals in the final week.

The work performed *in vitro* had a paradoxical effect to the *in vivo* analysis because both pyrogallol and methyl-pyrogallol-sulfate were found directly within the cancer cells. Pyrogallol was present in the first hour, but had been completely metabolized to methyl-pyrogallol-sulfate by the second hour. This rapid conversion of pyrogallol was similar to what was reported *in vivo*. When the concentration of pyrogallol was increased to 50 mg/L there was an increase in pyrogallol concentration and a decrease in the metabolite concentration. This indicated that the sulfation/methylation is rate limiting once the system is saturated with pyrogallol.

In conclusion, pyrogallol is a rapidly sulfated polyphenol with the ability to transcend the phospholipid membrane to enter cancer cells. Further investigation should focus on the metabolites and determine if they have any bioactive properties. The

sulfated metabolite instead of the parent compounds, at least *in vivo*, was the only pyrogallol derivative present in plasma, and therefore the only compound bioavailable to elicit an effect. It would be interesting to investigate in a future study if changing the method of delivery from oral to either intraperitoneal or intravenous injection could increase pyrogallol concentration in tumor tissue.

CHAPTER VI

CONCLUSION AND FUTURE STUDIES

This research focused on the antiproliferative activities of mango polyphenols and their central microbial metabolite pyrogallol in ductal carcinoma *in situ* stage model of breast cancer. Previous studies have demonstrated the cancer-cytotoxic activities of many polyphenols but their microbial metabolites have only infrequently been investigated. Increased consumption of polyphenols has been linked to a decreased incidence in chronic diseases. Epidemiological studies and meta-analyses have correlated the intake of polyphenols from fruits and vegetables, teas and spices to the incidence of chronic disease, but only some show a significant inverse correlation to certain types of chronic diseases. Factors that are responsible for the high variability in human clinical and epidemiological studies include other nutritional factors such as intake of carcinogens (e.g. polycyclic aromatic hydrocarbons), overall lifestyle, phase I and phase II metabolism, microbial metabolism of polyphenols and other factors. Upon ingestion, polyphenols are rapidly metabolized by host and microbial enzymes that are characterized by significant inter-individual differences. Few studies have been performed with microbial metabolites of polyphenols. Pyrogallol is a central metabolite of dietary tannins that occur in most fruits, vegetables, teas and spices.

Several studies investigated polyphenol effects on breast cancer, where the information on polyphenol efficacy on DCIS breast cancer is very limited. DCIS research is important because is a unique form of breast cancer that captures a late, pre-

invasive stage. In the initial study of this dissertation, *in vitro* assessment of a mango extract that is low in tannins but rich in small phenolic acids, pyrogallol, gallic acid, and methyl gallate were investigated. Additionally, potential synergistic activities with 5-FU were assessed. The concentration (10 mg/L) was selected for a majority of the work due to reproducible effects without cytotoxicity non-cancerous cell lines. A limitation of this concentration is that some of the cellular mechanisms were not as clearly identified as might have been at higher concentrations. While this dose is similar to what is achievable through diet, it has its limitation for mechanistic evaluations. A higher dose may have elicited a more pronounced mechanistic response in the selected models. Yet, a clinical systemic exposure to this higher level might yield similar cytotoxic side-effects as they are experienced with conventional chemotherapy.

Pyrogallol was selected as central study treatment for the following reasons: a) pyrogallol is the main central metabolite of microbial metabolism of tannins that occur in mango, most fruits, vegetables, teas and spices, b) it had the greatest antiproliferative effect of any individual polyphenol screened and reduced DCIS proliferation by more than 50%. Following compound selection, concentration-dependency of cytotoxic activities were investigated in both cancerous and non-cancerous breast cell lines. A dose range of 10 mg/L-20 mg/L of pyrogallol was not cytotoxic in non-cancer cells but caused significant anti-proliferative activities in cancer cells.

Initially, it was hypothesized that pyrogallol or the polyphenolic extract may have a synergistic effect with the cancer drug, 5-FU, based on previously published work with other polyphenols. 5-FU was also included as a positive control *in vitro* and in the

initial animal study. Non-significant interactions were noticed between 5-FU and the polyphenol-based treatments. 5-FU is a genotoxic compound whose main mechanism of action is being misincorporated into macrostructures such as DNA and RNA resulting in inhibition of DNA replication and problems in protein translation. The dysregulation in protein production eventually leads to cell death, and this treatment is not targeted to only affect cancer cells, but also affects normal cells resulting in unwanted side effects. It was hypothesized that there would be an additive or synergistic interaction between the polyphenol treatment and 5-FU, because a major mechanism of polyphenol-induced suppression of proliferation seemed to be based on interactions with the mTOR pathway. The differences in mechanism were hypothesized to produce at least additive effects when they were co-administered; however, at the concentration used, no interactions with 5-FU were detected.

Based on its central role in inflammation and proliferation, the mTOR pathway was selected as the primary pathway of investigation. PG had a more pronounced effect on mRNA levels, and reduced IGF1-R, P13K, HIFα, AKT, and mTOR. ML reduced PI3K, AKT, and mTOR mRNA levels compared to non-treated controls. Both treatments reduced protein expression of IGF1-R, IRS1, IF, AKT, and P70S6. PG also reduced total mTOR levels. Both treatments also had an effect on phosphorylated protein levels, with PG significantly reducing IGF1-R, AKT, and P70S6 levels. ML had a similar effect and significantly decreased IR, AKT, and P70S6 phosphorylation levels. The main conclusion elucidated was the PI3k/AKT/mTOR signaling axis appears to be

at least in part cause for decreased proliferation induced by dietary-relevant concentrations of ML and PG.

