POLYPHENOL-INDUCED ANTI-INFLAMMATORY AND CYTOTOXIC ACTIVITIES IN BREAST AND COLON CANCER: POTENTIAL ROLE OF MIRNA'S IN CELL SURVIVAL AND INFLAMMATION

A Dissertation

by

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ABSTRACT

Cancer is a major cause of death worldwide. Hence, there is a great need to develop novel therapeutic agents for the prevention and treatment of cancer in addition to conventional therapies. Dietary polyphenols are known to be effective in the prevention and treatment of several chronic diseases such as cancer, cardiovascular diseases, and diabetes. Particularly in carcinogenesis, polyphenols are known to suppress cancer growth, angiogenesis, and metastasis. Several studies have demonstrated that polyphenolics, ellagitannins, gallotannins, and chlorogenic acid from pomegranate, mango, and plum juice, respectively, are potent inhibitors of cancer cell proliferation and induce apoptosis and cell cycle arrest as well as decrease inflammation in vitro and vivo.

The therapeutically relevant compounds in pomegranate are ellagic acid, ellagitannins, flavonoids, and 3-glucosides/3,5-diglucosides of the anthocyanins delphinidin, cyanidin, and pelargonidin that exerted antioxidant, anti-inflammatory, and anticarcinogenic activities in vitro and vivo. Mango pulp extract contains gallotannins, gallic acid, galloyl glycosides, and flavonoids such as quercetin and kaempferol glycosides, which showed antioxidant, anti-inflammatory, and anticarcinogenic activities in vitro and in vivo. Chlorogenic acid and neo-chlorogenic acid are contained in plum juice and are also known to function as chemoprevention and chemotherapeutic agents.

The overall objective of this work was to investigate the underlying anti-inflammatory and cytotoxic mechanisms involving miR-27a-ZBTB10-Sp and miR-155-SHIP-1-PI3K axes, miR126-VCAM-1, miR126-PI3K/AKT-mTOR and

miR143/PI3K/AKT/mTOR axes in polyphenol-mediated anti-inflammatory and anticarcinogenic activities in vitro and vivo.

Pomegranate and Mango polyphenols exhibited antioxidant, anti-inflammatory, anticarcinogenic, and antiproliferative activities in vitro and in vivo. Polyphenols inhibited cell proliferation of breast cancer cell line BT474 and suppressed tumor growth in athymic BALB/c nude mice with BT474 xenografts. Interactions of Pg with miR-27a-ZBTB10-Sp and miR-155-SHIP-1-PI3K axes and mango miR126/PI3K/AKT axis were identified. In addition, pomegranate and plum polyphenols exerted cytotoxic and anti-inflammatory effects in azoxymethane AOM-treated rats and colon cancer cells. Interactions of Pg with miR126/VCAM-1 and miR126/PI3K/AKT/mTOR axes and plum with miR143/PI3K/AKT/mTOR were identified as mechanisms that at least in part appear to be involved in the anti-inflammatory and antiproliferative activities of pomegranate and plum polyphenolics.

The presented research was conducted in order to understand the efficacy of polyphenols present in pomegranate, mango and plum and their underlying molecular mechanisms in different cancer models.

DEDICATION

For being my constant encouragement

To my father Prasanta Kumar Banerjee and mother Bithi Banerjee

To my husband Dr. Arnab Chakrabarty

To my wonderful little cutie, nine month old son, Niladri Chakrabarty

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CHAPTER I

INTRODUCTION: SIGNIFICANCE OF POLYPHENOLIC COMPOUNDS IN CANCER THERAPY

Background

Cancer is a major health care problem in the United States and worldwide and is the second most common cause of death in the United States, exceeded only by cardiovascular disease. Currently, 1 in 4 deaths in the United States is related to cancer and 2012, it was estimated that approximately 1,638,910 new cancer cases will have been diagnosed; among these, about 577,190 deaths are estimated. [1-3]. From 1998 to 2007, the overall cancer death rates decreased both in men and woman across all racial and ethnic groups [1-4]. Survival statistics are significantly influenced by demographic factors such as gender, age and country or region, as well as the type of cancer and stage at diagnosis [1-3].

Between 2004 and 2008, the overall cancer occurrence rates decreased slightly in men (by 0.6% per year) but were stable in women and the cancer death rates were decreased by 1.8% per year in men and by 1.6% in women. Between 1999 and 2008, death rates from cancer decreased by more than 1% per year in men and women of every racial/ethnic group except for American Indians/Alaska whose death rates were unchanged [4]. These improved survival rate can be attributed to screening for certain cancers and detection at their early stages and in improved treatment regimens.

The most common cancers among men are cancers of the prostate, lung, and bronchus, as well as colon and rectal cancers and these accounted for about 52% of all newly diagnosed cancers in 2012. Among women, the three most commonly diagnosed types of cancers are breast, lung, and bronchus, followed by colon and rectal cancer, which combined accounted for 52% of estimated cancer cases in women in 2012 (Figure 1) [4].

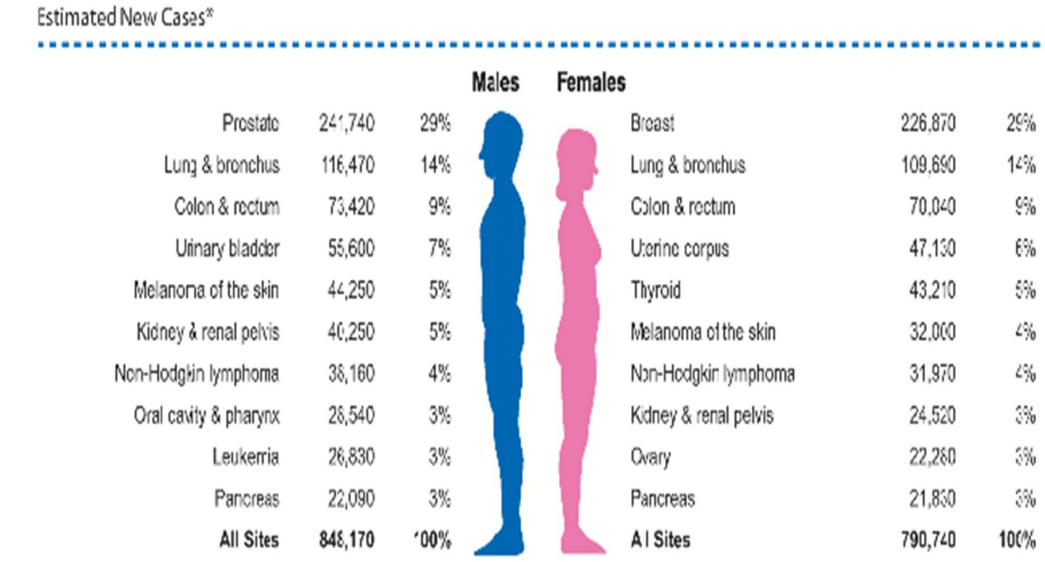

Figure 1 Estimated new cancer cases for each of ten major cancer types in the United States (by sex), 2012 [4]

Death rates decreased for all 4 major cancer sites (lung, colorectum, breast, and prostate), with lung cancer accounting for almost 29% of the total decline in men and breast cancer accounting for 26% of the total decline in women (Figure 2). Further reduction in death due to cancer can be achieved through the improvement of cancer

prevention and treatment, with emphasis on groups in the lowest socioeconomic bracket [4].

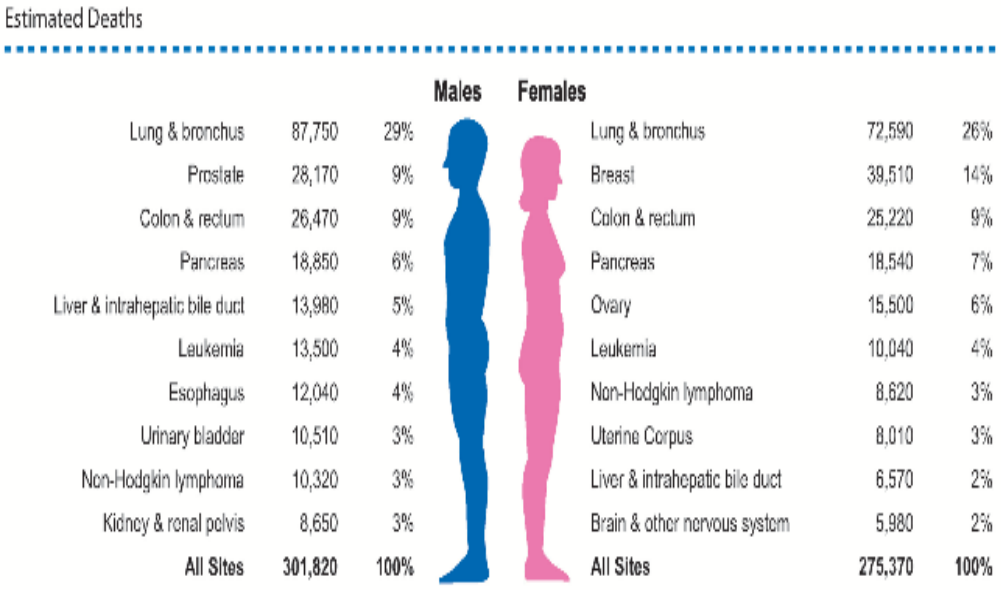

Figure 2 Estimated new cancer deaths, by gender, for ten major cancer types in United States, 2012 [4]

Carcinogenesis is a multistep process involving uncontrolled cell proliferation, cellular transformation, and angiogenesis, in addition many tumors metastasize for distal sites where they continue to grow [4-9]. Carcinogenesis includes three steps tumor initiation, promotion and progression. Carcinogens are agents involved in cancer initiation which can be influenced by inflammatory agents, genetic and epigenetic alterations or that can activate of oncogenes and initiate tumor suppressor genes. Cancer progression leads to gradual phenotypical changes, from a normal to a pre-neoplastic cell that may be further transformed into a neoplastic cell [4-9].

Breast cancer

Breast cancer is one of the leading causes of cancer death among women worldwide and the American Cancer Society has estimated of 226,870 new cases of breast cancer with 39,510 deaths from breast cancer in 2012 [1-4]. Breast cancer is the most common cancer after skin cancers in women throughout the world [1-4].

The lifetime risk of developing breast cancer is about 1 in 8 (12%) among women in the United States. Interestingly breast cancer has accounted for 34% of the total decline in cancer in recent years and started the declining in 2000 after increasing for more than two decades .The use of hormone therapy is believed have contributed to this large decrease in breast cancer incidence.

The chance of death from breast cancer among women in the United States is about 1 in 36 (about 3%) and eath rates from breast cancer have been decreasing since about 1989, with a larger decline in women younger than 50 years of age [4]. These decreases are thought to be the result of detection at earlier stages through improved screening and treatment [4]. The incidence rate of breast cancer appears to be highest among whites, followed by African-Americans, whereas the mortality rates were highest among African Americans, followed by whites (Table 1) [4]. Of all breast cancer patients, the main cause of death is metastasis at distant sites [10]. Since the risk of metastasis cannot be predicted in patients with primary tumors, most patients undergo adjuvant therapy to eradicate breast tumor cells. Nevertheless some breast cancer patients die from metastasis, and from the toxic effect of extensive chemotherapy [10].

Table 1 Incidence and mortality rates by demography in the United States between 2004-2008 [4]

Incidence						
		White	African American	Asian American or Pacific Islander	American Indian or Alaska Native	Hispanic/Latino
All Sites	Male	546	626.2	332.4	427.8	423.4
	Female	420.8	394.2	284	362.1	333.5
Breast (Female)		122.8	116.1	84.9	89.2	92.3
Colorectum	Male	54.6	66.9	42.4	51.5	48.6
	Female	40.8	49.7	32.7	41.5	34.2
Kidney & renal Pelvis	Male	20.8	22.6	9.9	27.4	19.4
	Female	10.9	11.7	4.9	16.8	11.2
Liver & bile duct	Male	8.6	14.1	21.7	15.8	17
	Female	2.9	4	8.2	7.6	6.4
Lung & bronchus	Male	63.7	102.7	49.8	71	46.8
	Female	57.2	51.4	28.1	51.7	27
Prostate		142.8	230.8	79.7	101.2	126.7
Stomach	Male	8.5	16.4	16.8	13.9	13.8
	Female	4	8.2	9.4	6.8	8.4
Uterine Cervix		7.7	10.6	7.4	9.8	12.2

Table 1 Continued

Mortality						
		White	African American	Asian American or Pacific Islander	American Indian or Alaska Native	Hispanic/Latino
All Sites	Male	222	295.3	184.7	190	149.1
	Female	152.8	177.7	94.1	138.4	101.5
Breast (Female)		22.8	32	12.2	17.2	15.1
Colorectum	Male	20.1	30.5	18.8	19.8	15.5
	Female	24	20.4	9.9	14	10.3
Kidney & renal Pelvis	Male	6	6	2.6	8.9	5.2
	Female	2.7	2.6	1.2	4.1	2.8
Liver & bile duct	Male	7.2	11.5	14.7	11.9	11.6
	Female	8	8.9	6.9	6.7	5.2
Lung & bronchus	Male	86.9	85.4	86.7	80.5	31.9
	Female	41.2	38.8	38.5	33.9	14.3
Prostate		22.4	54.9	10.5	20.7	18.5
Stomach	Male	4.6	10.7	9.2	8.5	7.7
	Female	2.8	5	5.4	3.9	4.5
Uterine Cervix		2.2	4.8	2.1	3.4	3.1

Colon cancer

According to the American Cancer Society, colorectal cancer is the third leading cause of death in the United States and then estimate 143460 new cases of colorectal cancer and 52,690 death in 2012 [1-3]. Colorectal cancer occurs equally in both men and women.

The incidence of colorectal cancer and associated mortality rate is increasing worldwide and the lifetime risk of developing colorectal cancer is about 1 in 20 [1-3]. Colorectal cancer incidence rates have been decreasing for the past two decades from

66.3 cases per 100,000 in 1985 to 46.4 per 100,000 in 2005. According to cancer statistics, this decrease is attributed to improved diagnosis, which helps early detection and removal of colorectal polyps before tumor formation.

Death rates for colorectal cancer have decreased in both men and women over the last two decades, with a rapid decrease of 4.3% per year from 2002 to 2005 in both men and women, compared to 2.0% per year from 1990 to 2002 in men and 1.8% per year from 1984 to 2002 in women (1-3). The incidence and mortality rate of colorectal cancer appeared to be highest among African-Americans (Table 2) [4].

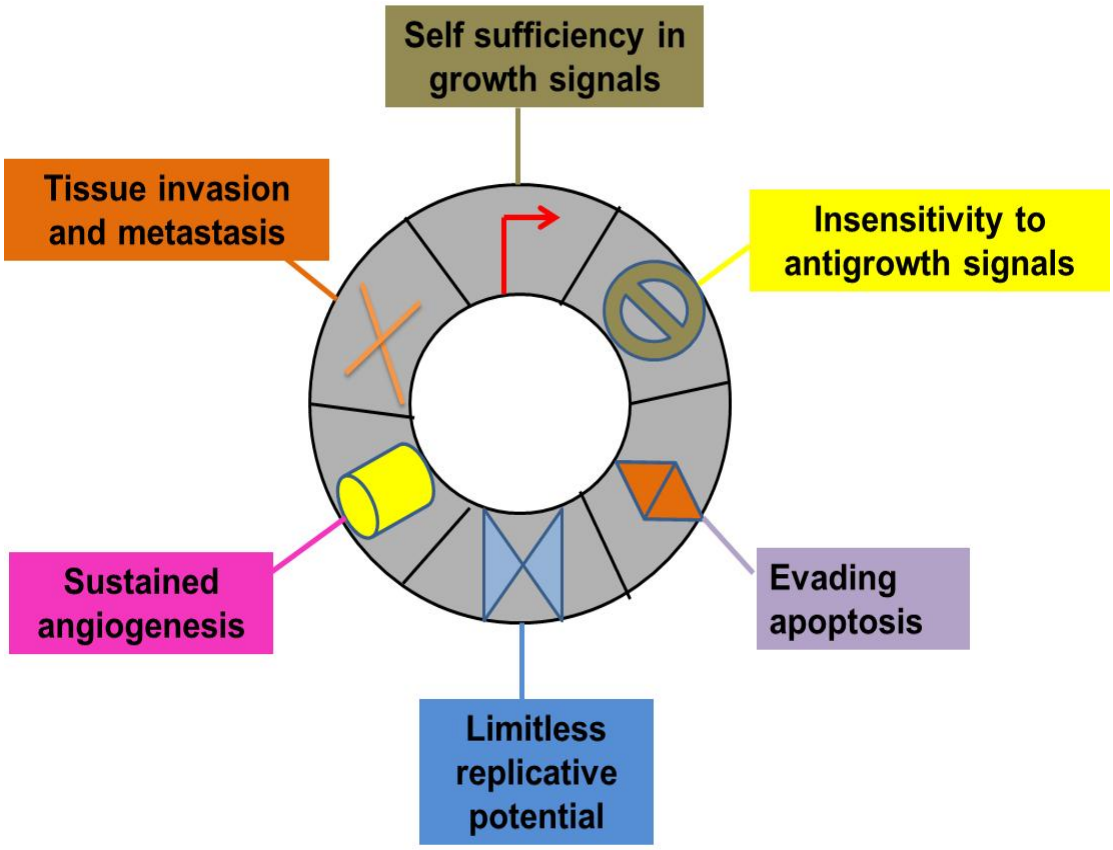

Figure 3 Hallmarks of cancer [11]

Hallmarks of cancer

Tumor formation is accompanied by gradual phenotypic changes, from a normal to a preneoplastic cell that transforms to a neoplastic cell. Six hallmarks in cancer progression have been defined (Figure 3) self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

1. Self-sufficiency in growth signals: In order to maintain normal cell homeostasis, non-transformed cells depend on growth factors to proliferate. These growth signals are transmitted through transmembrane receptors that are targets for various growth factors and extracellular matrix components (Figure 3). In contrast, cancer cells exhibit decreased dependency on exogenous growth factors. And became autonomous through auto-activation of several growth factors such as platelet derived growth factor (PDGF) vascular endothelial growth factor (VEGF), transforming growth factor, (TGF) and epidermal growth factor (EGF). Production of these growth factors result in autocrine stimulation through a positive feedback signaling loop [11].

2. Insensitivity to anti-growth signals: Antigrowth signals can inhibit cell proliferation either by moving cells out of the active proliferation cycle into a temporarily quiescent (G0) state or by permanently inhibiting cell proliferation whereby cells remain in a postmitotic state. Cancer cells escape these anti-proliferative signals. For example, Retinoblastoma (Rb), a tumor suppressor gene, plays a key role in the

transition from the quiescent (G0/G1) phase to the replicating (S) phase of the cell cycle and in cancer cells Rb can become inactivated[11].

3. Evading apoptosis: A crucial mechanism of cell death is apoptosis which is a form of programmed cell death. In cancer, there is an imbalance between cell proliferation and cell death that can result in tumor growth of cells that escape apoptosis. In cancer cells the balance between antiapoptotic and pro-apoptotic signals is shifted and cancer cells can proliferate under adverse environmental conditions such as hypoxia, and chemotherapy which are toxic to normal cells [11].

4. Limitless replicative potential: After a number of replications and cell divisions, normal cells stop growing and this stage is called senescence. However cancer cells acquire immortalization and fail to undergo cell death by increasing the activation of telomerase (hTERT) enzyme which is required for cell proliferation and excess of telomere length [11]. This property is important for cancer cell progression and metastasis[11].

5. Sustained angiogenesis: Neovascularization or formation of new blood vessels is enhanced during cancer progression. New blood vessels play important role for cancer cell survival and are essential for supplying oxygen and nutrients to the cancer cells. For example, increased expression of VEGF is observed in several cancer cells [11].

6. Tissue invasion and metastasis: Cancer cells have the capability of invasion and metastasis and can migrate to distant organs and develop metastatic tumors and cancer cells. Metastasis of cancer cells is the major cause of death in cancer patients.

Cell-to-cell adhesion molecules such as cadherins and integrins influence the invasive and metastatic properties of cancer cells [11].

Animal models used for breast cancer research

The prevention and treatment of breast cancer, with various cytotoxic and endocrine therapies, have been somewhat successful however it is also important to develop novel therapies. [12-14]. In addition to progress in screening and assessment, the development of mouse xenograft models had played an important role in understanding breast tumor formation and progression. [12-14] and the heterogeneity of this diseases and these models are essential for drug development and determines their effectiveness in different types and subtypes of tumors [12-14]. The xenograft model provides reproducible characteristics and a unique opportunity to understand tumor growth regulation and metastasis in an in vivo environment [13, 14].

It has been previously shown that some established human breast cancer cell are tumorigenic in immuno-compromised nude mice [12-14]. Studies have shown that human breast cancer cell xenografts can serve as an effective model of breast cancer growth and provide insight on mechanism of tumor growth and effectiveness of anticancer agents .[12, 13]. Breast cancer xenografts are implanted into the subcutaneous tissue of the flanks (Figure 7) or mammary fat pad (orthotopic site) [13, 14].

Figure 4 Breast cancer xenografts are implanted into the subcutaneous tissue of the flanks

Transgenic breast cancer model

p53 knockout ($p53^{-/-}$) animal model

The first identified tumor suppressor gene was p53, which prevents neoplastic development by inhibiting abnormal cell proliferation [15]. p53 activation is mediated through several dependent pathways, such as kinases or independent pathways and also in response to cellular stresses [15]. p53 activity, decreased through mutations in tumor suppressor genes exhibits oncogenic activity through increased cell survival and genomic instability [15, 16]. p53 knockout model predicts significant possibility of developing human tumors with p53 mutation. Close association between p53 mutation with both spontaneous and inherited human tumors creates a better chance to develop a representative transgenic animal model for p53-related cancers [16]

PTEN knockout ($PTEN^{-/-}$) animal model

The PTEN (*p*hosphatase and *ten* sin homolog deleted from chromosome 10), another important tumor-suppressor gene, also known as *MMAC1* (*m*utated in *m*ultiple *a*dvanced *c*ancers) is involved in cell cycle processes [17]. Mutations or somatic deletions of PTEN, may result in increased cell proliferation and leads to tumor growth. PTEN appears to be mutated in human cancers, such as prostrate breast cancer. PTEN controls AKT expression which reveals PTEN function as tumor suppressor gene through control of cell survival [17]. Studies have shown that mutation of one allele of PTEN can result in impaired apoptosis in a mouse model. Similarly loss of one allele plays an important role for early selection and detection of cells during transformation [17]. Thus PTEN knockout ($PTEN^{-/-}$) animal model is used to study mammary tumorigenesis [17].

ErB2 overexpression transgenic mice model

The ErbB2 receptor tyrosine kinase gene (also known as HER-2; Human Epidermal Growth Factor Receptor 2; family or NEU, the rat homologue of ERBB2,) is overexpressed in many human invasive breast cancer [18]. Breast cancer cells with overexpression of ErbB2 showed resistance to some chemotherapeutic agents [18, 19]. Breast cancer patients receive a combination of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) as chemotherapeutic agents [18]. However ErbB2-overexpressed breast cancer patients showed less response to CMF- chemotherapy agents than their counterpart with normal ErbB2 expression [18, 19]. To understand the molecular basis

of ERBB2-influenced mammary tumorigenesis, ErB2 overexpressed transgenic mice model have been developed [19].

The carcinogen-induced mammary carcinoma model

Several carcinogen-induced mammary carcinoma model has been reported such as methylcholanthrene (MCA) is a highly carcinogenic polycyclic aromatic hydrocarbon and 7,12-dimethylbenz(a)anthracene- (DMBA) used as potent carcinogens for mammary tumorigenesis, DMBA treated animal model closely mimics human mammary tumorigenesis as DMBA metabolites can cause DNA damage, which is a key event in carcinogenesis initiation [20].

Animal models used in colon cancer studies

Chemical carcinogen models

Experimental study of colon cancer in animal models has a long history of 80 years [21, 22]. Several studies have shown the induction of colon tumorigenesis when mice or rats have been administered chemical carcinogens. For example, Lorenz et al., have shown that mice fed with the polycyclic aromatic hydrocarbon methylcholanthrene develop gastric and intestinal tumorigenesis. Lisco et al., have demonstrated that rats fed with radioactive yttrium develop colon tumorigenesis [22] and the most commonly used colon cancer carcinogens are 1,2-dimethlyhydrazine (DMH) or a zoxymethane (AOM) [22].

An early population study shows that the consumtion of cycad flour, a source of hydrazine, a colon carcinogen, caused colon carcinogenesis in the animals [22]. Other studies show that rats, fed with hydrazine rich cycad flour, developed adenocarcinomas

[22]. Cycad flour contains cycasins a form of methylazoxymethanol (MAM). Earlier studies have shown that DMH, a metabolic precursor of MAM, induce colon tumors in rats. It has been demonstrated that, as a carcinogen, AOM is more stable and more potent than DMH and several thousand studies have been performed using AOM [22].

There are a a number of advantages in using the carcinogen-induced colon cancer rodent model, the results are highly reproducible, and the animals with different genetic background can be used [22]. AOM-induced colon cancer pathogenesis re-capitulates early stages of the human adenoma-carcinoma. So far, the majority of carcinogen-induced colon cancer models have been carried out in rats and in mouse models and studies with chemopreventive agents and dietary factors have also been performed in carcinogen-induced colon cancer models [21, 22].

Xenograft colon cancer model

Another major model used for investigating colon cancer pathogenesis is the mouse xenograft model. Human colon cancer cells are grafted into immuno-compromised nude mice predominantly into subcutaneous tissue [23]. Although subcutaneous sites are non-invasive and do not metastasize, these sites are optimal for the observation of tumor growth and progression [23]. Transplanting tumors into their orthotopic site such as in the cecum of nude mice may induce liver metastases. Human colon cancer cell suspensions have also been injected into the cecal wall. Colon cancer tissue implantation at the orthotopic site were found to be more metastatic than tumors obtained after tumor cell injections [23].

Transgenic APC $^{min/+}$ colon cancer model

Adenomatous polyposis coli (APC) is a tumor suppressor gene encoded by the *APC* gene in human [24]. APC regulates the uncontrolled cell growth which causes cancerous tumors. *APC* gene protein controls cellular processes and function as a "gatekeeper" to inhibit colon tumor development [24-26]. Mutation in the adenomatous polyposis coli (*APC*) gene may result in sporadic colorectal tumors and also familial adenomatous polyposis (FAP), a hereditary form of colon cancer [24-26]. Hundreds to thousands of colorectal polyps developed in FAP human patients, may further develop to colon carcinomas if not removed in a timely manner [27]. There is much interest in transgenic APC $^{min/+}$ mouse for studying intestinal tumorigenesis underlying mechanisms and potential cancer prevention and treatment methods [25]. APC $^{min/+}$ mouse survive about 120 days due to intestinal tumors which has a mutated APC gene, similar to FAP colon cancer. This animal model mimics the colon carcinomas and rapid development of polys in patients due to mutation of one APC gene [25].

Types characteristics and classification of breast cancer

There are different types of breast cancer with distinct characteristics and occurrence frequency.

Ductal carcinoma

Mammary ductal carcinoma is the most common type of breast cancer of ductal carcinomas are predominant a) Ductal carcinoma in situ (DCIS) which is non-invasive, with cancer cells inside the milk ducts (lactiferous ducts) that do not spread through the wall of the ducts into the surrounding breast tissue. b) Another form is

invasive ductal carcinoma (IDC), in which infiltrating, malignant neoplastic cells spread in the breast tissue [28].

Ductal carcinoma in situ

Ductal carcinoma in situ (DCIS) is the most common form of non-invasive breast cancer. The meaning of “in situ” is in its original place." DCIS is appeared to be "non-invasive" and does not spread beyond the milk duct into surrounding breast tissue. Most women diagnosed at an early stage of breast cancer ductal carcinoma in situ can be successfully treated. This has been shown About 1 in 5 new DCIS breast cancer cases. A mammogram is often the best way for early detection of DCIS. Later on DCIS can increase the risk of developing an invasive breast cancer [28].

Invasive (or infiltrating) ductal carcinoma

The second most common type of breast cancer is an invasive (or infiltrating) form of ductal carcinoma (IDC). IDC develops inside a milk duct of the breast, spreads through the wall of the duct, and further develops and grows within the fatty tissue of the breast. It also has the potential to spread (metastasize) to other parts of the body through the lymphatic system and bloodstream. About 8 of 10 invasive breast cancers are IDC.

Lobular carcinoma in situ

Invasive (or infiltrating) lobular carcinoma

Invasive lobular carcinoma (ILC) [28] initiates in the milk-producing glands (lobules) like IDC, and can also metastasize to other tissue of the body. About 1 in 10 invasive breast cancers are ILC and diagnosis by mammograms has a lower success rate than for IDC.

Less common types of breast cancer

Inflammatory breast cancer

Inflammatory breast cancer accounts for about 1 to 3% of all breast cancers and this type of cancer usually develops without the formation of palpatable tumors. Instead, IBC makes the skin on the breast thick, look red and feel warm. Mammogramdo not detect this tumor and IBC readily metastasize

Triple-negative breast cancer

Triple-negative breast cancers (invasive ductal carcinomas) cells do not express estrogen or progesterone receptors, or human epidermal growth factor receptor 2 (HER2) proteins on their surface. This type of breast cancer occurs more often in younger women and in African-American women and is known to spread more rapidly than most other types of breast cancer. Because the tumor cells lack of hormone receptors, hormone therapy or drug therapy that targets HER2 are not effective and alternative treatments are necessary.

Paget disease of the breast

Paget disease is a rare form of breast cancer which account for only 1% of all breast cancer cases. It starts in the breast ducts and spreads to the skin of the nipple and then to the areola.

Phyllodes tumor

This is very rare form of breast cancer that develops in the stroma (connective tissue) of the breast. Unlike carcinomas, it does not form in the ducts or lobules. These tumors are usually benign but in rare cases may be malignant.

Angiosarcoma

Angiosarcoma initiates in cells that line blood vessels or lymph vessels. Although it rarely occurs in the breast when it does, it generally develops as a complication of previous irradiation treatments.

Risk factors of breast cancer

Genetics and specific mutated genes

A woman with first degree relatives (such as mother, sister, or daughter) who has been diagnosed with breast has a higher chance of developing breast cancer than a woman without a family history of cancer. Cancer in the family h doubles the chance of being diagnosed with cancer because cancer is usually caused by an inherited gene mutation. Among the several breast cancer associated that are inherited; the most common are BRCA1 and BRCA2 [28]. Inherited mutations in these genes cause hereditary breast and ovarian cancer syndrome (HBOC). It is more likely that someone has inherited a particular form of breast cancer if the person is diagnosed at an early age, especially if they have relatives with the disease.

Environmental and dietary factors

Dietary and environmental factors have a strong influence on development of several types of cancer. For instance meat consumption of red meats that are associated with a higher incidence of colon cancer and the consumption of fat associated with breast cancer Dietary and environmental variables have strong influences on several types of cancer. For instance, meat consumption is often linked with colon cancer and fat consumption is often linked with breast cancer [29, 30].

Increased risk of breast cancer has been associated with a diet high in saturated fat during early adulthood [31, 32]. The fatty acid composition in dairy or animal sources is significantly different from vegetable sources. Animal fat consists of saturated and monounsaturated fatty acids, but vegetable fat is primarily polyunsaturated and/or monounsaturated. there is a significant correlation between the intake of animal fat and the incidence of mammary tumorigenesis [31, 32].

Types characteristics and classification of colon cancers

Adenocarcinoma

The most common type of colon cancer is the adenocarcinoma that originates in glands and accounts for 90-95 % ofall colorectal cancers. About 90% of colorectal adenocarcinoma starts as adenomas and theseare which is a type of polyp that may lead totumor formation. Adeocarcinomas in the colon are divided into two types a) mucinous and b) signet ring cell type. The mucinous colon tumor comprises about 10-15 of adenocarcinomas, whereas the signet ring type represents less than 0.1 % of adenocarcinomas [25, 33, 34].

About 20% of colon cancers are inherited or are linked to a family history of colon cancer. The main types of inherited colon cancers are hereditary nonpolyposis colorectal cancer syndrome (HNPCC) and familial adenomatous polyposis (FAP). HNPCC or Lynch syndrome is an autosomal dominant genetic condition that increases the risk for colon cancer (Figure 4A). [25, 33, 34] HNPCC accounts for 5 7% of all colon cancers. HNPCC is featured as \early-onset of colon cancer and is characterized by defective mismatch repair genes [35-37]. Another inherited colon cancer is familial

adenomatous polyposis (FAP), which develops in childhood and is characterized by development of hundreds to thousands of polyps in the gastrointestinal tract.

Leiomyosarcomas

Leiomyosarcomas are a type of colon cancer that develops in the smooth muscle of the colon. It accounts for less than 2% of colorectal cancers and has a higher chance of metastasizing [25, 33, 34].

Lymphomas

Colorectal lymphomas are a less common form of colon cancer which initiate in the rectum then move to the colon. However, if lymphomas develop in the other part of the body, this spread to the colon and then move to the rectum. Non-Hodgkin's lymphoma accounts for 0.5% of all colorectal cancers. [25, 33, 34].

Melanomas

Melanoma colon cancer generally develops from a melanoma that started in some other distal organ and then spread to the colon or rectum. Melanoma colon cancer is not common and accounts for less than 2% of colorectal cancers. [25, 33, 34].

Risk factors of colon cancer

Genetics and specific mutated genes

FAP can lead to hereditary colon cancer. FAP patients develop colon polyps at an early age and gradually develop hundreds of polyps in the colon. The gene involved in this disease is called Adenomatous polyposis coli (APC) and if not treated at the early stage these polyps can develop into cancer.

HNPCC, or Lynch syndrome paitents also occurs at an early age andPolyps develop in HNPCC to a much lesser extent than in FAP. Mutations in one of the DNA repair enzyme genes such as MutL homolog 1, colon cancer, nonpolyposis type 2 (MLH1), DNA mismatch repair protein Msh2 (MSH2), MSH6 or mutS homolog 6 (MSH6), PMS1 protein homolog 1 (PMS1), or mismatch repair endonuclease PMS2 (PMS2) can lead to hereditary nonpolyposis colorectal cancer (HNPCC) [25, 34].

According to Kinzler and coauthors APC gene functions as the "gatekeeper" of epithelial cell proliferation in colon. Generally, gatekeeper genes play an important role in maintaining cell numbers and mutation of the gatekeeper gene is responsible for an imbalance between cell proliferation and cell death [34].

The multi-hit hypothesis has shown that about 4-10 mutational events are required for the development of malignant tumors. As for example, events such as mutations in APC and p53 (two alleles) and also in one copy of the RAS oncogene (Figure 5) may result in colorectal adenocarcinoma [38]. The multi-hit hypothesis is common in many cancers where development of preneoplastic cells and tumors growth occur due to clonal expansion regulated by successive mutation. First mutation leads to limited growth of the cells followed by the second mutation resulting in benign growth of the cells. Finally if one of the cells undergoes third mutation results in transformation of these cells into a malignant invasive cancer cells [38].

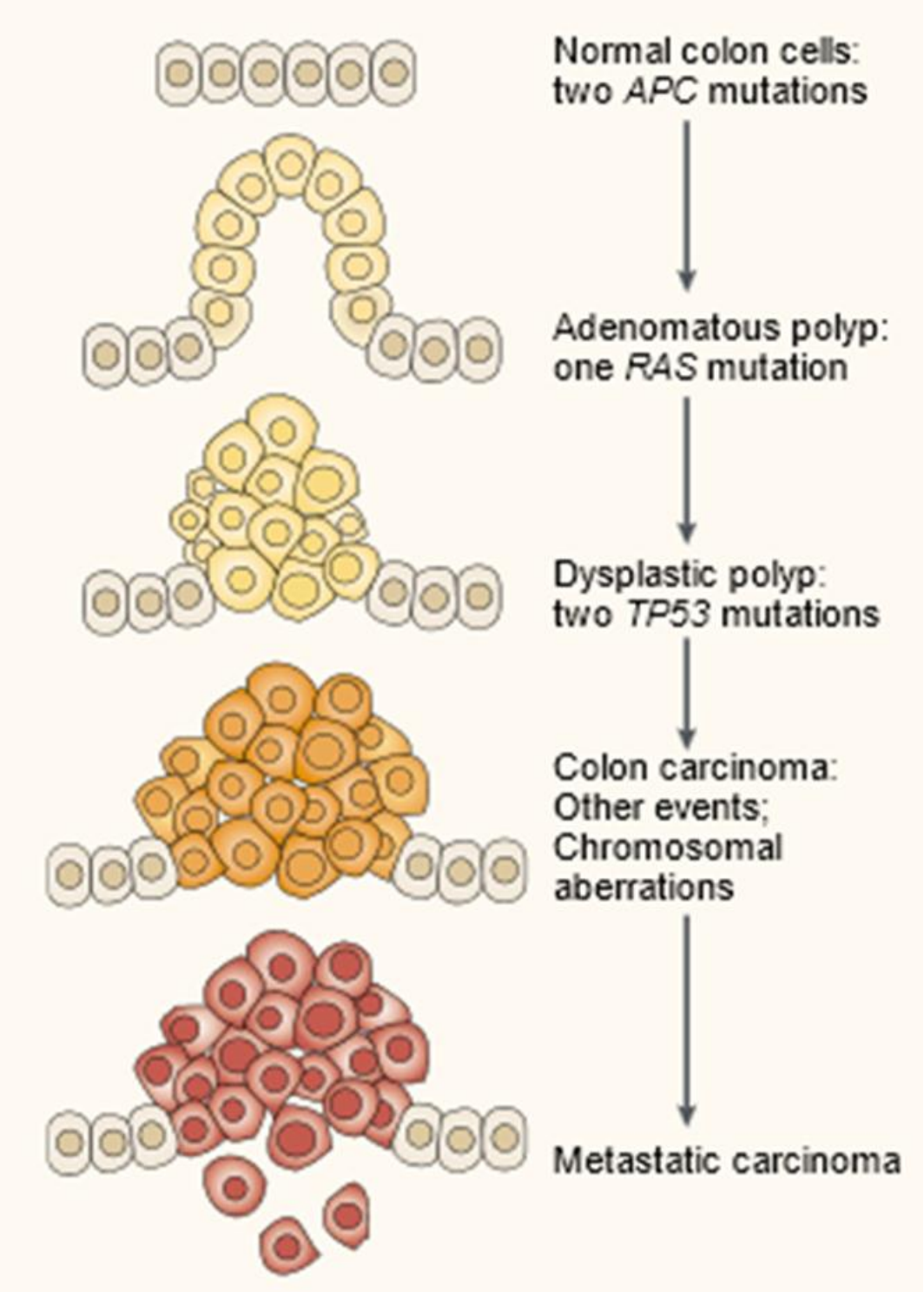

Figure 5 Five hits hypothesis for development of colorectal cancer [38]

Environmental and dietary factors

Environmental factors, specifically diet, play a crucial role in the incidence of colorectal cancer [39]. Intake of sucrose or being tall or obese increases the risk for colon cancer [39]. High sucrose intake is an important factor in contributing to colorectal cancer [39]. Uncooked sucrose increased aberrant crypt loci formation and upregulated colonic epithelial cell proliferation in mice.[40]. Studies have shown that cooked sucrose may be genotoxic and a common side-product of the cooking process is 5-hydroxymethyl-2-furaldehyde, a compound that may cause the formation of microadenoma [41, 42].

Although several studies have been conducted investigating the correlation of sucrose intake and the incidence of colon cancer, no significant correlation has been observed [43] [39, 42]. The intake of red meat and fat increases the risk of colon cancer. Fat intake increased the formation of bile acids and the bowel mucosa is exposed to the toxic effects of bile acids. Levels of carcinogenic heterocyclic amines (from red meat proteins) are increased in a high fat diet and cooking food such as meat at high temperature also increases the amount of heterocyclic amine formation. A significant correlation between the intake of red meat and the incidence of colon carcinogenesis was observed in several studies [39, 42].

Epidemiological, studies suggest that a diet rich in polyphenolics may protect against colonic inflammation and colon cancer [44-46]. Clinical and preclinical studies have reported that polyphenolics from pomegranate exert anti-carcinogenic, anti-inflammatory and antioxidant activities [47-50].

Polyphenols

The large group of polyphenols is the most abundant and ubiquitous group of plant secondary metabolites and constituent of the human diet and is comprised of several structurally distinguished subgroups. In plants, polyphenols protect the plants from pathogens and environmental hazards. Polyphenols are the most important compounds mainly produced to defense against UV radiation, pest and predators [9, 51]. Polyphenols also play an important role in the maintenance of plant physiology, morphology, growth and development.[52].

Sources of polyphenols

Polyphenols are widely distributed across plant species. Polyphenols belong to a diverse group characterized by the presence of phenolic group and also classified by their source of origin, chemical structure and biological function. These highly diverse and widely distributed polyphenols are plant based and are mainly abundant in fruits vegetables, spices chocolate, coffee, tea, wine and grain [9, 51].

Biosynthesis and chemistry of polyphenols

More than 8000 polyphenolic compounds have been identified [52]. The Shikimate and acetate pathways are the main two pathways involved for the origin of secondary plant metabolite polyphenols. Polyphenols occur as simple molecules such as phenolic acid or as polymerized compounds such as tannins. The polyphenols can conjugated with one or more sugar such as monosaccharide, disaccharides or oligosaccharides linked with hydroxyl groups. Polyphenols either linked with glucose or other residues such as galactose and also glucuronic acid. Accordingly polyphhenols are

divided into different classes such as simple phenols, benzoquinones, phenolic acid, acetophenones xanthones, stilbenes, flavonoids, lignins etc [9] (Figure 6, Figure 7).

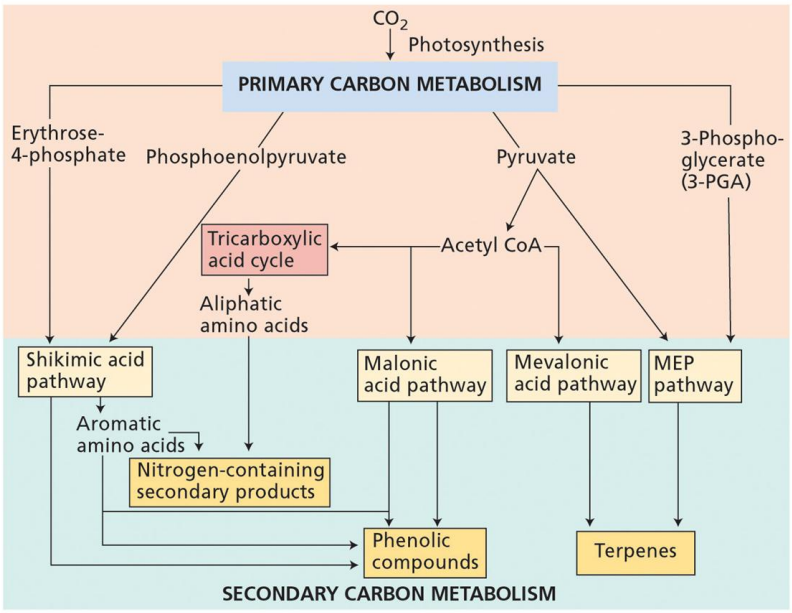

Figure 6 Schematic overview of acetate and shikimate pathway [52]

The most common low molecular weight phenolic compounds identified are simple phenolic acids with typically one ring flavonoids that contain three benzoic rings. Simple phenolics with C_6 structure includes phenol, thymol hydroquinone etc. Phenolics with (C_6- C_1) structures including phenolic acid such as gallic acid, p-hydroxybenzoic. Among many others, the most important phenylpropanoid are the hydroxycinnamic acids such as caffeic acid Ferulic acid and derivatives [9]. The most important plant phenolics identified as flavonoids (C_6- C_3- C_6). Based on the basic structure flavonoids

forms an oxygenated heterocyclic where two aromatic rings linked though three carbon atoms [9].