This initial cell based assays concluded that mango polyphenols had an effect on DCIS breast cancer, and therefore supplied justification for further investigation. A xenograph model was employed because it was deemed a suitable model to further examine the anti-proliferative activities of the selected polyphenol-treatments in breast cancer development. DCIS cells were xenographed into the mammary pads of female athymic mice, and treated for 4 weeks with either a mango extract or pyrogallol at 0.8 and 0.2 mg/d by oral gavage. Treatments significantly reduced tumor volumes compared to vehicle treated controls. Treated animals did not display any signs of toxicity and had similar body and liver weights compared to controls.

Tumor-tissue was the main focus for a majority of the molecular work and much of what was discovered from the *in vitro* exploration was confirmed in the tumor tissue. Major genes along the mTOR pathway was significantly reduced, greater than 40% for both treatments, and included IRS1, IGF1-R, PI3K, AKT, ERK, and mTOR. Results also support the *in vitro* findings that polyphenols have anti-inflammatory properties and reduced CRP and TNFα with MG treatment and IL-1β in PG treated tumors. Total protein levels for IRS1, AKT, and mTOR were reduced more than 20% in polyphenol administered tumors. A downregulation in protein phosphorylation was also noted with MG including IR, IRS1, IGF1-R, and mTOR phosphorylation. PG treatment had a similar effect; however, p-mTOR was not significantly reduced. Further, p70S6K, an indicator of mTOR activity was reduced along with p-ERK. Currently mTORC1 is

known to have 4 phosphorylation sites (S1261, T2446, S2448, and S2481), but the activity of each of those sites has not been characterized completely. In this instance it may have been more advantageous to examine each of the phosphorylation sites instead of just one in order to better elucidate the effect PG had on p-mTOR.

While the effects to the mTOR signaling axis explain the anti-proliferative activities of polyphenols both *in vitro* and *in vivo*, it was hypothesized that potentially other mechanism affecting mTOR could be involved. A further exploration *in vitro* revealed that both treatments acted as ROS generators in DCIS breast cancer cells. This led to the hypothesis that possibly AMPK, a pathway induced by ROS might be involved in the inhibition of the mTOR pathway. AMPK, a kinase involved in cellular energy homeostasis, can be causally involved in the inhibition of mTOR upon activation. Western blot results indicate that AMPK pathway constituents including Sestrin, Becklin, LKB1, and ULK were upregulated in tumor samples. Polyphenol treatments induced phosphorylation of AMPKα at Thr 172 which is found on the catalytic subunit. Overall, the activation of AMPK through phosphorylation at Thr 172 caused a reduction in mTOR and consequently downregulated proliferation in DCIS cancer cells. Further analysis *in silico* revealed PG ability to directly bind with the allosteric binding site of AMPKα which would result in its activation. Consequently PG may activate AMPK either through ROS generation, or through direct allosteric activation.

A follow-up *in vivo* study was performed in order to compare a higher dose PG treatment of 0.8 mg/day to the previously used dose of 0.2 mg/day. The major objectives were: 1) to further investigate the anti-proliferative mechanisms at two different

concentrations with the hypothesis that the higher dose of PG would induce autophagy through the activation of AMPK, while the lower dose mainly reduced proliferation. 2) To evaluate the bioavailability of pyrogallol and determine if it was reaching the site of action or if the anti-proliferative activities might have been induced systemically.

Anti-proliferative activities of PG were comparable to findings from the previous study with treatments animals having reduced tumor volumes for the duration of the study. The tumors of the treatment animals continued to grow throughout the treatment, but at a less rapid pace than control tumors. There was no significant difference in tumor volumes between the two different PG concentrations. This is advantageous, because a reduced dose that is more realistically achievable had the same effect as an elevated dose which could be less achievable through diet or supplementation.

Genes along both the AMPK and mTOR pathways were significantly decreased in PG treated tumors, and mirrored the results previously described adding additional support that these are the major pathway being affected by PG. Both tumor and plasma samples collected at time of necropsy were analyzed for the presents of PG and PG metabolites. The tumors did not contain any measurable concentrations of PG or metabolites commonly found systemically. Pyrogallol sulfate was detected and characterized in the plasma samples. This may suggest that the observed anti-proliferative activities may be due to either extracellular or systemic activities. The PG-sulfated metabolite present in the plasma samples indicated that PG is sulfated rapidly, presumable through SULTs in the liver. This is consistent with currently ongoing human clinical studies focusing on the bioavailability of PG following 400 grams of mango

consumption. Cell culture analysis of samples treated with PG indicated that both PG and methylated PG were present intracellularly at an absorption rate of 0.3 and 0.7% at 20 mg/L for 1 h. Potential follow up research should focus on PG metabolites, both the sulfate and methylated forms and determine if they have an effect in DCIS cancer.

Another interesting observation from the xenograph model is that the treatments only slowed the tumor development. Instead, the difference in tumor-size between controls and treatment was decreasing after the initial treatment phase over the duration of the study. Since pyrogallol is rapidly metabolized and detoxified by SULTs and SULTs are present in multiple tissue types, it is plausible that sustained exposure to PG increased the detoxifying activity of SULTs through upregulation of SULTs expression, causing a more rapid excretion of pyrogallol metabolites.

In conclusion, the research presented within has shown polyphenols associated with mango consumption delayed proliferation in DCIS breast cancer both *in vitro* and *in vivo*. Further research needs to be conducted to determine if these results translate to an effect in humans at a dose achievable through either dietary intake or through dietary supplementation.

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