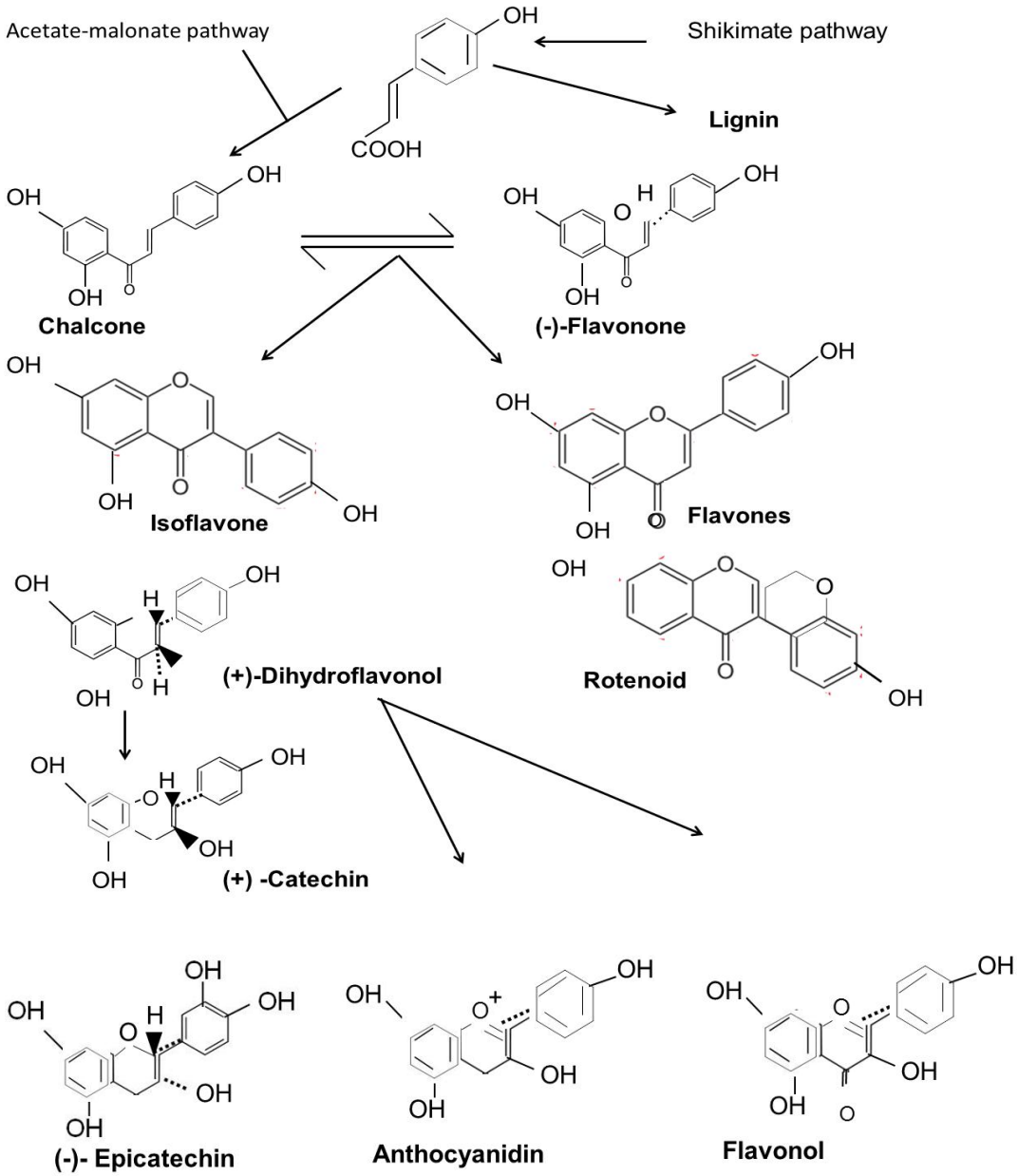

Figure 7 Flavonoid classes biosynthesis pathways [52]

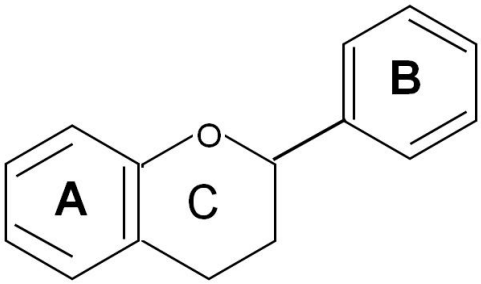

Figure 8 The basic structure of flavonoids[52]

The flavonoid ring A is derived from the acetate pathway and ring B is originated from the shikimate pathway [9] (Figure 8).

Flavonoids and simple phenols comprise the major class of plant polyphenols. Based on their chemical structure some of the plant phenolics can be linked to the cell wall components such as polysaccharides and lignin. Flavonoids mainly exist as free monomers. Among the other flavonoids anthocyanins is highly water soluble and responsible for plant pigmentation which gives bright color to flowers and fruits. The glycosides of anthocyanidin (eg cyanidin, delphinidine etc) are referred by the term anthocyanin [9].

Classification of dietary polyphenols

Secondary plant components are ubiquitous in plants have been used throughout times for medicinal purposes and many are known to be pharmacologically safe and [52] effective. Polyphenols have drawn great attention due to the potential to reduce various diseases including cardiovascular disease, cancer, and diabetes.

The more than 8000 polyphenols have been grouped as follows Flavonoids including flavonlos (e.g. quercetin), flavones (e.g. apigenin), flavanol (e.g..epicatechin)

flavanones (e.g. naringenin) and anthocyanidines (e.g. Cyanidin) present in pomegranate, berries, tea, parsley, soyabean (Table 2 and Table 3) and other polyphenols were also identified in turmeric (curcumin), Pterostilbene (blueberries), Resveratrol (red wines) (Table2) . Generally, most of the flavanoids identified exist as glycosides conjugated with different sugars or polymerized with other polyphenolics [53].

Flavanoids

These groups are mainly predominant in plants. As mentioned in the literature about 2000 naturally occurring flavonoids were identified. Flavonoids groups were classified into following classes' flavonlos, flavones, flavanols, flavanones and anthocyanidines. Flavonols (e.g. quercetin, kaempferol), flavones (e.g. apigenin) along with their glycosides comprises the most common flavonoid group (Table 2).

Flavonol quercetin: The main flavonol available in human diets is quercetin (3, 5, 7, 3 '4' pentahydroxyflavone), (9). It is widely distributed in various fruits and vegetables, particularly in citrus fruits, apples, onions, parsley, dark berries. Quercetin is one of the major bioactive compounds found in fruits such as apple, berries, and wine [53].

Table 2 Polyphenols grouped under flavonoids, different classes, compounds and dietary sources [53]

Under Flavonoids Group Class	Compound	Dietary Sources
Flavonols	Quercetin	Berries; Apples
Flavones	Apigenin	Parsley
Flavanol	Epicatechin Epigallocatechin-3- gallate (EGCG)	Tea
Flavanones	Naringenin	Orange Peel
Anthocyanidines	Cyanidin Delphinidin	Cherries; Strawberries Pomegranate Dark fruits
Isoflavonoids	Genistein	Soybean

Table 3 Polyphenols grouped carotenoids under different compounds and dietary sources [53]

Group:	Compound	Dietary Sources
Other polyphenolic compounds	Curcumin	Turmeric
	Resveratrol	Grapes; red wine
	Pterostilbene	Blueberries

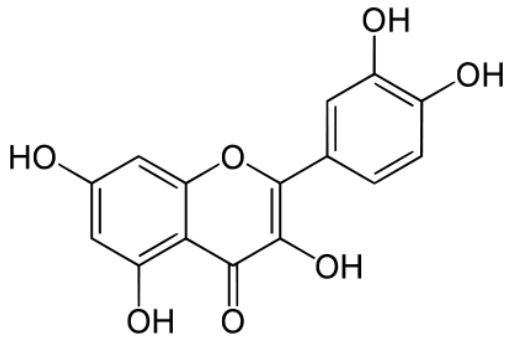

Figure 9 The basic structure of quercetin [51]

Flavones apigenin: The natural occurring flavones present in the human diet are apigenin (4',5,7-trihydroxyflavone) (Figure 10). Apigenin available in fruits and vegetables such as grapefruit , parsley, onions, oranges [54].

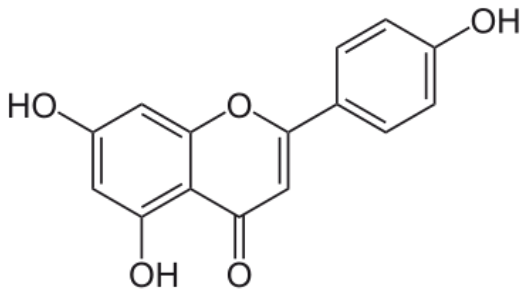

Figure 10 The basic structure of apigenin [51]

Flavanol catechins: The flavanols catechins (Figure 11) and epicatechins, their gallate esters comprises the major constituent of green tea (Figure 12). Three hydroxyl groups (OH) groups are arranged adjacently on the B ring of flavonoids to form their gallos [55].

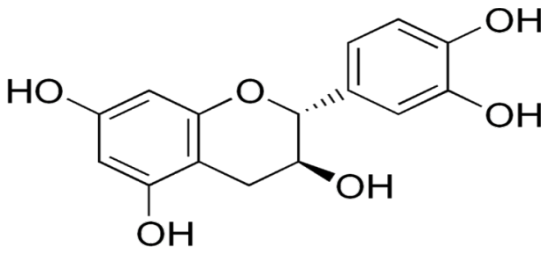

Figure 11 The basic structure of -(+) -catechins [51]

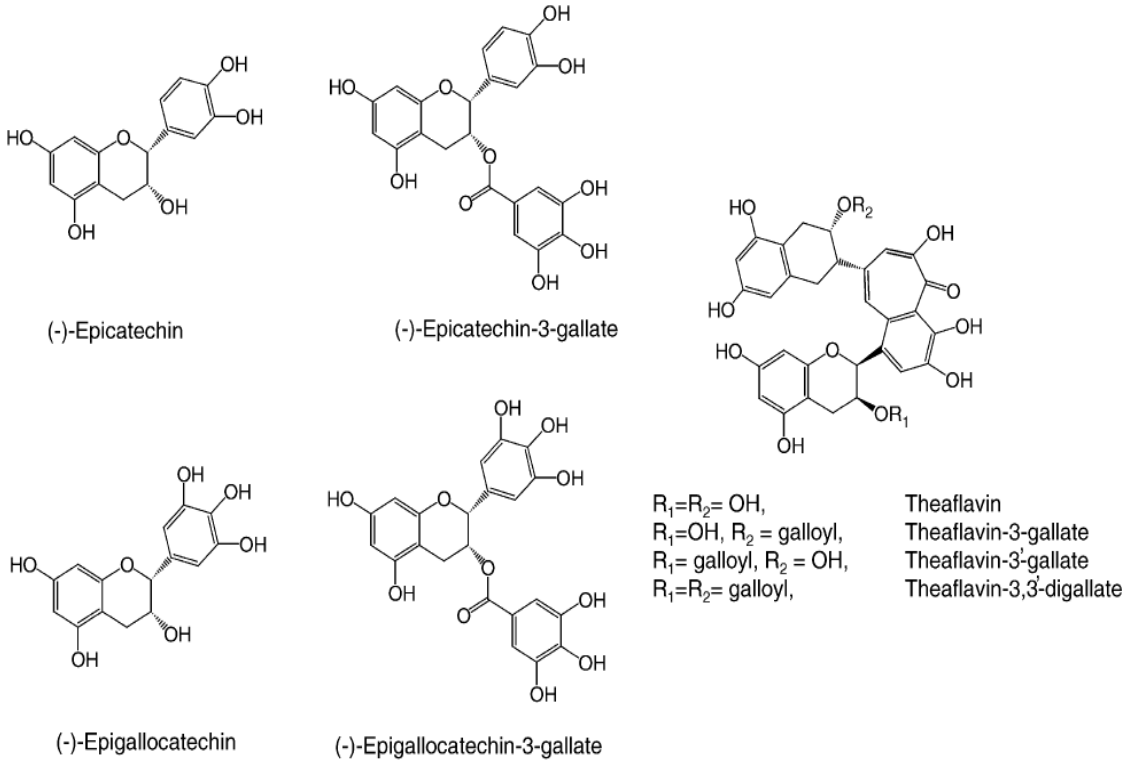

Figure 12 Tea polyphenol [56-58]

Flavanone naringenin: The main flavanone available in human diet is naringenin (Figure 13) an available in orange peel, involved detoxifying phase II enzymes; it induces apoptosis in several cancer cells and also HaCaT p53-mutant human keratinocyte cells [59].

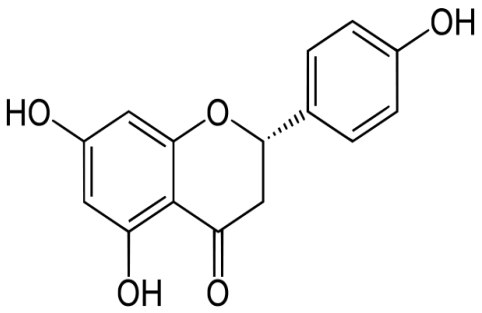

Figure 13 The basic structure of naringenin [51]

Anthocyanidins: Anthocyanins are an abundant group of flavonoids (Figure 14) present in several fruits and vegetables such strawberries, cherries pomegranate, red cabbage, sweet potato, red radish and other bright color fruits [7]. Different anthocyanidin such as cyanidine, delphinidin and pelargonidin comprises the phenolic group called flavonoids [7, 60-63]. Structural differences between anthocyanin groups depend on the presence of hydroxyl groups and also number, nature and position of the sugar-group attached [64].

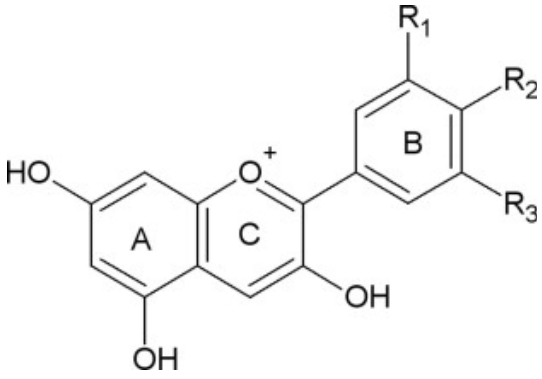

Figure 14 The basic structure of anthocyanidine [10]

Delphinidin, is a major anthocyanidin present in several pigmented fruits and vegetables. Delpinidine is a diphenylpropane-based polyphenolic ring structure with positive charge in its central ring [7]. Cyanidine (Figure 15a) is another important anthocyanidin present in pigmented fruits and vegetables. Delphinidin appeared to have stronger biological activity due to the presence of three hydroxyl group in β-ring [7] (Figure 15b). Pelargonidin is present in pomegranate (Figure 15c).

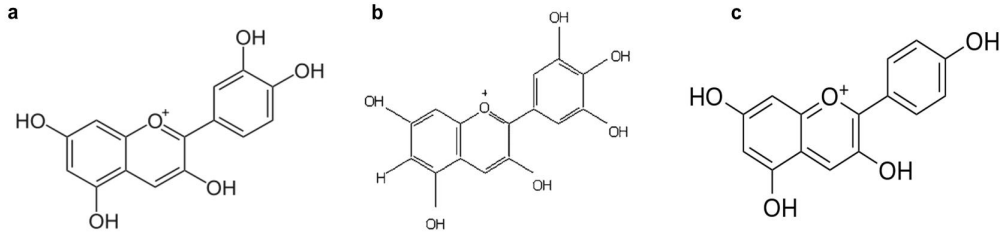

Figure 15 The basic structure of a. cyanidine; b. delphinidin and c. pelargonidin [51]

Other polyphenolic compounds Curcumin, Resveratrol and Pterostilbene Besides flavonoids other dietary flavonoids compound exists in fruits, vegetables and spices shows health benefits (Figure 16).

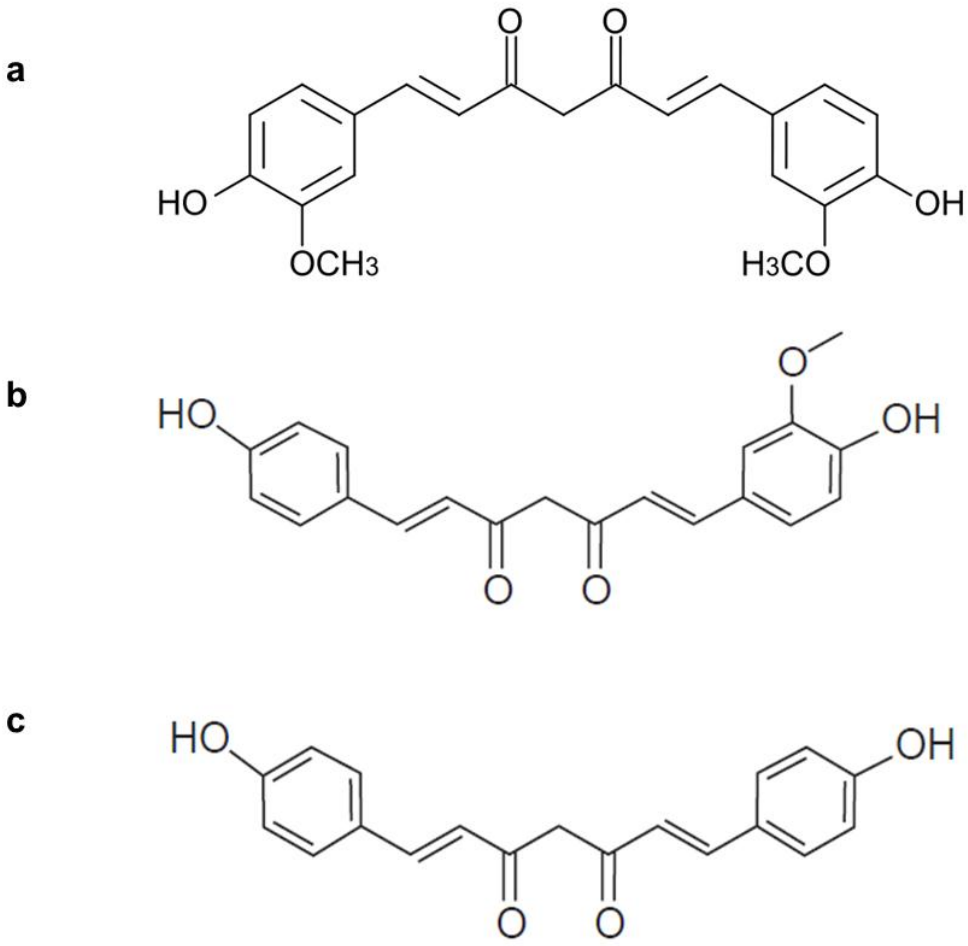

Figure 16 The basic chemical structures of (a) Curcuminoids curcumin (Diferuloyl methane) (b) Demethoxy Curcumin (p-Hydroxy-cinnamoyl-feruloyl-methane) (c) Bisdemethoxy Curcumin (p-Didydroxy-dicinnamoyl-methane) [6, 8, 65]

Curcumin Curcumin (diferuloylmethane) derived from the root of Curcuma longa L. (Figure 16) basic chemical structure Curcuminoids curcumin (Diferuloyl methane, Demethoxy Curcumin (p-Hydroxy-cinnamoyl-feruloyl-methane) and Bisdemethoxy Curcumin (p-Didydroxy-dicinnamoyl-methane) mainly used as spices.

Resveratrol Resveratrol (3,4',5-trihydroxy-trans-stilbene), a phytoalexin exists in two isoform trans- resveratrol and cis- resveratrol (Figure 17) has been shown to

exhibit a wide range of medicinal and biological properties [8]. Resveratrol is found in grapes, red wine etc. Resveratrol is absorbed in human liver as resveratrol- glucuronide. Trans- resveratrol is known to be more stable form.

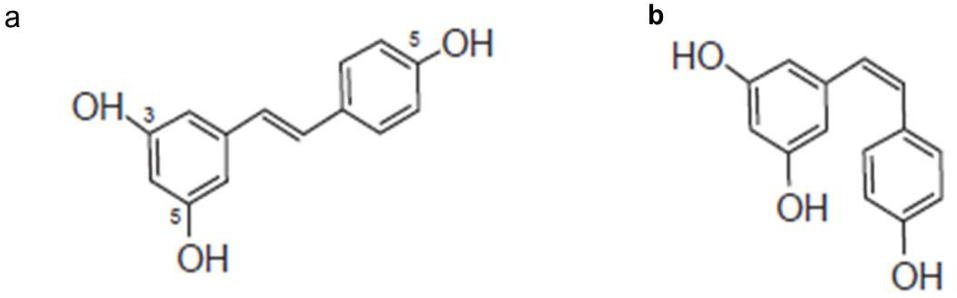

Figure 17 The basic chemical structure of resveratrol (a) trans- resveratrol and (b) cis-resveratrol[53]

Pterostilbene It is a natural dimethylated analogue of resveratrol derived from blueberries and grape. The chemical structure is trans-3,5-dimethoxy-4'-hydroxystilbene (Figure 18).

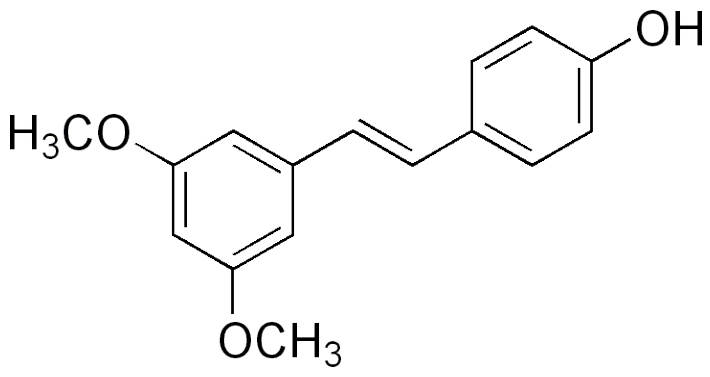

Figure 18 The basic chemical structure of Pterostilbene [53]

In vitro and in vivo studies showed that pterostilbene suppresses tumor growth and metastasis without toxicity. Pterostilbene shown much higher bioavailability compared to other stilbene compounds. However the molecular mechanism of pterostilbene and its effects in humans are not well understood [66].

Polyphenols in pomegranate, mango and plum

Pomegranate

Pomegranate (Punica granatum L.) which is found mainly in the Mediterranean region, India and China, is rich in polyphenols. Pomegranate fruit have been historically used for medicinal purposes. It has drawn attention due to its potential health benefits that have been demonstrated in several studies [53, 67-69]. Some of the most important biochemical components of pomegranate are hydrolyzable tannins ellagitannins, such as punicalagins and punicalins, and flavonoids (Figure 19). Polyphenols in pomegranate can be consumed in the form of whole fruits, dietary supplement or juice[69].

Predominant and therapeutically relevant compounds present in pomegranate, other than ellagic acid and hydrolysable tannins ellagitannins, are the flavonoids anthocyanins delphinidin, cyanidin, and pelargonidin; conjugated 3-glucosides/3,5-diglucosides such as pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucoside, pelargonidin 3,5-diglucoside, cyaniding 3,5-diglucoside, and delphinidin 3,5-diglucoside (Figure 20) [70]. Ellagitannins (ETs) (Figure 21) are the most common polyphenols found in pomegranate and are also available in other berry fruits such as red raspberries, black raspberries, strawberries, muscadine grapes, and some nuts [69].

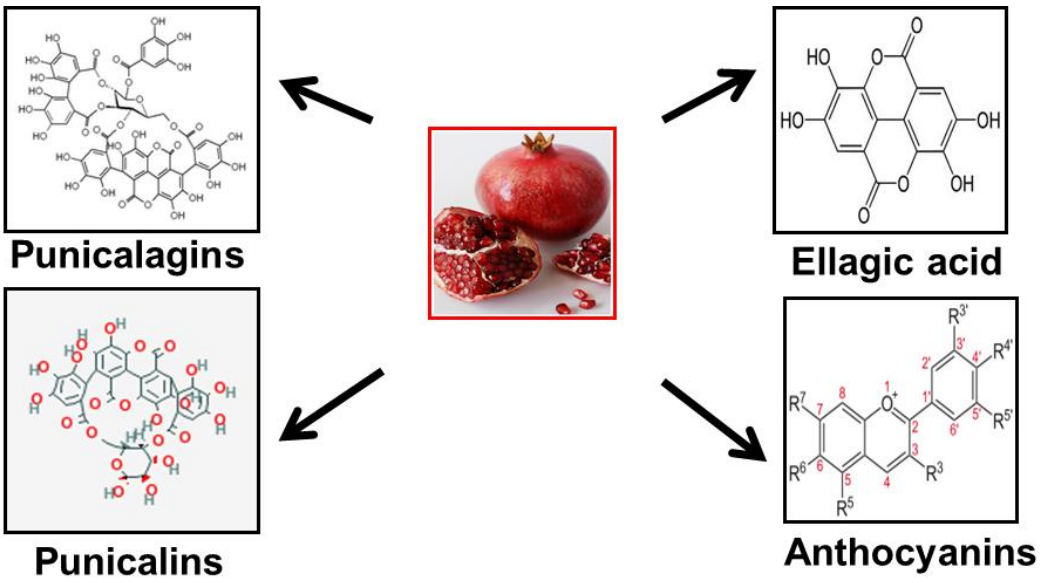

Figure 19 Chemical structure of polyphenols presents in pomegranate ellagitannins, punicalagins and punicalins, free ellagic acid and anthocyanins [53]

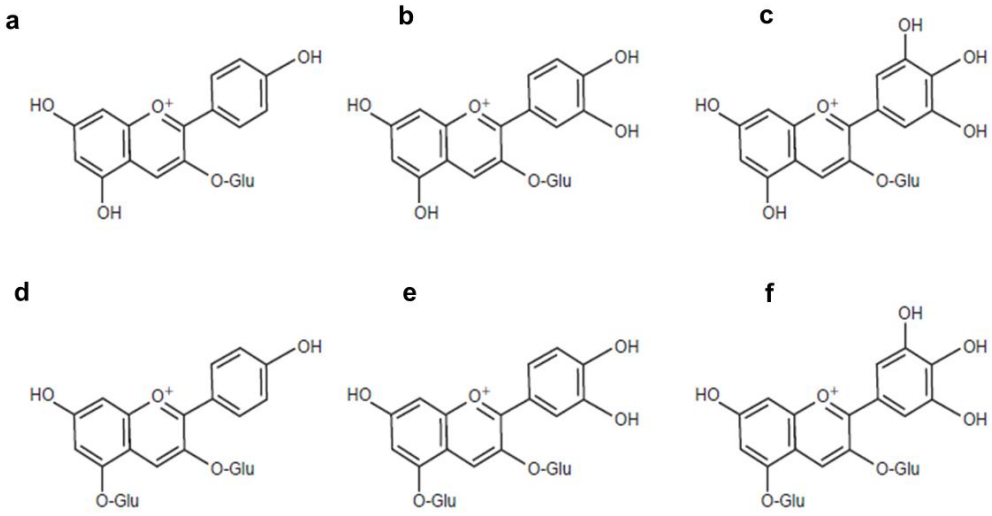

Figure 20 Chemical structure of anthocyanins presents in pomegranate (a) pelargonidin 3-glucoside, (b) cyanidin 3-glucoside (c) delphinidin 3-glucoside (d) pelargonidin 3,5-diglucoside, (e) cyaniding 3,5-diglucoside, and (f) delphinidin 3,5-diglucoside) [53]

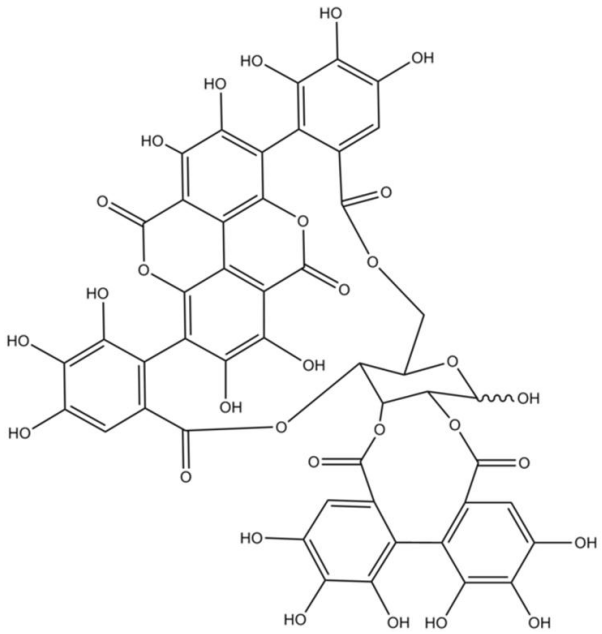

Figure 21 Chemical structure of punicalagin the hydrolysable tannins [53]

Mango

Mango (Mangifera indica L.) is a worldwide consumed tropical fruit. Mango pulp, peel and seeds are rich in bioactive compounds (Figure 22). The most predominant and therapeutic compounds present in mango are gallotannins, gallic acid, galloyl glycosides and flavonoids such as quercetin and kaempferol glycosides. Mango extract also contains mangiferin and complex mixture of other antioxidant polyphenols [71, 72]. Ripe mango contains the highest amount of gallic acid content and polyphenolics compared to other fruits.

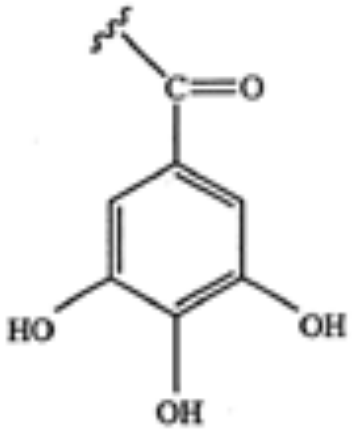

Figure 22 Chemical structures of gallotannins the hydrolysable tannins [53]

Plum

In plums, therapeutically relevant compounds are flavonols, (the flavan 3-ols catechin, and epicatechin; the flavonols quercetin 3-glucoside), anthocyanins (cyanidin 3- galactoside, 3- glucoside, 3- rutinoside, and 3-acetylglucoside), procyanidins (dimers and trimmers), phenolic acids (chlorogenic acid and neo-chlorogenic acid) and carotenoids [73-75] (Figure 23).

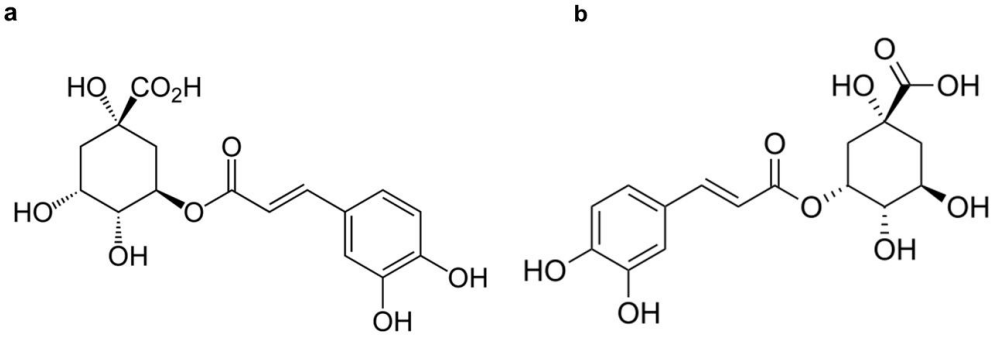

Figure 23 Chemical structures of (a) chlorogenic and (b) neo-chlorogenic acid presents in plums

Pharmacokinetics of polyphenolics

In general, polyphenolics are poorly absorbed in animals and humans as indicated by several absorption and bioavailability studies and this presents a limitation to their efficacy in animal and human clinical trials [76-78]. Pharmacokinetic studies are crucial in the evaluation of the efficacy and safety of polyphenolics in chemoprevention and chemotherapy [76-78].

Pharmacokinetics of pomegranate polyphenolics

Previous studies have shown that the pharmacokinetic profile of ellagic acid indicated limited absorption and rapid elimination after oral administration of pomegranate polyphenols [76, 77]. Seeram et al., have shown that with intake of 180mL pomegranate juice containing 318 mg punicalagins and 12 mg of free ellagic acid (EA), EA concentration were increased in the plasma and cleared from the plasma within 5h. The pharmacokinetic profiles of the EA are shown in Figure 24[77].

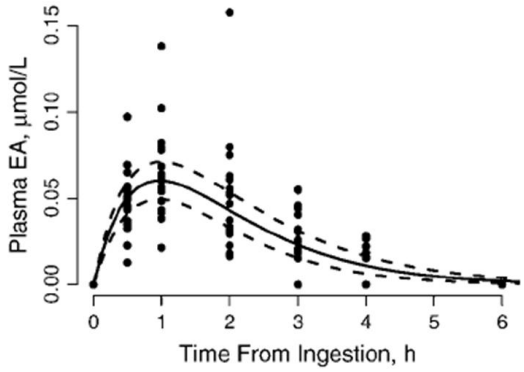

Figure 24 Pharmacokinetic profile of ellagic acid (EA) from pomegranate juice [77]

The process of absorption, metabolism, distribution, and excretion (ADME) of ellagitannins, punicalagins rich pomegranate has been studies in animals and in humans

[79-81]. Dietary polyphenols when interfered with the carcinogenesis process either interact with phase-I reacted and suppress it or activate phase-II reaction and detoxify the carcinogens forming conjugates of phase-I metabolites. Thus the balance between carcinogen activating enzyme and detoxifying enzyme determines the interaction of the cell to carcinogens. Literature studies have shown that ellagitannin punicalagins rich pomegranate juice was absorbed and transformed to ellagic acid and cleared from the circulation rapidly.

Absorption of anthocyanin remains unmodified and can be uptake by cultured cells both in cytoplasm or membrane [82] [7]. In vitro and in vivo studies didn't reveal the formation of anthocyanin residue after treatment [7]. However, pharmacokinetics studies have shown that in human the bio absorption value of anthocyanin is generally poor.

The absorbed pomegranate polyphenols undergo metabolism and detoxification regulated by phase II enzymes such as glucuronosyl transferases and sulfo-transferases that increased water solubility. Less water soluble metabolites were also formed by enzyme such as catechol-O-methyl transferase (COMT). The activity of these metabolizing enzymes depends upon its gene expression [76, 77, 83-85]. The metabolities of pomegranate polyphenols were ellagic -acid, dimethylellagic acid glucuronide, urolithin A , urolithin B were identified [76, 77].

Pharmacokinetics of mango polyphenolics

The most important and therapeutic compound polyphenols present in mango are gallotannins, gallic acid, galloyl glycosides and flavonoids such as quercetin and

kaempferol glycosides. Higher molecular weight gallotannins rich mango juice is absorbed and transformed to gallic acid [86, 87]. Previous studies have emphasized the hydrophilic composition of mango plyphenolics which are more compatible with cell-based assays and may have a higher bioavailability as opposed to hydrophobic compounds [86, 87].

Pharmacokinetics of plum polyphenolics

Previous studies have shown the pharmacokinetics study of chlorogenic acid after intravenous administration [88]. The antioxidant and antitumorigenic properties of chlorogenic acid and caffeoylquinic acid derivatives have been demonstrated in vitro and preclinical studies. In order to emerge as an effective chemopreventive and chemotherapeutic agent, chlorogenic acid neo-chlorogenic acid and caffeoylquinic acid rich plum juice need to be absorbed and metabolized. Pharmacokinetics studies demonstrated a quick absorption and rapid distribution of chlorogenic acid followed by a slow elimination [89]. In human clinical trials, it has been found that chlorogenic acid is differently absorbed, distributed, metabolized and eliminated (ADME) [90].

Polyphenols in cancer prevention and treatment

In addition to conventional therapies there is a need to develop novel therapeutic agents in the prevention and treatment of cancer [91-93]. In order to improve therapy success and survival rates it is crucial to develop novel therapeutic agents in addition to conventional treatments. Diet containing polyphenol that are present in vegetables, fruits, spices, green tea play a crucial role in the prevention of cancer. Dietary polyphenols are known to prevent carcinogenesis, inhibit cancer growth and

suppress transformation, inflammatory process, angiogenesis and metastasis in vitro and animal studies [5, 94]. Epidemiological and experimental studies illustrate the anti-tumorogenic, anti-inflammatory and anti-proliferative effects of a polyphenol-rich diet including cruciferous vegetables, curcumin, grapes, pomegranates that all contain complex mixtures of polyphenols[5, 94].

Polyphenols have drawn some attention due to their chemopreventive properties and chemotherapeutic potential without side effects that are known from conventional cancer therapeutics [52]. In addition to their potential as anti-cancer, anti-oxidant, anti-inflammatory agents in the treatment of hypertension, cardiovascular disease, cancer, diabetes and others it is also used in the industries such as cosmetics, paints, food industry as additive agents [9]. The nutritional significance of natural polyphenols such as flavonoids, tannins and other small polyphenols in the epidemiological, preclinical and clinical trials for the prevention and treatments of chronic diseases such as cancer has been demonstrated [45, 46, 93, 95]. Polyphenols are known to interact at the tumor initiation, promotion and progression level (Figure 25) [52].

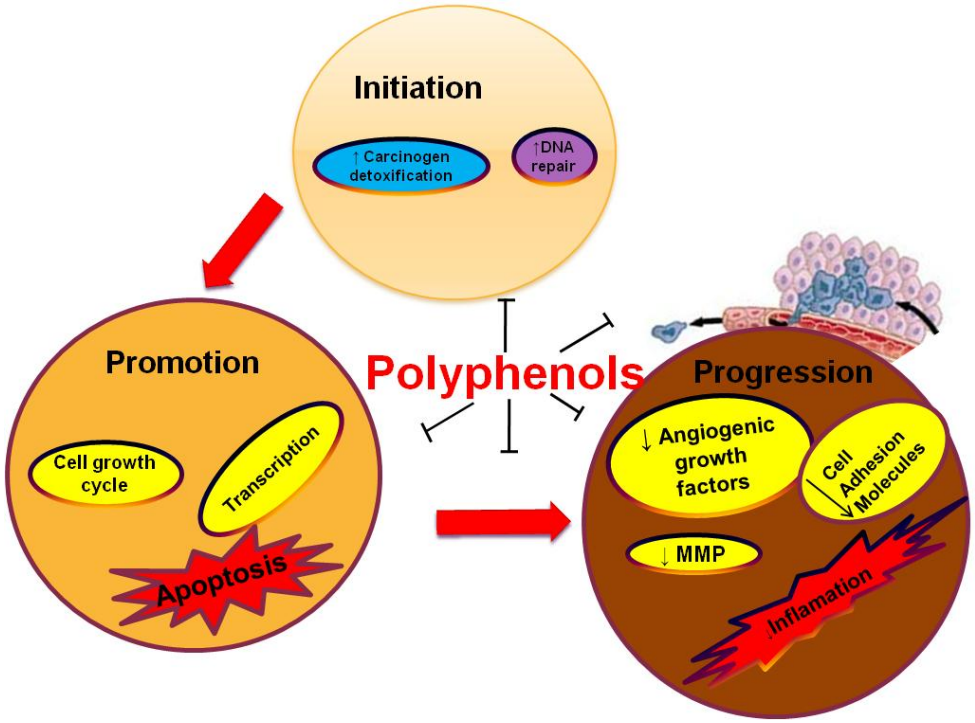

Figure 25 Polyphenolics interaction with multistage carcinogenesis process [52]

Polyphenols and their targeted molecular mechanism

Several studies are demonstrated that many classes of polyphenols alter signal transduction pathways by modulating the expression of key element involved in cell proliferation, apoptosis, differentiation, inflammation as well as angiogenesis and metastasis [52]. In cancer cells, the presence of excess reactive oxygen and nitrogen species such as hydrogen peroxide (H_2O_2), hydroxyl radical (^{-}OH), superoxide (O_2^{-}), and peroxynitrite (ONOO-) oxidative stress is increased [52]. According to several reports, the production of H_2O_2 can inhibit cell apoptosis, increase cell proliferation, angiogenesis, invasion and metastasis [52]. In general, cancer cells have a higher expression of H_2O_2 and this may contribute to malignant transformation by inducing

damage to DNA, proteins, and lipids, and this may contribute to uncontrolled cell proliferation [52].

Polyphenolic compounds modulate multiple molecular and cellular pathways inhibitingcarcinogenesis. Cell signaling kinases including MAPK, Phosphatidylinositol 3 kinase PI3K/AKT have been identified targets of several dietary polyphenols [69, 96-100].

Polyphenols have been demonstrated to act on transcription factor such as NFκB, Specificity protein (Sp) Sp1, Sp3 and Sp4, STAT's [101]. It also interfere cell cycle protein CDK's and Cyclins and apoptotic proteins such as Caspases, Poly (ADP-ribose)-polymerase1 (PARP), Bax. In addition polyphenols also targets anti-apoptotic marker such BCL-2, survivin and cell proliferation, angiogenesis and metastasis markers such as Ki-67, PCNA, iNOS, Cox-2, VEGF, CD-31 and MMPs are also identified as dietary polyphenols targets [101].

In order to understand the pathogenesis of cancer and to inhibit tumor development it is important to identify the gene involved and the interference of various dietary polyphenols at different molecular and cellular level. Thus polyphenols decrease DNA damage, increase detoxification of carcinogenesis at tumor initiation stage, inhibit cell proliferation, induce apoptosis and targets various transcription factor at tumor promotion stage or finally at progression stage polyphenols decreased expression of angiogenic marker, cell adhesion molecules and metastatic marker such as vascular endothelial growth factor (VEGF), vascular cell adhesion molecules (VCAM-1) and matrix metalloproteinases (MMPs) respectively (Figure 25).

Polyphenols possess the ability to inhibit precancerous and cancerous cells. Studies presented here investigated polyphenols from whole fruit pomegranate, mango or plum that are known to target several cells signaling pathway. Overall, the effects of pomegranate extract in miR-27a-ZBTB10-Sp and miR-155-SHIP1- PIP3-AKT-NF-kB interactions were investigated. Interactions of Pg with miR126/VCAM-1 and miR126/PI3K/AKT/mTOR axes were identified as the underlying mechanisms. Mango polyphenols targeting AKT/PI3K/p85 pathway and interactions of PL with thePI3K/AKT/mTOR axis were also investigated in this work (Figure 26).

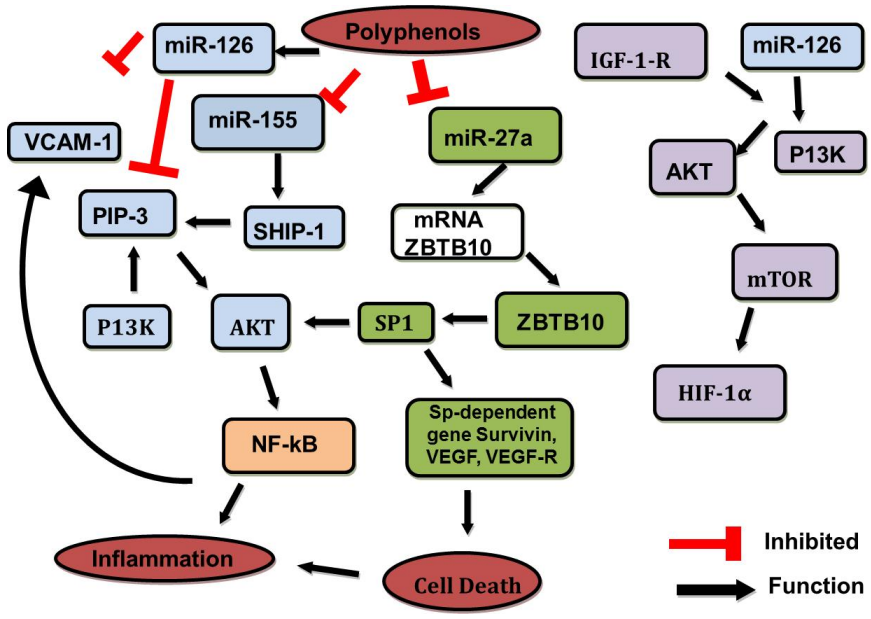

Figure 26 Schematic representation of polyphenols modulating several cells signaling pathway

Cytotoxic activities of polyphenols from fruits presented in this study

Previous studies have demonstrated that whole fruit polyphenols synergistically exhibit stronger anticancer, anti-inflammatory and anti-oxidative

properties in comparison to single compounds. In this work, we have presented a study of the therapeutic application of three different whole fruits, namely pomegranate, mango and plum. Diet consumed by humans with natural polyphenols antioxidants compounds has drawn immense attention for its chemopreventive and chemotherapeutic agents. Among many others, whole fruit juice and extract were commonly used in ancient times for various medicinal purposes [102]. The antioxidant properties of pomegranate juice have been found to be similar that of green tea and significantly higher than that of red wine [103].

Studies have shown that polyphenols is a potent inhibitor of cell proliferation in human breast, prostate, liver, lungs and colon cancer [69, 104-110]. The main objective of this study is to understand the underlying molecular mechanisms of the effect of whole fruit polyphenol extracts on breast cancer cells and on colon cancer. It has been shown that the polyphenol compounds reduce cancer risk, cardiovascular and several other chronic diseases. However, little research has been conducted to investigate the mechanism behind the efficacy of whole fruits. Compared to whole fruit polyphenols, individual isolated compounds appears to be less effective, as they remain at high levels than their actual appearance in plasma after consuming whole fruits [86, 87, 111].

We have investigated the pharmacological and molecular mechanism of Pomegranate whole fruit polyphenols and its beneficial effects. Pomegranate is consumed as fresh fruit, fermented juice, jam, wine, oil and also in extract supplements [102, 103]. The fruit is primarily cultivated in India, China, Afghanistan, Japan and

some parts of the United States. Pomegranate polyphenols (Pg) are a rich source of polyphenolic compounds including ellagic acid in its free and bound forms and hydrolyzable tannins such as, punicalagin and penicillin. These have been demonstrated to have beneficial effects in the treatment of several chronic diseases such as cancer and cardiovascular diseases [49, 69, 103, 110, 112].

Pomegranate polyphenols exert powerful antioxidant, anti-inflammatory, and anticarcinogenic activities in vitro and vivo [47, 48, 50, 113]. The most abundant among these polyphenols is punicalagin the hydrolysable tannins a strong bioactive constituent responsible for more than 50% of the pomegranate potent antioxidant and anticarcinogenic properties.[114]

It has been reported that Pomegranate polyphenols has a higher antioxidant capacity than both red wine and green tea [103]. Polyphenol rich dietary supplement Pomegranate juice scavenges free radicals and decreases macrophage induced oxidative stress, lipid peroxidation and antherogenesis in animals [115-118]. Several studies have demonstrated the anti-inflammatory effects of Pomegranate juice in atherosclerotic mice model [112, 119, 120]. Pomegranate wine is also known to prevent the activation of NF-kB in vascular endothelial cells [121]. Literature studies have shown that pomegranate polyphenols modulates several pathways in cancer models [47, 49, 50, 69, 104, 106, 108, 109, 122]

Commercial pomegranate juice exhibits potent antioxidant and antiatherosclerotic [103, 123-126] properties. Punicalagin is the most predominant polyphenols present in the fruit husk and during processing. Punicalagin is present in

pomegranate juice and is significantly more than 2 g/L concentration[103]. Single compound such as ellagic acid and tannins have been shown to possess anticancer properties, such as apoptotic, cell cycle arrest properties and suppression of tumor growth in animal models [127]. Recently, several studies have demonstrated the effect of pomegranate polyphenols on xenografts tumor growth, apoptotic, angiogenic, antiproliferative properties and suppression of nuclear factor- kB (NFkB) activity [47, 67, 69, 70, 95, 105, 106, 109, 119, 128-131]. Several preliminary data on the NFkB pathway targeted by pomegranate polyphenols have been shown but the exact molecular mechanism is still unknown. The findings however are consistent with other plant polyphenols such as red wine Resveratrol, green tea catechins, curcumin and betulinic acid, all of which exhibit antiproliferation and induce apoptosis by modulating NFkB pathways [132-139].

In this study our objective is to understand the modulation of NFkB and several cell signaling pathways in cancer models treated with whole fruit polyphenols (Figure 27). Nuclear factor-κB (NF-κB), a transcription factor plays a key role in the regulation of several genes important in cellular activity such as inflammation, cell growth, and progression. There is evidence that the specificity protein (Sp) transcription factors (Sp1, Sp3, Sp4), are overexpressed in tumors leading to the proliferative and the angiogenic phenotype associated with cancer cells and also induce inflammation by interacting with the p65 unit of NF-kB [50, 135, 138-141]. Several botanicals have been shown to interact with the specificity proteins, and Sp-repressor zinc finger protein ZBTB10, thus inhibiting cancer cell growth in vitro and in vivo. Specificity proteins regulated several

genes are important for angiogenesis, inflammation, and cell proliferation such as vascular angiogenic growth factor (VEGF), NF-kB and survivin, [50, 135, 138, 139, 141]

Similarly previous studies have demonstrated that pomegranate reduces inflammation by inhibiting PI3K dependent phosphorylation of AKT in lung cell lines and in vivo thus reducing the activation of NF-kB [69, 106]. Previously, resveratrol and quercetin, were demonstrated to reduce NF-kB-involved inflammation as well as induce apoptosis [142-145]. The objective of this present work is to understand the post-transcriptional targets involved in inflammatory and apoptotic pathways in estrogen receptor positive (ER+) cancer cells treated with polyphenolics extracted from pomegranate (Pg) and additionally in nude mice xenografted with cancer cells focusing on the anti-inflammatory and pro-apoptotic pathways involving ZBTB10-Sp transcription factor-axis and the PI3K-AKT-axis, both regulating inflammation through NF-κB as well as apoptosis.

We have also investigated the effect of pomegranate polyphenols in colon cancer cells and potent carcinogenesis azoxymethane (AOM) induced colon cancer rat model.

Previous studies have shown chemopreventive activity of pomegranate extract in colon cancer cells [95]. Pomegranate extract is known to suppress NF-κB activation and also down-regulates NF-κB dependent genes involved in proliferation (Cox-2) angiogenesis (VEGF), and metastasis (ICAM-1, VCAM-1) of the tumor [69, 106, 138, 146, 147]. Vascular cell adhesion molecule-1 (VCAM-1) is known to be a prognostic

marker for colon cancer study [148-150]. VCAM-1 is a cell-surface glycoprotein that is responsible for cell-to-cell interactions. Overexpression of VCAM-1 is observed in colon cancer [148-150]. These findings support our hypothesis that pomegranate extract inhibits NFκB activation and down-regulates the NFκB dependent gene VCAM-1 (Figure 27).

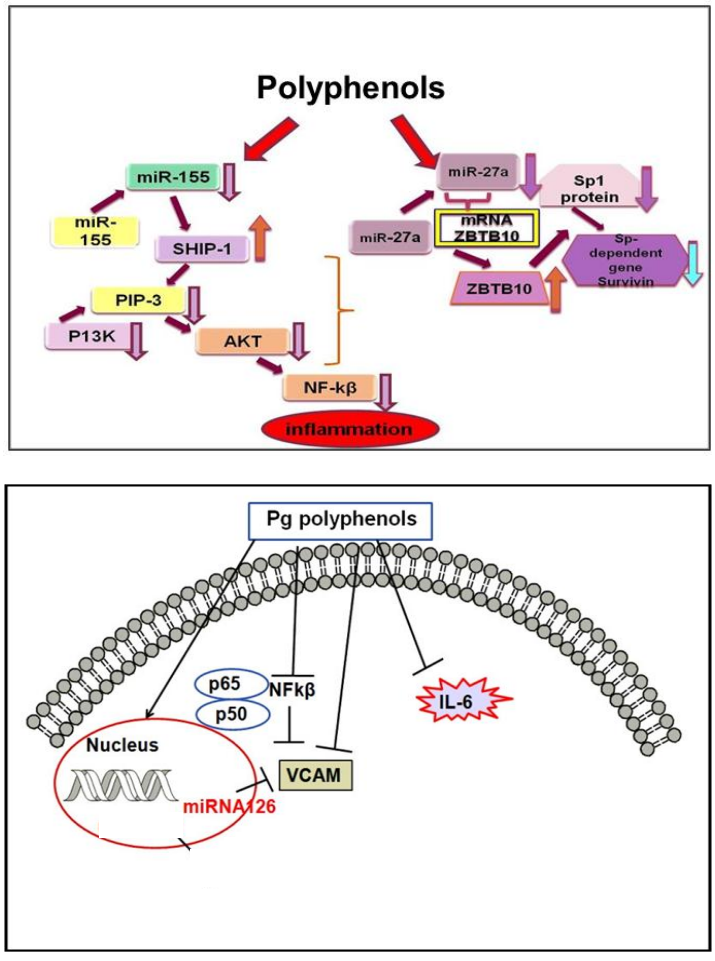

Figure 27 Schematic diagram of the relationship between inflammations, and apoptosis

Mango polyphenols are known to inhibit cell proliferation in several cancer cell lines such as colon, lung, prostate and breast, cancer cells [86, 87, 111, 151]. Previous studies have shown the antioxidant, anti-inflammatory, antitumorogenic and anti-proliferative properties of mango extract [86, 87, 111, 151]. In addition several literatures showed the potential role of mango polyphenols in modulating several cellular signaling pathways in cancer model.

Previous studies indicated the anti-proliferative, anti-inflammatory and anti-tumorigenic properties of mango extract [86, 87, 111, 151]. The potential role of polyphenols present in mango was shown beyond antioxidant and anti-inflammatory properties by modulating several cell signaling pathways in cancer model. Mg modulates NF-κB transcription factor, which regulated genes involved in cell proliferation, inflammation, angiogenesis and metastasis in cancer development [96, 138, 152, 153]. Mango polyphenol, mangiferin, have shown chemopreventive and chemotherapeutic action against benzo(a)pyrene induced lung cancer in swiss albino mice [154]. Similarly mango polyphenols are also known to reduced prostate cancer in mouse and inhibited human prostate LNCaP cell proliferation through apoptosis [111].

Previous studies have shown that polyphenolics derived from plums have chemoprevention and chemotherapy potential against cancer development. Plum polyphenols act as cancer inhibiting agents through scavenging free radicals and inhibiting tumor growth. Plum polyphenols suppress tumor development by inducing tumor cell apoptosis and by modulating different signal transduction pathways. Several literature studies have shown the chemoprevention and chemotherapeutic properties of

flavan 3-ols, flavonols, anthocyanins. However, not many studies have been performed on phenolic acids such as chlorogenic and neo-chlorogenic, which are known to be less potent polyphenols to inhibit cell cycle and induce apoptosis [73-75, 155].

Chlorogenic and neo-chlorogenic acids are the esters formed between caffeic acid and quinic acid. These compounds have shown a wide range of biological and pharmacological activities and have been used as a cancer blocking agent, suppressed reactive oxygen species (ROS) mediated inflammation and inhibited cancer growth in several cancer models such as azoxymethane (AOM) treated colon cancer, liver, breast, oral and tongue cancer [74, 155-161]. Regarding the cancer suppressing properties of chlorogenic acid, it targets different molecular pathways for different cancer models. In addition, Chlorogenic and neo-chlorogenic acid also acts as a strong antioxidant dietary compounds. In vitro, these compounds scavenge free radicals, increase the resistance of LDL to lipid peroxidation and inhibit DNA damage[74, 155].

As previous studies have shown, chlorogenic acid play an important role as an antioxidant agent and function as a repressor of metabolic activation of carcinogens. Chlorogenic acid also acts as a modulator of intracellular signaling pathways. In vitro and preclinical animal studies have established the antioxidant and anticarcinogenic activities of chlorogenic acid and neo-chlorogenic acid.

However, despite the high potential of polyphenolics in cancer prevention and treatment efficacy and molecular mechanisms are not conclusively investigated. Thus in the presented research it was the objective to understand the efficacy of polyphenols present in pomegranate, mango and plum and underlying molecular mechanisms.

CHAPTER II

CYTOTOXICITY OF POMEGRANATE POLYPHENOLICS IN BREAST CANCER CELLS IN VITRO AND VIVO - POTENTIAL ROLE OF MIRNA-27A AND MIRNA-155 IN CELL SURVIVAL AND INFLAMMATION *

Summary

Several studies have demonstrated that polyphenolics from pomegranate (Punica granatum L.) are potent inhibitors of cancer cell proliferation and induce apoptosis, cell cycle arrest, and also decrease inflammation in vitro and vivo. There is growing evidence that botanicals exert their cytotoxic and anti-inflammatory activities, at least in part, by decreasing specificity protein (Sp) transcription factors. These are overexpressed in breast-tumors and regulate genes important for cancer cell survival and inflammation such as the p65 unit of NF-κB.

Moreover, previous studies have shown that Pg extracts decrease inflammation in lung cancer cell lines by inhibiting phosphatidylinositol 3,4,5-trisphosphate (PI3K)-dependent phosphorylation of AKT in vitro and inhibiting the activation of NF-kB in vivo. The objective of this study was to investigate the roles of miR-27a-ZBTB10-Sp

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and miR-155-SHIP-1-PI3K on the anti-inflammatory and cytotoxic activity of pomegranate extract.

Pg extract (2.5-50 μg/ml) inhibited growth of BT-474 and MDA-MB-231 cells *but* not the non-cancer MCF-10F and MCF-12F cells. Pg extract significantly decreased Sp1, Sp3, and Sp4 as well as miR-27a in BT474 and MDA-MB-231 cells and increased expression of the transcriptional repressor ZBTB10. A significant decrease in Sp proteins and Sp-regulated genes was also observed. Pg extract also induced SHIP-1 expression and this was accompanied by downregulation of miRNA-155 and inhibition of PI3K-dependent phosphorylation of AKT.

Similar results were observed in tumors from nude mice bearing BT474 cells as xenografts and treated with Pg extract. The effects of antagomirs and knockdown of SHIP-1 by RNA interference confirmed that the anti-inflammatory and cytotoxic effects of Pg extract were partly due to the disruption of both miR-27a-ZBTB10 and miR-155-SHIP-1. In summary the anticancer activities of Pg extracts in breast cancer cells were due in part to targeting microRNAs155 and 27a. Both pathways play an important role in the proliferative/ inflammatory phenotype exhibited by these cell lines.

Introduction

Polyphenols from fruits, vegetables and spices have demonstrated anti-inflammatory and anticarcinogenic activities in vitro and vivo [67, 69, 104, 106, 129, 130, 138, 162-165] . This includes several reports on the cytotoxic activities of polyphenols in the prevention and treatment of breast and other cancer cell lines [8, 67, 104, 122, 129, 166-169]. Pomegranate (Punica granatum L.) is rich in polyphenols. The

predominant and therapeutically relevant compounds are ellagic acid, ellagitannins, flavonoids, and 3-glucosides/3,5-diglucosides of the anthocyanins delphinidin, cyanidin, and pelargonidin [70], that exert antioxidant, anti-inflammatory, and anticarcinogenic activities in vitro and vivo [47, 48, 113]. Polyphenolics from pomegranate juice and peels inhibited aromatase activity relevant to the prevention of breast cancer [104, 170] , exhibited cytotoxic activities in hepatocellular carcinomas in rats [109], and suppressed chemical-induced colon cancer in rats [165, 171]. The inhibition of NF-κB and other inflammatory markers by pomegranate polyphenolics have been reported for breast [104, 130], lung [69, 106] and prostate cancer cell lines [49, 67, 99].

In previous studies, we have demonstrated that the cytotoxicity of several botanicals is due, in part, to downregulation of specificity protein (Sp) transcription factors (Sp1, Sp3, Sp4). These transcription factors are widely overexpressed in tumors and regulate genes required for cell proliferation, survival and angiogenesis [139, 140, 172-176]. Sp-1 is also involved in the regulation of NF-κB through both a GC-rich binding site in the promotor region of the NF-κB p65 subunit and agents such as curcumin that downregulate Sp transcription factors also decreased NF-κB [138].The high expression of Sp1, Sp3 and Sp4 in breast and other cancer cell lines and the effects of botanical drugs on downregulation of Sp transcription factors is due to several pathways, including both proteasome-dependent and independent mechanisms [138, 141]. In breast cancer cells, the high expression of Sp is due, to suppression of ZBTB10, a Sp-repressor by miR-27a. It has been shown that several anticancer agents act through downregulation of miR-27a which is accompanied by induction of ZBTB10 and

downregulation of Sp proteins [138-140, 175-177] and our preliminary screening of miRNAs indicated that miR-27a expression was significantly decreased by pomegranate extract.

Previous studies on the anti-inflammatory properties of pomegranate indicate that pomegranate extract decreased NF-κB [178] [179]. Our preliminary screening of inflammation-involved microRNAs showed that pomegranate extract decreased the expression of miR-155 and this was accompanied by induction of the miR-155-regulated inositol 5'-phosphatase SHIP-1. This phosphatase is a crucial regulator of phosphatidylinositol-3,4,5-trisphosphate (PI3K), a second messenger in the activation of AKT and nuclear translocation of NF-κB [180] [181]. Previous studies reported that Pg extracts decreased inflammation and repressed lung-tumor growth in mice by inhibiting PI3K-dependent phosphorylation of AKT and decreasing the activation of NF-κB in vivo [69, 106]. Moreover, the polyphenols resveratrol and quercetin decreased miRNA-155 and inhibited NF-κB-involved inflammation in a preclinical murine model and in vitro cell study [142, 182-184]. This suggests that pomegranate polyphenolics may act through miR-155, SHIP-1 and AKT associated pathway [185].

Hence, the objective of this study was to investigate the role of miR27a-ZBTB10 and miR-155-SHIP-1 in mediating the anti-inflammatory and cytotoxic effects of pomegranate polyphenolics.

Materials and methods

Botanical extracts

Stiebs Pomegranate Products (Madera, CA) provided the pomegranate juice concentrate (Pg) utilized in this study from the 2009 California crop (Sample # 0622-33808). Pg was stored at 4°C upon receipt and isolated within 48 h. Polyphenols from Pg were diluted with water to facilitate loading and partitioning from a Sep Pack Vac 20 g C18 cartridge (Waters Corp. Milford, MA) previously conditioned with 100% methanol containing 0.01% HCl. Compounds were eluted with 100% methanol and solvent removed by rotary evaporation (Büchi Labortechnik AG, Flawil, Switzerland) at 40°C. Residual water was removed on a speedvac system (Savant, Thermo Scientific Inc, Pittsburgh, PA). The dried extract was stored at -80°C prior to weighing and dissolution in dimethyl sulfoxide (DMSO) for cell culture and analytical procedures.

HPLC-PDA analysis

The pomegranate polyphenolics were analyzed in negative ESI-MS and quantified against a standard of punicalagins A and B and cyanidin-3-gluoside. Separations were made in a SunFire^{TM} C_{18} column (Supelco Inc. Bellefonte, PA) (250 x 4.8 mm, 5 μm) at room temperature. A mobile phase of water/acetic acid was run (98:2) to Phase A and acetonitrile/water/acetic acid was run (68:30:2) in Phase B. A gradient program at 0.4 mL/min initially ran Phase B at 0%, from 0 to 5% Phase B in 1 min, from 5 to 30% Phase B in 15 min, from 30 to 65% Phase B in 40 min, and 65 to 95% Phase B in 50 min before returning to initial conditions. Detection was at 280 and 520 nm for ellagitannins and anthocyanins, respectively. Compounds were tentatively identified

based on mass spectrometric analysis. This was performed on a Thermo Finnigan LCQ Deca XP Max ion trap mass spectrometer with an electrospray ionization probe in negative ion mode under the following conditions sheath gas (N_2), 60 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 3.3 kV; capillary temperature, 250°C; capillary voltage, 1.5 V; tube lens offset, 0 V. Standard compounds for the identification and quantitative analysis of ellagitannins and anthocyanins were obtained from Acros Organics (Morris Plains, NJ.) and ChromaDex (Irvine, CA), respectively.

Reagents

Antibodies against cleaved caspase-3, poly-(ADP-ribose)-polymerase (PARP), NF-κB (p65), phosphorylated NF-κB (p65) as well as SHIP-1 were purchased from Cell Signaling Technology (Beverly, MA). All other antibodies and SHIP-1 small interfering RNA (SiRNA) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Reporter lysis buffer and luciferase reagent for luciferase studies were obtained from Promega (Madison, WI). Invitrogen (Grand Island, NY) supplied LipofectAMINE 2000 reagent. Western lighting chemiluminescence reagent was purchased from Perkin-Elmer Life Sciences (Waltham, MA). Primers for Sp1, VEGF, VEGFR-1, survivin, ZBTB10, SHIP-1 were purchased from Integrated DNA Technologies (San Diego, CA). Primers for Sp3 and Sp4 were obtained from Qiagen; antagomers of miR-27a (inhibitor) and miR155 (inhibitor), as well as scrambled miRNA were from Dharmacon, Inc. (Lafayette, CO). mirVana TM extraction kit, reverse transcription (RT) and real-time PCR amplification kits were purchased from Applied Biosciences (Foster City, CA). Sp1, Sp3

and NF-κB promoter constructs were kindly provided by Dr. Yanan Tian (Texas A&M University).

Cell culture

Human mammary carcinoma cell lines BT474 and MDA-MB-231 as well as non-cancer breast fibroblasts MCF-10F and MCF-12F, were obtained from ATCC and maintained according to the supplier guidelines (American Type Tissue Collection (ATCC, Manassas, VA).

Cell proliferation

The cell proliferation was assessed with an electronic cell counter at 48 h (Z1™ Series, Beckman Coulter, Inc, Fullerton, CA), as previously described, [139] and are presented as net growth.

Real-time PCR analysis of mRNA and miRNA

BT474 and MDA-MB-231 cells were seeded (3 x 10^5 cells onto a 6-well plate) and incubated for 24h to allow cell attachment. Cells were treated with Pg extract and after 24 h mRNA was extracted for gene expression analysis as previously described [139]. RNA-quality and quantity was assessed using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription (Invitrogen Corp., Grand Island, NY) and qRT-PCR were carried out as previously described [139] on an ABI Prism 7900 Sequence Detection System (Applied Biosystems Inc, Foster City, CA). Primers for TBP, ZBTB10, Sp1, Survivin, VEGF and VEGFR-1 have been previously described [139]. The primer-sequences for SHIP-1were F:5'-GTG-GAG-AGA-TCT-GGC-CTC-AG-3' and R:5'-GGG-AGC-AAC-AGC-AAA-GAC-TC-

3', for VEGF, the sequence were F:5'- AAG GAG GAG GGC AGA ATC AT-3', and R:5'- ATC TGC ATG GTG ATG TTG GA-3'. Integrated DNA Technologies, Inc. (Coralville, IA). Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA), homology-searched by an NCBI BLAST and examined by dissociation curve analysis. Primers for Sp3 and Sp4 were purchased from Qiagen. microRNA was extracted, reverse transcribed, and qRT-PCR reaction was performed as previously performed [139, 140].

Transfection with antagomers of miR-155 and miR-27a (inhibitors), miR-27a mimic, or small interfering RNA (siRNA) against SHIP-1 gene

Cells seeded (1x105) into 12-well plates were incubated for 24h to allow cell attachment. Transfections with 10nM siRNA against SHIP-1 gene Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and antagomers (inhibitors) of 20 nM, 40 nM and 80 nM miR-27a and miR-155, and also mimic of 20 nM miR-27a (Dharmacon, Lafay ette, CO) were performed as previously described [138, 139]

Reporter gene transfection and luciferase assays

Cells were transfected with constructs as previously described [138, 139, 164].

Western blotting

Cells (4 × 105) were seeded in 6-well plates and incubated 24 h to allow cell attachment. They were also treated with Pg extract (0-10μg/mL) for 24 h. Cells were harvested and prepared for Western Blotting as previously performed [139, 140]. Proteins were visualized with a Kodak Molecular Imaging System (Carestream Health, Rochester, NY).

Xenograft study

Female athymic BALB/c nude mice (aged 3- 4 weeks) from Harlan laboratory (Houston, TX) were implanted with BT474 cells ($2X10^6$ cells) in matrigel (BD Bioscience San Jose, CA) s.c into the flank [139]. After 10 days mice were treated with either 100 µl vehicles (1% DMSO in corn oil) by oral gavage, or 0.8mg gallic acid equivalent (GAE)/kg/day of Pg extract/d for 35 days after approval by the Institutional Animal Care and Use Committee at Texas A&M University (College Station, TX). Final body and tumor weights were determined. Tissues were flash-frozen in liquid nitrogen and stored at -80˚C for mRNA and protein analysis. 4-AM-thick paraffin-embedded tumor sections were cut and stained with Hematoxylin and Eosin (H&E) for bright field microscopy.

Statistical analysis. Quantitative data represent mean values with the respective standard deviation (SD) or standard error of the mean (SE) corresponding to 3 or more replicates. Data were analyzed by one-way analysis of variance (ANOVA) using SAS version 9.3 (SAS Institute Inc., Cary, NC). Tukey's Post Hoc multiple comparisons were used ($p<0.05$) to establish significant statistical difference.

Results

Polyphenolic composition of pomegranate extract determined by HPLC-MS

The polyphenolic profile of pomegranate varieties was previously reported as being rich in ellagitannins and anthocyanins [47-49, 103]. The primary ellagitannin in pomegranate juice is punicalagin [103], that can be converted into free ellagic acid upon hydrolysis. In this study, a polyphenolic extract was prepared from a pomegranate juice

extract (Pg) that was kindly provided by Stiebs LLC (Kirkland, WA). The chromatographic profiles (Figure 28a and b) show the ellagitannins punicalagin A (peak 1), punicalagin B (peak 2), anthocyanins delphinidin 3-glucoside (peak 3) and cyanidin-3-glucoside (peak 4). Low concentrations of ellagic acid glucoside (peak 5) and free ellagic acid (peak 6) were also detected.

Anti-proliferative and pro-apoptotic activities of pomegranate extract

The cytotoxic activities of Pg were investigated in human BT474 and MDA-MB-231 breast cancer cell-lines and non-cancer breast fibroblasts MCF-10F and MCF-12F (Figure 29a). In both cancer cell lines, there was a concentration-dependent decrease in cell viability after treatment with Pg (2.5-25 μg/ml) after 48 h. In contrast, no significant cytotoxic effects were observed in non-transformed cells treated under the same conditions (Figure 29a). Similar results were observed after 72 h (data not shown). Pg-induced cytotoxicity was accompanied by activation of capase-3, a primary apoptosis-executing enzyme, and the cleaved product of the substrate Poly (ADP-ribose)-polymerase1 (PARP) [186]. Full lengths PARP protein was not decreased by Pg whereas the highest concentration of Pg decreased full length Caspase-3 (Figure 29b).

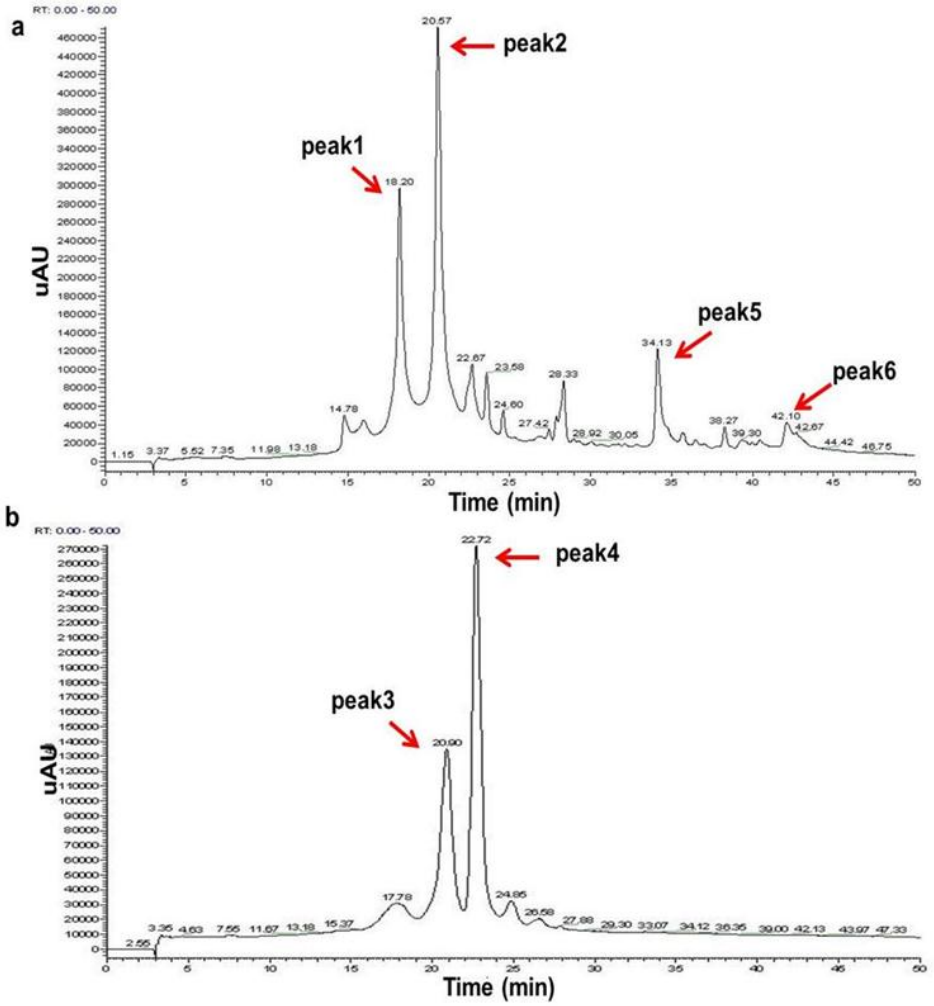

Figure 28 Representative chromatogram of polyphenolic compounds in an extract of mango juice concentrate (a) ellagitannins at 280 nm, tentative peak assignments. 1, punicalagin A; 2, punicalagin B; 5, ellagic acid glucoside and 6, ellagic acid; (b) anthocyanins at 520 nm, tentative peak assignments. 3, delphinidin-3-glucoside; 4, cyanidin-3-glucoside

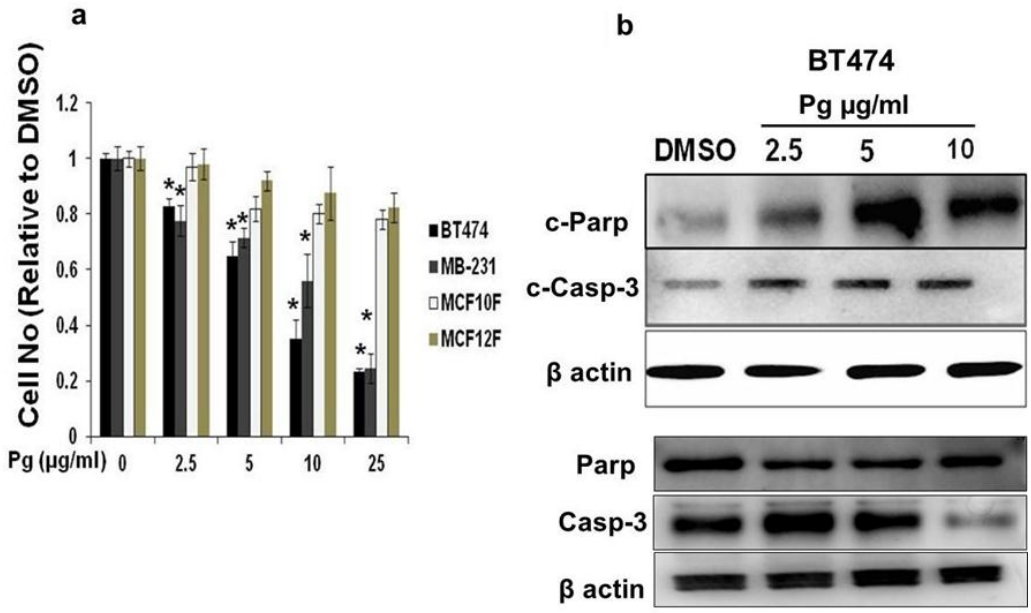

Figure 29 Pomegranate extract (Pg) inhibited proliferation and induced apoptosis in cancer cells (a) cells were seeded and treated with DMSO (vehicle control) or different concentrations of Pg (2.5-10 µg/ml treatment) for 48 h. (b) western blot analysis of apoptosis-associated proteins (Full length capase-3 and cleaved capase-3; full length PARP and cleaved PARP). BT474 cells were treated with DMSO (control vehicle) or different concentrations of Pg extract 2.5-10 µg/ml for 24 h, and whole-cell lysates were analyzed by western blot analysis. All experiments were performed as described in the material and method section and were performed at least three times, and the result was expressed as mean ± SE. * indicates significant changes at $p<0.05$.

Modulation of Sp transcription factors and Sp-regulated genes and disruption of miR-27a:ZBTB10

Treatment of BT474 breast cancer cells with Pg (2.5-10 µg/ml) decreased expression of Sp1, Sp3, and Sp4 mRNA (Figure 30a) and protein (Figure 30b). Pg also decreased luciferase-activity in BT474 cells transfected with a plasmid construct containing the GC-rich regions from the Sp1 and Sp3 promoters [139] (Figure 30c). Additionally, Pg induced expression of the Sp-repressor ZBTB10 (Figure 30d) and

decreased VEGF, VEGFR-1 and survivin mRNA and protein (Figure 30e) and (Figure 30f). [139].

The basal expression of miR27a was higher in BT474 and MDA-MB-231 breast cancer cells compared to the non-cancer breast fibroblasts (Figure 30g). Pg (2.5-10 μg/ml) significantly decreased miR-27a expression only in the cancer cell lines in a concentration-dependent manner, in the non-cancer fibroblasts minimal effects on miR-27a were observed (Figure 30h). When BT474 cells were transfected with the antagomir (Ant.) of miR-27a, miR-27a was decreased and ZBTB10 mRNA was induced and this was comparable to the effects of Pg extract (Figure 30d and h) Both the miR-27a antagomir and Pg extract alone and in combination decrease miR-27a (Figure 30i) and induce ZBTB10 (Figure 30j). Transfected of BT474 cells with miR27a mimic increased miR-27a level and this was partially reversed by the treatment with Pg, indicating that Pg directly or indirectly targets miR27a (Figure 30k). We also observed the Pg extract decreased Sp1, NF-κB (p65) and VEGF protein (Figure 31a) and mRNA (Figure 31b) expression in MDA-MB-231 cells and this was accompanied by induction of ZBTB10 mRNA (Figure 31c) and this is similar to the effects of Pg extract in BT474 cell.

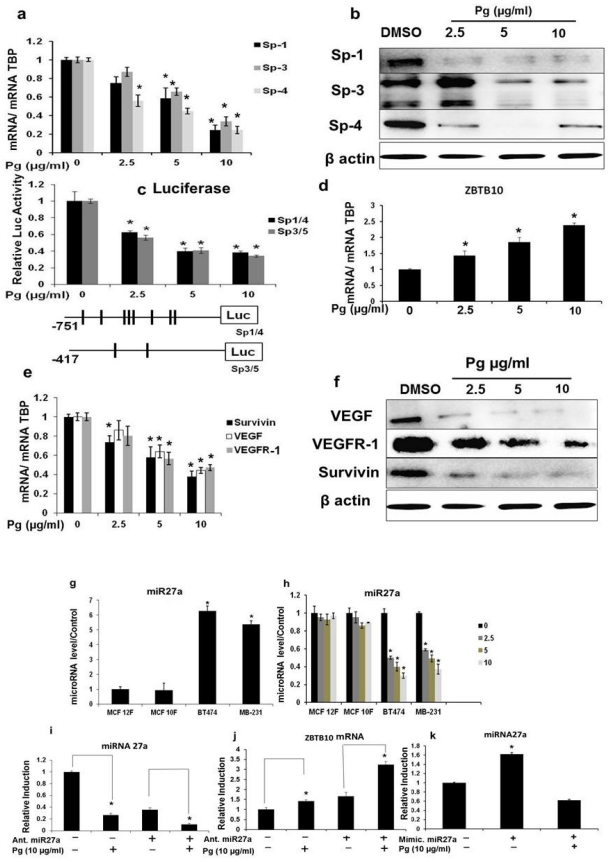

Figure 30 Effects of Pg on Sp1, Sp3, Sp4 and Sp-regulated genes in BT474 cells Pg extract decreased Sp1, Sp3 and Sp4, and Sp-regulated genes and induced apoptosis. Cells were treated with solvent DMSO (control vehicle) or different concentration of Pg (2.5-10 µg/ml) for 24h. (a) expression of mRNA and (b) protein of Sp1, Sp3 and Sp4. (c) the luciferase activity of Sp1 and Sp3 promoter constructs transfected into BT474 cells. The data represent ratios of luciferase/β-gal activity in transfected cells treated with DMSO (control) or Pg (2.5-10µg/ml) for 24h. (d) mRNA levels of ZBTB10 of cells treated with DMSO (control) or Pg (2.5-10µg /ml) for 24h. (e) Survivin, VEGF and VEGFR-1 mRNA and (f) protein levels of cells treated with DMSO (control) or Pg (2.5-10µg /ml) for 24h. (g) miR-27a and (h) miR-27a in different breast cancer and non-cancer breast fibroblasts (i) effects of Pg with and without the antagomir (Ant.) for miR-27a on the expression of miR-27a, (j) on the expression of ZBTB10 mRNA and (k) Effects of Pg with and without the mimic for miR-27a on the expression of miR-27a. All experiments were performed as described in the material and method section and were performed at least three times, and the result was expressed as mean ± SE. * indicates significant changes at $p<0.05$

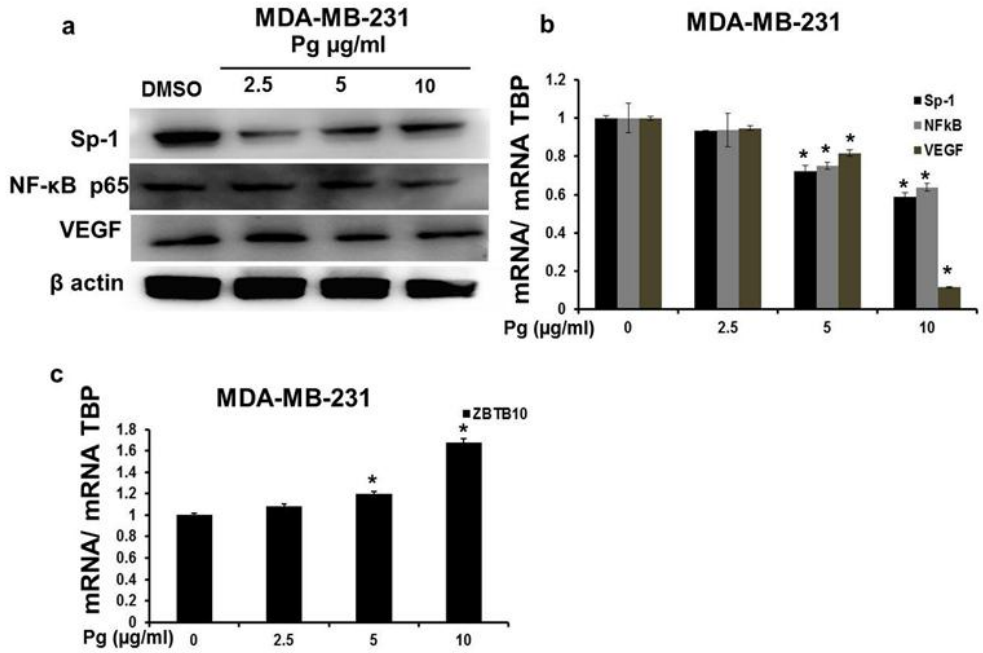

Figure 31 Effects of Pg on Sp1 and Sp-regulated genes in MDA-MB-231 cells (a) expression of protein and (b)mRNA of Sp1, NF-κB and VEGF (c) mRNA levels of ZBTB10 in cells treated with DMSO (control) or Pg (2.5-10µg /ml) for 24h. All experiments were performed as described in the material and method section and were performed at least three times, and the result was expressed as mean ± SE. * indicates significant changes at $p<0.05$.

Pg-induced modulation of SHIP-1, PI3K and AKT and involvement of SHIP-1 and miR-155 in the modulation of AKT and NF-κB

Pg also induced the upregulation of SHIP-1 mRNA and protein in BT474 (Figure 32a and b) and this was accompanied by a decrease in pPI3K and pAKT while but not AKT (total protein) (Figure 32b). Pg extract also caused similar effects on SHIP-1, pAKT, pPI3K and AKT in MDA-MB-231 breast cancer cells (Figure 32c). To better understand the effects of Pg on SHIP-1, PI3K and AKT, BT474 cells were transfected with the siRNA of SHIP-1. SiRNA SHIP-1 increased pAKT protein levels. In contrast,

Pg decreased pAKT protein expression (Figure 32b) and these effects were partially reversed by siRNA SHIP-1 (Figure 32d). MiR-155 is overexpressed in several breast cancer cell lines [187-190]. It was previously reported that polyphenolic extracts decrease miR-155 [142, 182, 191]. Basal expression of miRNA-155 was elevated in BT474 and MDA-MB-231 cells compared to the non-cancer cell lines (Figure 32e). Pg (2.5-10 μg/ml) significantly decreased miR-155 (Figure 32f) in the cancer cell lines (compared MCF-10F and MCF-12F) and this correlates with the upregulation of SHIP-1 mRNA levels in BT474 and MDA-MB-231 cells (Figure 32a and c). Transfection of BT474 cells with miR-155 antagomir of treatment with 10 μg/ml Pg alone or in combination decreased miR-155 expression (Figure 32g) and increased SHIP-1 mRNA level (Figure 32h). Further confirming that Pg extract disrupts miR155: SHIP-1 interaction in BT474 cells.

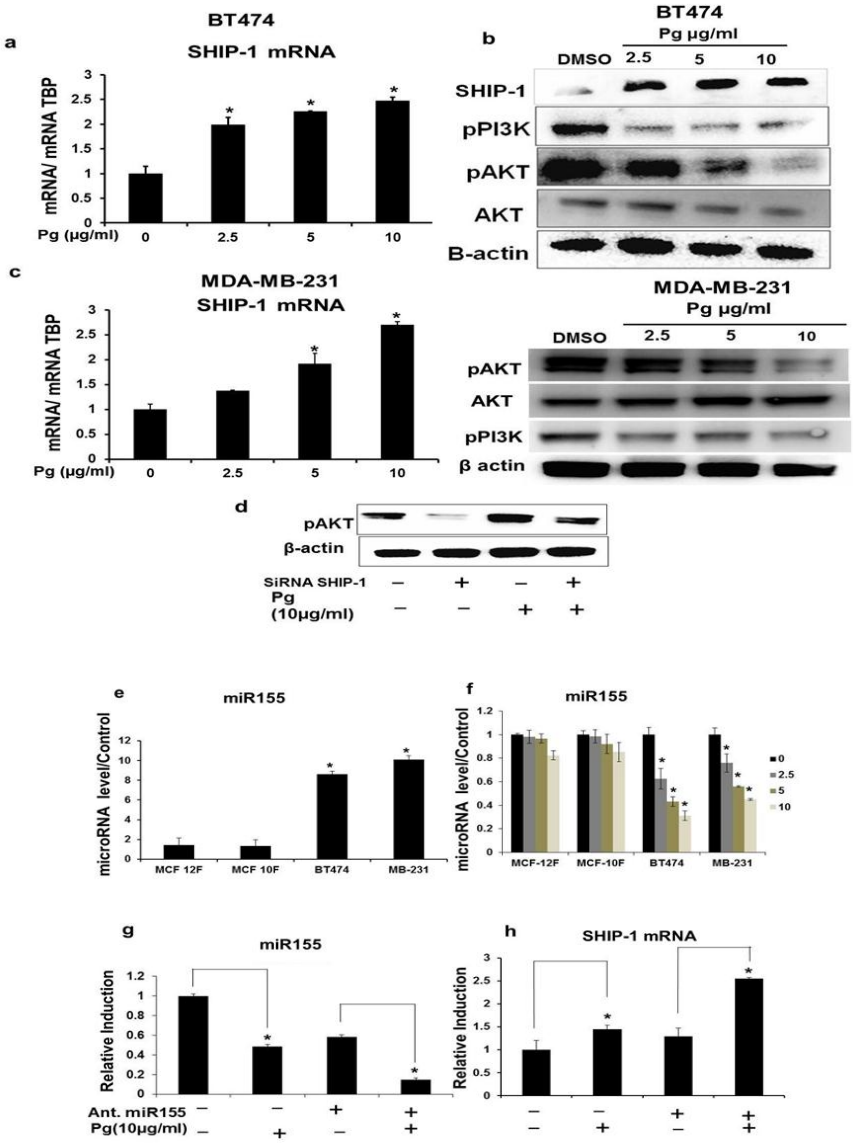

Figure 32 Effects of Pg on AKT-associated kinase pathways in BT474 and MDA-MB-231 cells (a) effects of Pg on SHIP-1 mRNA levels and (b) SHIP-1, pP13K and pAKT protein expression in BT474 cells. Cells were treated with solvent DMSO (control) or different concentration of Pg (2.5-10 µg/ml) for 24 h (c) SHIP-1 mRNA levels and protein expression of SHIP-1, pP13K and pAKT in MDA-MB-231 cells. Cells were treated with solvent DMSO (control) or different concentration of Pg (2.5-10 µg/ml) for 24 h (d) effect of SiRNA SHIP-1 with and without Pg on pAKT protein (e) basal levels of miR-155 in different breast cancer and non-cancer breast fibroblasts. (f) effects of Pg on miR-155 levels in different breast cancer and non-cancer breast fibroblasts. (g) effects of Pg with and without Ant. miR155 in BT474 cells on the expression of miR155 and (H) SHIP-1 mRNA levels. All experiments were performed as described in the material and method section and were performed at least three times, and the result was expressed as mean ± SE. * indicates significant changes at $p<0.05$.

Effects of Pg on NF-κB

Pg significantly inhibited the constitutive expression and phosphorylation of NF-κB p65 in BT474 cells (Figure 33a) and in MDA-MB-231 cells was also decreased by Pg (Figure 31a) Furthermore, Pg extract decreased luciferase activity in BT474 cells transfected with pNF-κB-Luc (Figure 33b) and similar results were observed in cell co transfected with miR-155 (Figure 33c) or miR-27a (Figure 33d) antagomirs. These results suggest that both microRNAs are involved in Pg-induced repression of NF-κB.

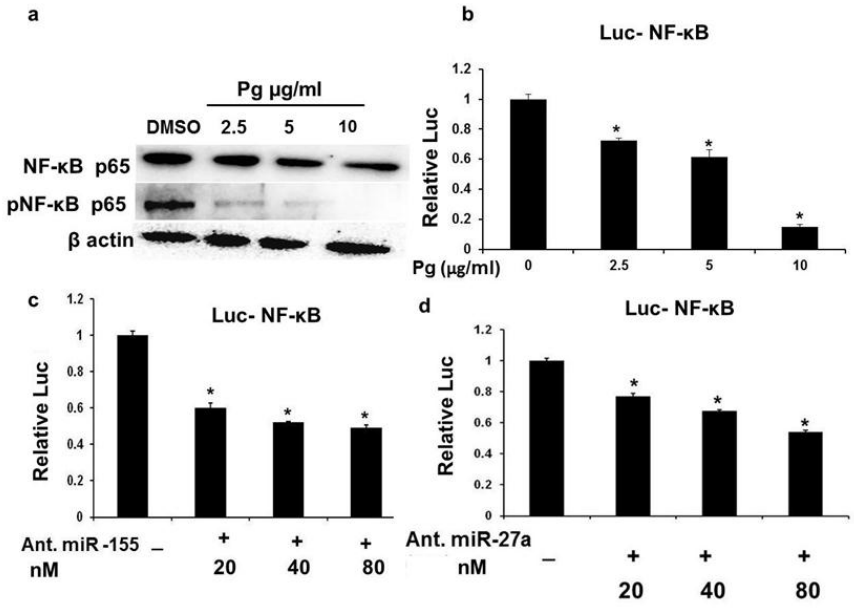

Figure 33 NF-κB expression and activity in BT474 cells (a) effect of Pg on NF-κB and pNF-κB protein levels and (b) luciferase activity in a plasmid containing a NF-κB binding site in the luc-promoter region. Cells were treated with solvent DMSO (control) or different concentration of Pg (2.5-10 µg/ml) for 24h. Effects of (c) miR-155 antagomers (Ant.) and (d) miR-27a antagomers (Ant.) on luciferase activity of the NF-κB promoter construct. Luciferase activity is expressed as ratio to β-gal. All experiments were performed as described in the material and method section and were performed at least three times, and the result was expressed as mean ± SE. * indicates significant changes at $p<0.05$.

Xenograft study in athymic female nude mice

The clinical relevance of the cytotoxic activities of Pg observed in vitro was investigated in an orthotopic model of breast cancer in athymic female nude mice with BT474 cells as xenografts. Treatment with Pg (0.8mg GAE/kg/day/) significantly decreased tumor volume and weight (Figure 34a and b). Histopathologic evaluations of tumors indicated that tumors of Pg-treated animals exhibited with increased apoptotic lesions compared to the control group (Figure 34c). Moreover, Pg decreased the expression of Sp1, mRNA and Sp1, Sp3, and Sp4 protein level (Figure 34d and e) and significantly decreased miR-27a (Fig. 7f) whereas ZBTB10 mRNA was upregulated (Figure 34d). In addition, Sp-regulated genes including VEGF, survivin and NFkB p65 were also decreased at the protein and mRNA level in tumors from mice treated with Pg compared to the control tumors (Figure 34d and e).In addition SHIP-1 mRNA (Figure 34d) and protein (Figure 34e) levels were induced in tumors. This was accompanied by a reduction of SHIP-1-regulated proteins pAKT and pPI3K in mice exposed to Pg (Figure 34e) and miR-155 (Figure 34f) was also decreased.

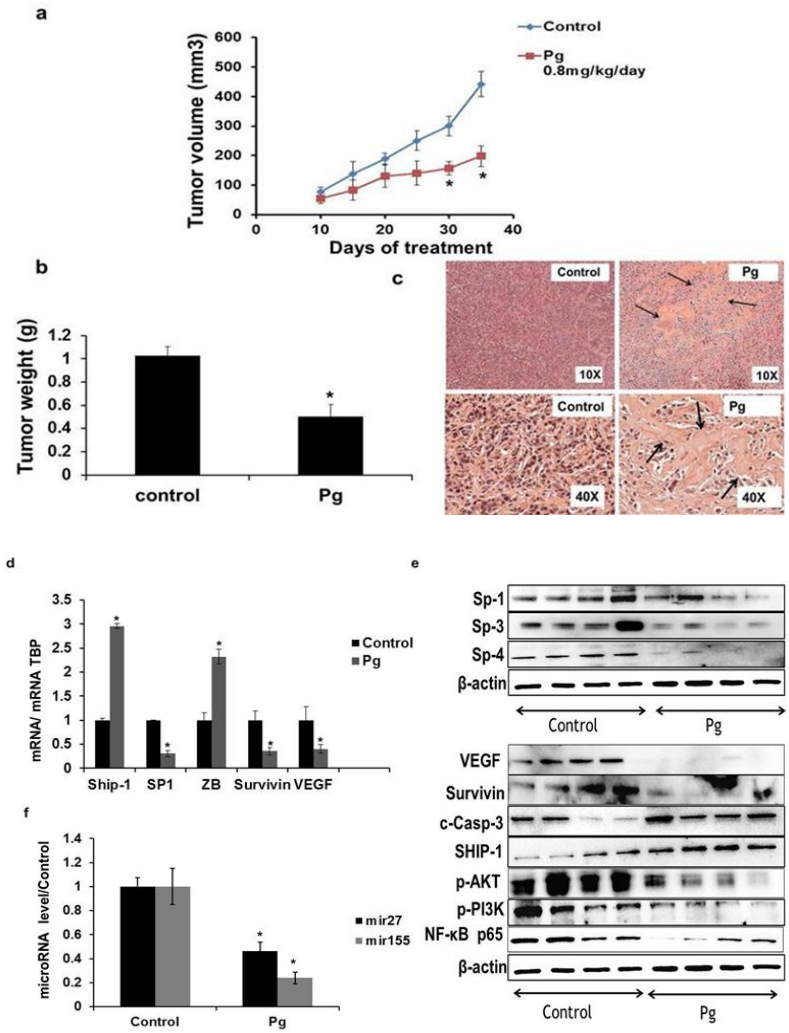

Figure 34 Antitumorigenic activity of Pg extract in vivo (a) in vivo studies athymic nude mice (10 per group) bearing BT474 cells as xenografts were treated with corn oil (control) or Pg extract in corn oil (0.8 mg Gallic Acid Equivalent (GAE)/kg/day) every day as described in the materials and methods reduced the tumor size, tumor area (b) and tumor weights (c) were determined as described in material and method section. (d) Histopathologic evaluation of tumors. Tumor from corn oil (control) and Pg extract treated mice were fixed, stained with hematoxylin and eosin (H&E) and examined histopathologically as described in the material and methods section. Pg extract decreases Sp- regulated genes and induces apoptosis in tumors. (e) effect of Pg on SHIP-1, Sp1, survivin, VEGF and ZBTB10 mRNA levels. (f) effect of Pg on Sp1, Sp3 and Sp4, VEGF survivin (Sp-dependent genes) and SHIP-1, pAKT, pPI3K and NF-kB protein levels. (g) in-vivo results showed miR-27a and miR-155 expression were significantly reduced in dosed (Pg treated) mice than the control. All experiments were performed as described in the material and method section and were performed at least three times, and the result was expressed as mean ± SE. * indicates significant changes at $p<0.05$.

Discussion

This study has investigated the cytotoxic and anti-inflammatory activities of a pomegranate extract in breast cancer cells and in an animal model [104, 165, 170]. Pg decreased breast cancer cell growth, and tumor volume and activated caspase-3). Polyphenolic pomegranate has previously been shown to induce apoptosis in cancer cell lines including breast, prostate, liver, lung and skin cancer involving multiple pathways and inducing apoptosis involving caspase-3 [69, 102, 104, 106, 109, 186]. Previous studies have observed resistance of non-cancer cells to the effects of polyphenolics observed in this study has been previously reported with delphinidin (anthocyanin) from pomegranate extracts [104, 192, 193]. The effects of Pg extracts on inhibition of tumor growth in anthymic nude mice bearing BT474 cells as xenografts has also been observed in nude mice bearing prostate, pancreatic and breast cancer cells [69, 138, 139, 163, 175].

Sp transcription factors (Sp1, Sp3, Sp4) that are overexpressed in multiple tumors and regulate genes required for cell survival and angiogenesis and it has been reported that the suppression of Sp transcription factors results in growth inhibition and apoptotic cell death [138, 140, 175]. Overexpression of Sp in cancer cell lines and tumor is usually accompanied by upregulation of genes involved in cell survival, growth promotion and angiogenesis [139, 140]. In this study, Pg extract decreased Sp1, Sp3, and Sp4 mRNA as well as protein levels and also decreased Sp-regulated genes involved in cell proliferation, angiogenesis and inflammation, namely cyclin D1, bcl2, survivin VEGF and its receptor (VEGFR-1) and NF-κB.These results are consistent with

previous reports, where secondary plant compounds, such as curcumin and a pentacyclic triterpene betulinic acid , decreased Sp1, Sp3 and Sp4 and Sp-dependent genes [138-140, 175] . We previously investigated the regulation of Sp transcription factors by microRNA-27a, that is also overexpressed in breast cancer and other cancer cell lines [139, 140, 176, 194, 195]. A specific antagomir for miR-27a (as-miR-27a) increased the expression of ZBTB10, a zinc finger protein [139, 140, 176] that in turn suppresses Sp and Sp-dependent mRNA/protein expression [139, 140]. The regulation of Sp and Sp-dependent genes through the miR27a-ZBTB10-Sp-axis by both botanical compounds and synthetic derivatives, has been shown in cancer cells and tumors derived from multiple sites [139, 164, 175, 176, 195, 196]. Pg also significantly decreased miR-27a in breast cancer cells, but not in non-transformed cell lines and upregulated ZBTB10 mRNA expression in BT474 and MDA-MB-231 cells. When cells were transfected with the antagomir of miR-27a, the expression of miR-27a was decreased and mRNA of ZBTB10 increased where Pg further enhanced as-miR27a-induced effects. This indicates that miR-27a-ZBTB10-Sp is involved in the Pg-induced downregulation of NF-kB and genes involved in survival and angiogenesis. Moreover, when cells were transfected with the mimic of miR-27a, the effects of Pg, were reversed by the mimic indicating that Pg either directly or indirectly targets miR-27a. Previous reports indicate that botanicals decreased miR-27a expression however, this has not been confirmed for a complex polyphenolic extract [139] [176, 196]. Results observed in athymic female nude mice study correspond to the in vitro studies where miR-27a was decreased and ZBTB10 was increased by the treatment of mice with Pg.

Pg extracts decreased luciferase-activity in BT474 cells transfected with pNF-κB-Luc indicating a decrease of overall NF-kB activity and a miR-27a, antagomir also decreased luciferase activity in BT474 cells transfected with pNF-κB-luc , confirming a role of this microRNA in Pg-induced suppression of NF-kB activity. In vivo, the expression of Sp transcription factors Sp1, Sp3 and Sp4 and Sp-regulated genes survivin and VEGF were significantly decreased in Pg-treated animals and this was accompanied by a decreased expression of miR-27a. Our previous studies with betulinic acid showed a significant decrease in tumor size in a model of ER-negative breast cancer in athymic female nude mice xenografted with MDA-MB-231 cells, where also miR-27a-ZBTB10-Sp was involved in the underlying mechanism [139]. This further confirms the role of miR-27a in the cytotoxicity of Pg in this study.

Several studies show that NF-kB –activity, at least in part, is mediated through AKT, a kinase that in part is regulated through PI3K a key regulator in cell survival and cell function [69, 106]. Inositol 5'- phosphatase (SHIP-1) a 145-kDa protein that contains a Src homology 2 domain is a regulator of phosphatidylinositol 3,4,5-trisphosphate (PI3K) [197-199]. SHIP-1 has been identified as a tumor suppressor in hematopoietic cancer [200-202] but the role of SHIP-1 in solid tumors has not been thoroughly investigated. In this study, Pg induced activation of NF-kB by inhibiting the phosphorylation of p65 as well as the phosphorylation of PI3K/p85, and AKT (Ser473). These findings are inconsistent with previous studies where it was demonstrated that pomegranate extracts decreased inflammation and repressed lung tumors in mice and also decreased PI3K-dependent phosphorylation of AKT phosphorylation and activation

of NF-κB were decreased [69, 106]. Based on these reports one of the objectives of this research was to determine whether the inhibition of NF-kB activity by Pg, was due, in part by SHIP-1-PIP3-AKT-NF-kB interactions. Pg extract increased expression of SHIP-1 protein which was accompanied by down-regulation of miRNA 155). SHIP-1 is regulated by miR-155 via a target-binding site in the 3' UTR region of the SHIP-1 mRNA [191]. Previously, the polyphenolics resveratrol and quercetin decreased miR155 levels in THP-1 monocytic cells (36). In this study Ant-miR-155 and Pg decreased miR-155 and increased SHIP-1 mRNA [185, 191] and si-SHIP-1 increased the phosphorylation of AKT, while Pg (and also as-miR-155) partially reversed this effect. Ant-miR-155 also decreased the activity of NF-kB in a concentration-dependent manner in cells transfected with pNF-κB-luc and in vivo, Pg extract decreased the expression of miR155. Pg increased both, SHIP-1 mRNA and protein expression where the phosphorylation of AKT and PI3K protein expression compared to tumors from control animals. Based on result of our mimic and inhibitor studies and on previous reports, we conclude both miR-27a and miR-155 are targets of Pg and their downregulation plays a significant role in the anti-inflammatory and cytotoxic efficacy of pomegranate extract and possibly other botanicals (Figure 35).

Conclusion

In summary, pomegranate extract exhibited cytotoxic and anti-inflammatory activities in breast cancer cells in vitro and in vivo. In addition there is evidence that these activities are significantly mediated in part through the effects of pomegranate

extract in miR-27a-ZBTB10-Sp and miR-155-SHIP1- PIP3-AKT-NF-kB interactions (Figure 36).

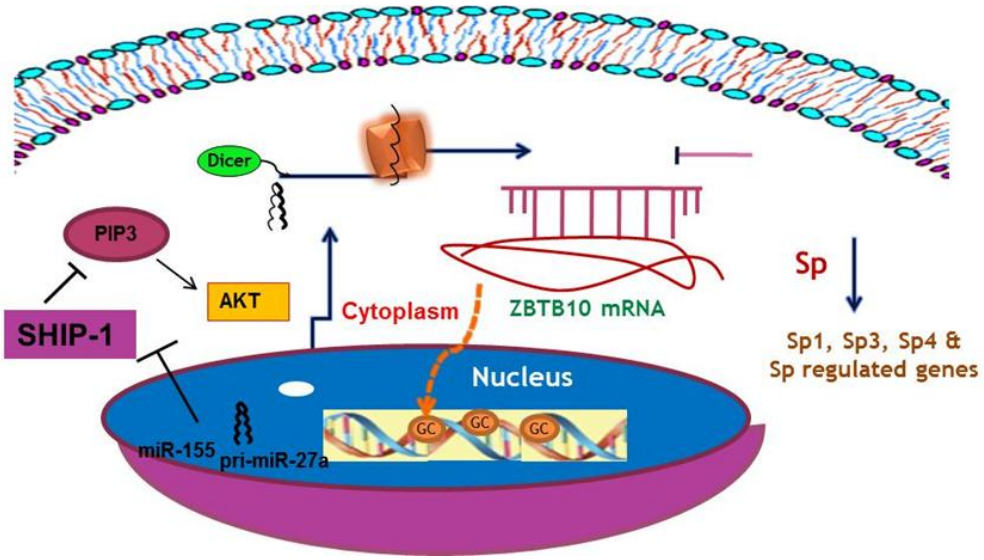

Figure 35 Schematic representation showing molecular mechanisms involved in breast cancer treated with Pg polyphenols Pg targeted both miR-27a and miR-155 by modulating both miR-27a-ZBTB10-Sp and miR-155-SHIP1- PIP3-AKT-NF-kB axes

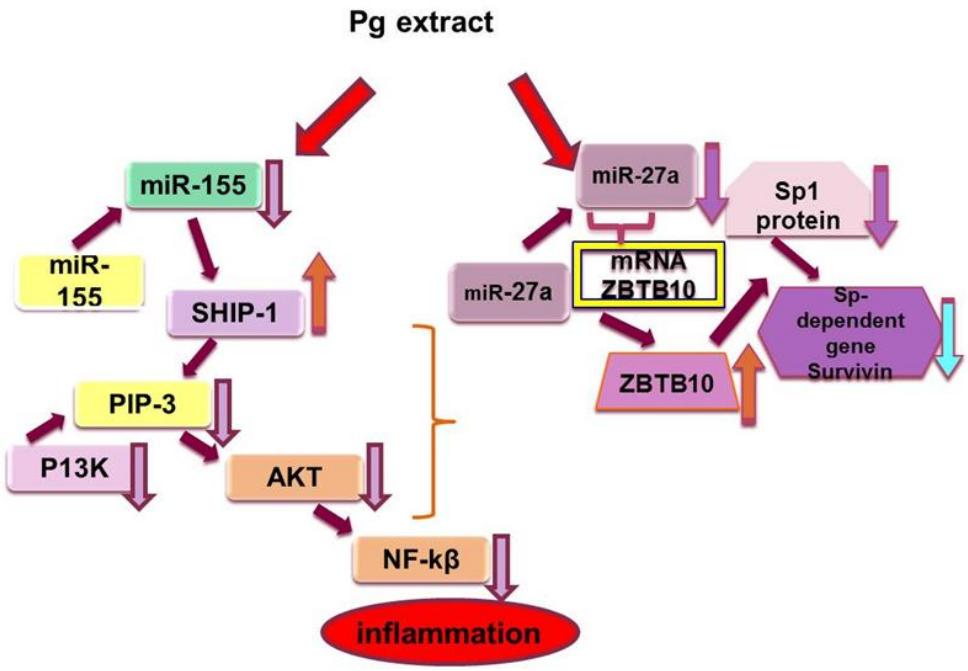

Figure 36 The effects of Pg extract in miR-27a-ZBTB10-Sp and miR-155-SHIP1- PIP3-AKT-NF-kB interaction

CHAPTER III

GALLOTANNINS AND GALLIC ACID CONSTITUENTS OF MANGO JUICE (MANGIFERA INDICA L) SUPPRESSED BREAST CANCER TUMOR GROWTH BY TARGETING PHOSPHATIDYLINOSITOL3-KINASE (PI3K)-AKT-NF-KB PATHWAY AND ASSOCIATED MICRORNAS

Summary

The cytotoxic and anti-inflammatory properties of polyphenolics have been demonstrated in numerous types of cancer. The objective of this research was to understand the post-transcriptional targets involved in anti-inflammatory and pro-apoptotic pathways in the breast cancer cells BT474 treated with a polyphenolic extract (2.5-40μg/ml) rich in gallotannins and gallic acid from mango fruit in vitro and in athymic nude mice bearing BT474 cells as xenografts.

Results show that cell proliferation as well as tumor size and weight in vivo was decreased by polyphenolics 60% and 70% in vitro and vivo, respectively. NF-kB protein and mRNA expression as well as activation were decreased in a dose-dependent manner (0.6 fold).

The extract also inhibited PI3K-dependent phosphorylation of AKT and expression and this was accompanied by down-regulation of miRNA 126 of BT474 (2.25 fold) and in vivo. NFκB-dependent genes involved in apoptosis-inhibition,

metastasis and angiogenesis, such as survivin, VCAM-1, and VEGF were also decreased, in vitro and in vivo.

A microRNA-array was also used to investigate whether microRNAs with target regions within genes were affected by the polyphenolic extract and results show that miR-126 Let7a and Let7b significantly increased with our extract.

In summary the anti-carcinogenic and anti-inflammatory activity of mango polyphenolics in breast cancer cells were at least in part due to targeting miR-126 - Phosphatidylinositol 3-kinase (PI3K)-AKT -NF-kB which plays an important role in the proliferative/ inflammatory phenotype exhibited in this breast cancer cell line.

Introduction

Breast cancer is one of the leading causes of cancer death among women worldwide. According to the American Cancer Society, 226,870 new cases of breast cancer with 39510 deaths from breast cancer were diagnosed among women in 2012. Epidemiological studies have suggested dietary polyphenols from fruits and vegetables reduced the risk of cancer and cardiovascular diseases [87, 104, 129, 203, 204]. In addition to available therapies, several alternative natural dietary polyphenols have been proposed as chemopreventive and chemotherapeutic agents for breast cancer [151, 205, 206].

Mango (Mangifera indica L.) is a tropical fruit, rich in bioactive compounds consumed worldwide. Polyphenols are present in mango pulp, peel, and seed. Mango polyphenols have shown potential health benefits and exert antioxidant, anti-inflammatory and antitumorogenic effects. Additionally mango polyphenols inhibit cell

proliferation in several cancer cell lines such as colon, lung, prostate and breast, cancer cells [86, 87, 111, 151]. Chemical analysis showed that the most predominant and relevant therapeutic compounds present in mango pulp extract are gallotannins, gallic acid, galloyl glycosides and flavonoids such as quercetin and kaempferol glycosides. Mango extract also contains mangiferin and a complex mixture of other antioxidant polyphenols [71, 72]. Literature have shown antitumorogenic activity of mango polyphenols against the estrogen positive and negative breast cancer cell lines MCF-7 and MDA-MB-231 [205, 207]. Another important mango polyphenol, mangiferin, have shown chemopreventive and chemotherapeutic action against benzo(a)pyrene induced lung cancer in swiss albino mice [154]. Similarly, mango polyphenols were known to suppress prostate cancer in mice and in human prostate LNCaP cell line through apoptosis [111].

Previous studies have shown the anti-proliferative and anti-inflammatory properties of mango extract [86, 87, 111, 151]. The potential role of polyphenols present in mango showed antioxidant and anti-inflammatory properties by modulating several cell signaling pathways in the cancer model. The transcription factor NF-κB plays a central role between inflammation and cancer [103]. NF-κB regulated genes are involved in cell proliferation, inflammation, angiogenesis and metastasis in cancer development [96, 138, 152, 153]. In cancer, NF-κB remains activated and highly expressed [96, 138]. Several literatures showed that polyphenols were known to target the NF-κB pathway [69, 97, 99, 138, 208]. Similarly polyphenols have been shown to reduce inflammation and inhibit lung-tumor growth in mice by suppressing PI3K-

dependent phosphorylation of AKT and reduce the activation of NF-κB in vivo [69, 106]. In our previous study we demonstrated the cytotoxic effect of pomegranate polyphenols which suppressesd the phosphorylation of NF-κB expression [50].

In this study, our preliminary data showed that gallic acid, mono-gallyl glucoside, tetra -gallyl glucoside, penta- gallyl glucoside, OH-benzoic acid hexoside and unresolved gallotannins rich mango polyphenols (Mg) suppressed cell proliferation in breast cancer BT474 cell line and tumor growth in athymic BALB/c nude mice with BT474 xenografts. The overall antitumorgenic potential of Mg may at least in part be based on anti-inflammatory and cytotoxic properties that inhibit cell proliferation and modulate molecular pathways in breast cancer cells. Mg suppressed phosphorylation of NF-κB which is accompanied by the downregulation of PI3K-dependent phosphorylation of AKT. In addition, Mg also reduced the expression of mammalian target of rapamycin (mTOR) and its downstream transcription factor hypoxia-inducible factors (HIF-1α). Our preliminary microRNA profile screening indicated Mg targets several microRNA which are important in cancer cell proliferation.

Hence, the objective of this study was to investigate the anti-inflammatory and cytotoxic effects of mango polyphenols through PI3K-AKT- NF-κB pathways and associated microRNA's.

Materials and methods

Botanical extracts

Mango, variety Keitt, was sourced from Mexico, and were allowed to ripen and subsequently processed by removing peel and seed. Pulp was diced, vacuum sealed and

stored at -20° C until used. Mango pulp was extracted following the methods of [209]. Briefly, mango pulp was extracted with a mixture of ethanol, methanol, acetone (1:1:1) three times the weight of the mango at ambient temperature for 1 h. Solids were removed by filtration and extracted twice more following the same procedures described. The filtered extract was evaporated and further concentrated on a 20 g C18 cartridge (Waters Corp. Milford, MA). The unbound fraction from C18 was partitioned into ethyl acetate twice to capture polar compounds that were not retained by C18. Ethyl acetate fraction and C18 elutant were combined and the solvent removed under reduced pressure at 45°C. The dried extract was stored at -80°C prior to weighing and dissolved in dimethyl sulfoxide (DMSO) for cell culture and analytical procedures.

HPLC-PDA analysis

Separations were made in an Acclaim™ C_{18} column (Bannockburn, IL), (250 x 4.6 mm, 5 μm) at room temperature. The mobile phase consisted of 0.1% formic acid in water (Phase A) and 0.1% water (Phase B). A gradient program at 0.4 mL/min initially ran Phase B at 0%, for 3 minutes, 21% Phase B in 20 min, from 21 to 35% Phase B in 30 min, and 35 to 49% Phase B in 50 min, 49% to 70% Phase B in 70 minutes before returning to initial conditions. Detection was at 280 and 360 nm for benzoic and cinnamic acids/flavonoids, respectively. Compounds were tentatively identified based on mass spectometry analysis. This was performed on a Thermo Finnigan LCQ Deca XP Max ion trap mass spectrometer with an electrospray ionization probe in negative ion mode under the following conditions sheath gas (N_2), 40 units/min; auxiliary gas (N_2), 5

units/min; spray voltage, 4.5 kV; capillary temperature, 300°C; capillary voltage, 7.0 V; tube lens offset, 40.0 V, the source current at 80.0 μA.

Cell culture

Human mammary carcinoma cell lines were purchased from ATCC and maintained the cells according to the supplier guidelines (American Type Tissue Collection (ATCC, Manassas, VA).

Cell proliferation

The cell proliferation was measured using an electronic particle counter at 48 h (Z1™ Series, Beckman Coulter, Inc, Fullerton, CA), on BT474 cells after a 48h treatment with Mg extract as previously described, [50] and are presented as net growth.

Reagents

Primers for VEGF, PI3K, AKT and HIF-1α were purchased from Integrated DNA Technologies (San Diego, CA). Scrambled miRNA was obtained from Dharmacon, Inc. (Lafayette, CO). mirVana TM extraction kit, reverse transcription (RT) and real-time PCR amplification kits were purchased from Applied Biosciences (Foster City, CA). We have purchased antibodies against, poly-(ADP-ribose)-polymerase (PARP), NF-κB (p65), phosphorylated NF-κB (p65), PI3K, mTOR and HIF-1α from Cell Signaling Technology (Beverly, MA). Rest antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Western blotting chemiluminescence reagent was purchased from Perkin-Elmer Life Sciences (Waltham, MA).

Real-time PCR analysis of mRNA

As described in previous literature BT474 cells were seeded in 6 well plate for 24 h to allow cell attachment [50]. Cells were treated with Mg extract and after 24 h mRNA was extracted for gene expression analysis as previously described [50]. According to the manufacturer's recommended protocol total RNA was isolated by using the RNeasy Mini kit (Qiagen, Valencia, CA) for mRNA analysis and for micro-RNA analysis the mirVanaTM miRNA isolation kit (Applied Biosystems, Foster City, CA). RNA purity was assessed using spectrophotometry (260/280 > 1.7). Total RNA concentration was determined by the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). According to the manufacturer‟s protocol cDNA was synthesized from isolated RNA using a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY). SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA) were used to carry out qRT-PCR on an ABI Prism 7900 Sequence Detection System (Applied Biosystems Inc, Foster City, CA). as previously described [50] on an ABI Prism 7900 Sequence Detection System (Applied Biosystems Inc, Foster City, CA). The pairs of forward and reverse primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). All primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). Each primer homology-search was performed by an NCBI BLAST search to ensure that it was specific for the target mRNA transcript., Inc. (San Diego, CA). Product specificity was determined by dissociation curve analysis. We followed the protocol as described

previously [50, 140]. The primer-sequences for VEGF were F:5'- AAG GAG GAG GGC AGA ATC AT-3', and R:5'- ATC TGC ATG GTG ATG TTG GA-3'.

MicroRNA profiles screening

Total RNA was isolated from the tumor samples using the mirVana™ miRNA Isolation Kit. cDNA synthesis and real-time qPCR was performed as described in the manual of Megaplex™ Pools A without pre-amplification (Applied Biosystems, Foster City, CA, USA). In brief, Equal amount (500ng) of total RNAs (n=3/group) was reverse transcribed to cDNA using Megaplex RT Primers and TaqMan MicroRNA Reverse Transcription kit. The megaplex product was loaded onto the TaqMan Human MicroRNA Array (Applied Biosystems, Foster City, CA, USA) to quantify individual miRNAs. The array was performed using a 7900HT system with an SDS software v.2.3. Only miRNA showing >2-fold increases or <0.5-fold decreases were shown in the results section.

Western blotting

BT474 cells (4×10^5) were seeded in 6-well plates and incubated for 24 h. They were also treated with Pg extract (0-10μg/mL) for 24 h. Cells were harvested and prepared for western blotting using a Bio-Rad protein kit. (Bio-Rad, Hercules, CA) as previously performed [50, 140]. 60μg of cell lysate was resolved by electrophoresis in 10% sodium dodecyl sulfate (SDS) polyacrylamide gel (100 v, 1.5 h) in a running gel buffer (containing. 25 mM Tris, pH 8.3, 162 mM glycine, and 0.1% SDS). The samples were transferred to nitrocellulose membrane for 1h at 350 mA. The membrane blocked for 1h in 5% skimmed milk dissolved in a mixture of T-PBS with 0.1% tween. The

membranes were incubated overnight in a mixture of 3% BSA (fetal bovine serum) in T-PBS with 0.1% tween and primary antibody dilutions based on manufacturer protocol. Next day the membrane was incubated in secondary antibody for 1 h at room temperature. Proteins were visualized with a Kodak Molecular Imaging System (Carestream Health, Rochester, NY). β-actin was used as an internal control to ensure equal amount of protein was loaded per well.

Multiplex bead assay

By using the multiplex kits (#48-611, #48-612, Millipore) according to the manufacturer's protocol the protein extract (20ug) from each tumor tissue from mice bearing BT474 xenografts was used to determine the relative abundance of the phosphorylation status and total protein in the AKT/mTOR signaling (kit contained AKT, GSK3β, GSK3α, IGF1R, IR, IRS1, mTOR, p70 S6 kinase, PTEN, RPS6, and TSC2). Multiplex analysis was performed by the Luminex L200 instrument (Luminex, Austin, TX), and data was analyzed by Luminex xPONENT software [210].

Xenograft study

From Harlan laboratory (Houston, TX) female athymic BALB/c nude mice (age 3- 4 weeks) were purchased and implanted with BT474 cells ($2X10^6$ cells) in matrigel (BD Bioscience San Jose, CA) s.c into the flank [50]. After approval by the Institutional Animal Care and Use Committee at Texas A&M University (College Station, TX) mice were utilized after a 10 day equilibrium period. Mice were treated with either 100 µl vehicle 0.8mg Gallic acid equivalent (GAE)/kg/day of Mg extract/d or 1% DMSO in corn oil by oral gavage for 35 days. Final body and tumor weights were

determined. Tissues were flash-frozen in liquid nitrogen and stored at -80˚C for mRNA and protein analysis. 4-AM-thick paraffin-embedded tumor sections were cut and stained with Hematoxylin and Eosin (H&E) for bright field microscopy.

Statistical analysis

Quantitative data represent mean values with the respective standard deviation (SD) or standard error of the mean (SE) corresponding to replicate. Data were analyzed by one-way analysis of variance (ANOVA) using SAS version 9.3 (SAS Institute Inc., Cary, NC).Tukey's Post Hoc multiple comparisons was used ($p<0.05$) to develop significant statistical difference.

Results

Polyphenolic composition of pomegranate extract determined by HPLC-MS

The polyphenolic profile of mango was previously shown as being rich in gallotannins, gallic acid, galloyl glycosides and flavonoids such as quercetin and kaempferol glycosides. Mango has also been reported to contain a mangiferin and complex mixture of other antioxidant polyphenols [71, 72]. In this study, HPLC-MS analysis (Figure 37) indicated the presence of gallic acid, mono-galloyl glucoside, tetra –galloyl glucoside, Penta- Galleo glucoside , OH-benzoic acid hexoside and unresolved gallotannins as the major polyphenols present in the mango pulp extract.

Anti-proliferative activities of mango extract

Mango extracts exerted cytotoxic activity in human BT474 breast cancer cell-lines (Figure 38A) After treatment with Mg extract (2.5-25 µg/ml), there was a concentration-dependent decrease in cell viability after 48 h. Mg-induced cytotoxicity

was accompanied by activation of the cleaved product of the substrate Poly (ADP-ribose)-polymerase1 (PARP) [186] and suppressed the expression of survivin, important for cell proliferation (Figure 38B)

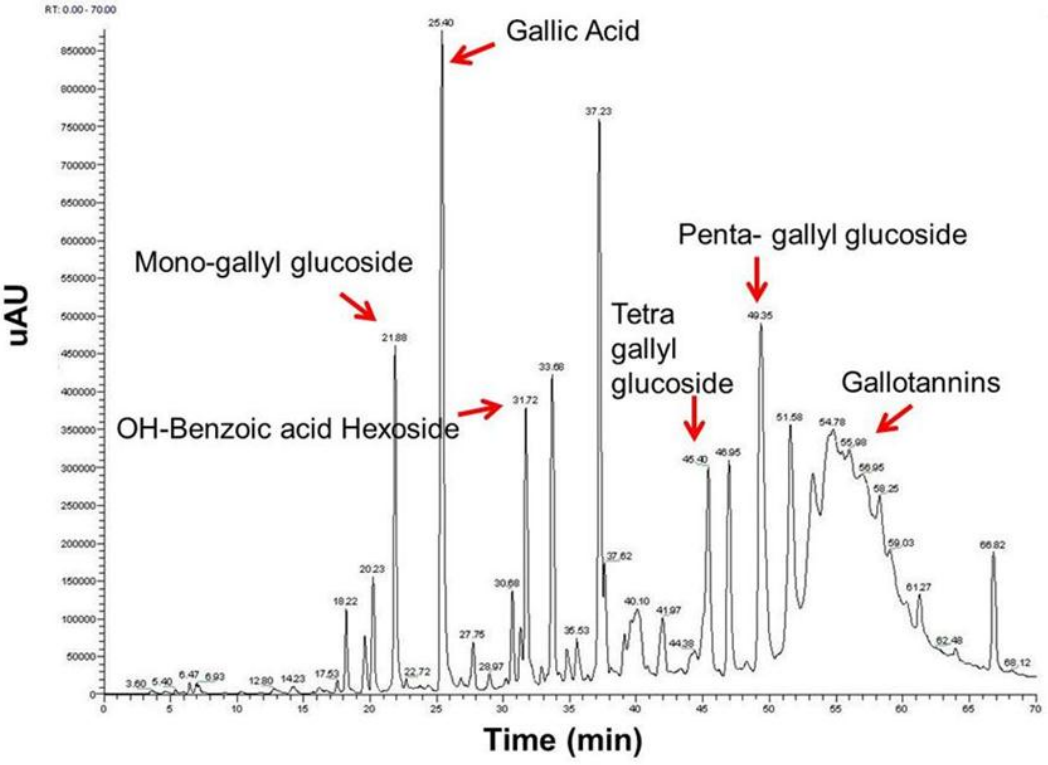

Figure 37 Representative chromatogram of polyphenolic compounds in an extract of mango juice concentrate gallic acid, mono-galloyl glucoside, tetra –galloyl glucoside, penta- gallyl glucoside , a OH-benzoic acid hexoside and unresolved gallotannins as the major polyphenols present in the mango pulp extract

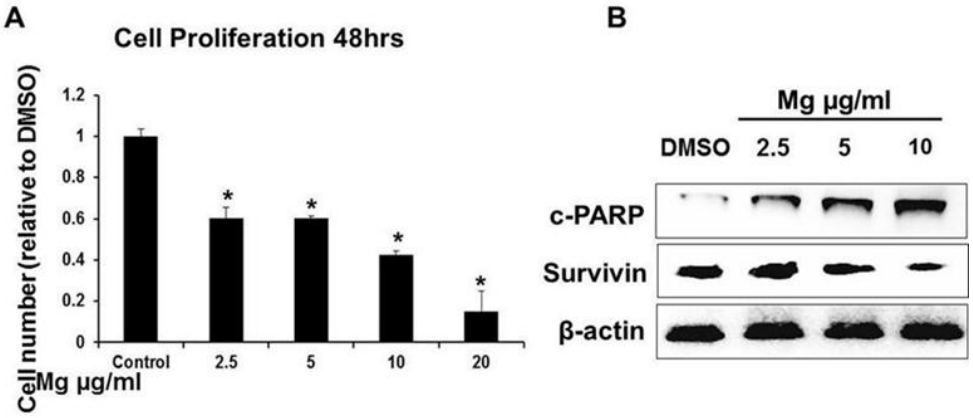

Figure 38 Mango extract (Mg) inhibits proliferation and induces apoptosis in cancer cells (A) cells were seeded and treated with DMSO (vehicle control) or different concentrations of Mg extract (2.5-20 µg/ml treatment) for 48 h as described in the materials and methods section. (B) western blot analysis of apoptosis-associated proteins (cleaved PARP) and cell survival protein (Survivin). BT474 cells were treated with DMSO (control vehicle) or different concentrations of Mg extract 2.5-10 µg/ml for 24 h, and whole-cell lysates were analyzed by western blot analysis as described in the material and methods section. All experiments were performed at least three times and the results were expressed as mean ± SE; * indicates significant changes at $p<0.05$.

Modulation of PI3K /AKT /mTOR pathway

The PI3K/AKT/ mTOR are well known for their role in carcinogenesis processes in several types of cancer such as colon, lung and breast cancer [211, 212]. PI3K/AKT controls cell proliferation and initiates cell survival pathways through phosphorylation of several downstream targets such as NF-κB and mammalian target of rapamycin (mTOR) [213-215]. Our previous study showed the cytotoxic effects of polyphenols from pomegranate in part mediated through PI3K/AKT in BT474 breast cancer cells in vitro and vivo [50]. In this study, mango polyphenols decreased HIF-1α,

pPI3K, pAKT and VEGF mRNA level (Figure 39 A) pPI3K, pAKT, AKT, VEGF and VEGFR protein levels (Figure 39B) .

Effects of Mg on NF-κB

In our previous study anti-inflammatory properties of pomegranate polyphenol were shown by inhibiting the expression of constitutive and phosphorylation of NF-κB p65 in breast cancer cells BT474 [50]. Literature showed other dietary polyphenols such as curcumin, suppressed NF-κB expression in pancreatic tumor growth and cancer cell lines [138]. Similarly, in this study we have investigated cytotoxic and anti-inflammatory properties of mango polyphenols. Mg suppressed the constitutive expression and phosphorylation of NF-κB p65 in BT474 cells (Figure 39C).

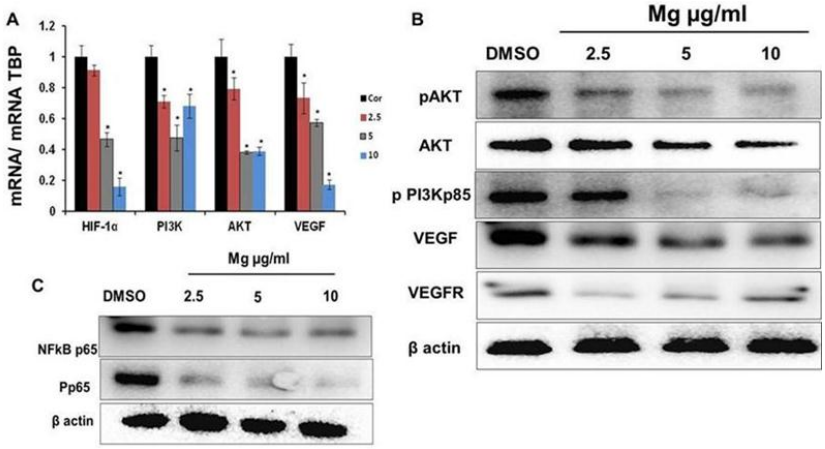

Figure 39 Mango extract (Mg) inhibits phosphorylation of AKT/PI3K/mTOR pathways and inhibits angiogenesis marker vascular endothelial growth factor (VEGF) and its receptor (VEGFR) in cancer cells (A) effects of Mg on HIF-α, PI3K, AKT and VEGF mRNA levels and (B), pP13K, pAKT AKT,VEGF and VEGFR protein expression (C) NF-κB (p65) and pNF-κB (p65) protein expression in BT474 cells. Cells were treated with solvent DMSO (control) or different concentration of Mg (2.5-10 µg/ml) for 24 h. All experiments were performed as described in the material and method section at least in triplicates and result were expressed as mean ± SE.* indicates significant changes from the control at $p<0.05$

Xenograft study in athymic female nude mice

Our previous study demonstrated cytotoxic activities of Pg in athymic female nude mice with BT474 cells as xenografts. Similarly in the present study, treatment with Mg (0.8mg GAE/kg/day/) significantly suppressed tumor volume (Figure 40A) and cleaved poly (ADP-ribose)-polymerase1 (PARP) upregulated (Figure 40B) Moreover, Mg decreased the expression of pPI3K pAKT mTOR protein level (Figure 40B) and significantly suppressed expression of HIF-1α downstream of mTOR pathway (Figure 40B). NFkB p65 protein levels were also inhibited in tumors from mice treated with Mg compared to the control tumors (Figure 40B). Similar results of pAKT and pmTOR protein level were observed through Luminex assay. VEGF protein expression was reduced in Mg treated group (.Figure 41A). Tissues collected from mice tumor were flash-frozen in liquid nitrogen and stored at -80˚C for mRNA and protein analysis. 4-AM-thick paraffin-embedded tumor sections were cut for immunohistochemistry and stained with VEGF primary antibody and images were taken for bright field microscopy (Figure 41B).

In addition our preliminary microRNA profile screening indicated that Mg targets several microRNA that are important in cancer cell proliferation. Tumor suppressor microRNA miR494, miR 31, let7b, let7a, miR708 and miR126 are upregulated whereas oncogenic miR221 expression was downregulated with the treatment of Mg (Table 4).

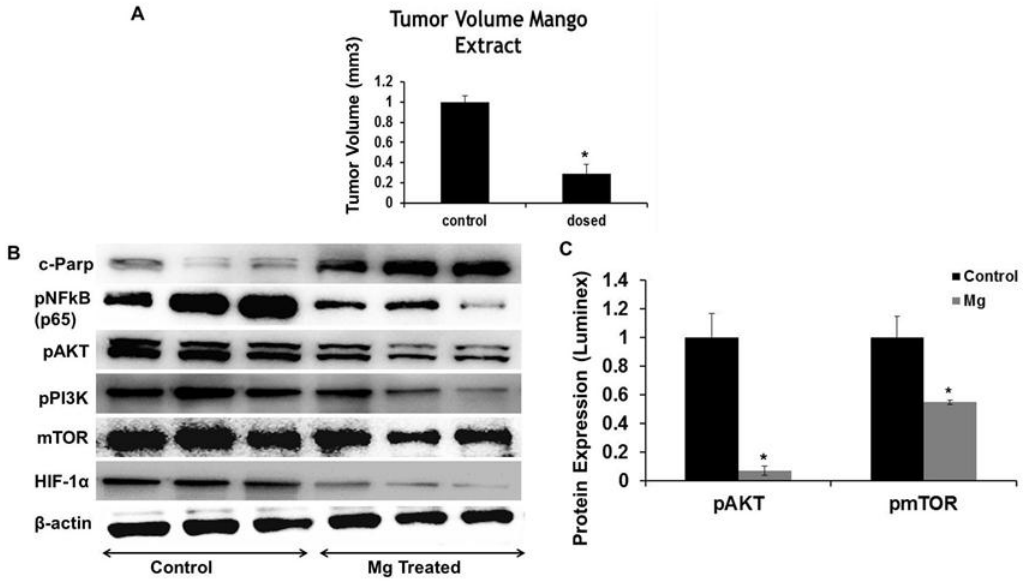

Figure 40 In vivo studies athymic nude mice A) tumor Volume (B) effects of Mg on apoptosis-associated proteins (cleaved PARP), pNF-κB (p65), pAKT, pPI3K, mTOR and HIF-α, protein expression through western blotting (C), pAKT and pmTOR protein expression through luminex assay. Athymic nude mice (10 per group) bearing BT474 cells as xenografts were treated with corn oil (control) or Mg extract in corn oil (0.8 mg Gallic Acid Equivalent (GAE)/kg/day) every day as described in the materials and methods. All experiments were performed as described in the material and method section at least in triplicates and result were expressed as mean ± SE.* indicates significant changes from the control at $p<0.05$.

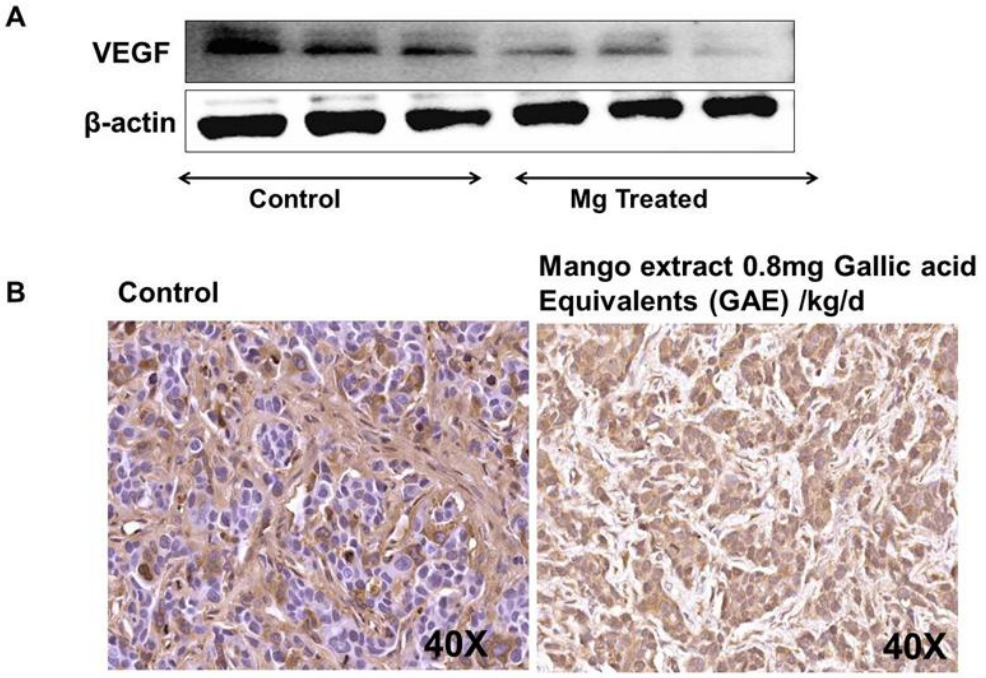

Figure 41 Effects of Mg on VEGF protein expression in expression in mice with BT474 xenografts (A) VEGF protein expression through western blotting in control and Mg treated group (B) immuno-histology staining with VEGF in control and Mg treated group. All experiments were performed as described in the material and method section at least in triplicates and result were expressed as mean ± SE.* indicates significant changes from the control at $p<0.05$

Table 4 The relative levels of miRNA gene expression from the mango group to control group were compared in mice with BT474 xenografts. MicroArray data

miRNA ID	Relative detection	FDR	Description	Function	Target mRNA
miR-494	3.49	0.00	Tumor suppressor	Suppress cell proliferation	PTEN
miR-31	192.54	0.00	Tumor suppressor	Inhibit breast cancer metastasis	FOXP3, P53
miR-221	34.17	0.04	Oncogene	Induce cell proliferation and angiogenesis	CD117
let-7b	2.53	0.05	Tumor suppressor	Reduce tumor growth	RAS, HMGA2, Casp3, Bcl2, Map3k1, Cdk5, IL6, IL10, TLR4
let-7a	5.78	0.10	Tumor suppressor	Reduce tumor growth	RAS, HMGA2, Casp3, Bcl2, Map3k1, Cdk5, IL6, IL10, TLR4
miR-708	2.72	0.10	Tumor suppressor	Induce cell proliferation	TMEM88, Wnt
miR-126	2.26	0.65	Tumor suppressor	Suppress angiogenesis	VEGF-A, IRS-1, AKT/PI3K

Discussion

Mango is a rich source of nutritive compounds, such as polyphenols, present in pulp, peel, and seed. The most predominant therapeutic compounds present in mango extract are gallotannins, gallic acid, galloyl glycosides and flavonoids, such as quercetin and kaempferol glycosides. Gallotannins, one of the main polyphenolics identified in mango, has been shown to modulate the pro-apoptotic and cell cycle related proteins in colon cancer cells [216].

In theory, these polyphenolic compounds reduce risk of cancer, cardiovascular and several other chronic diseases, yet little research has been conducted to investigate the efficacy of whole fruits. Compared to whole fruit polyphenols, individual isolated compounds appear to be less important, as they remain at higher levels than their actual appearance in plasma after consuming whole fruits [86, 87, 111].

Previous studies have shown potential health benefit of mango polyphenols, which exerts antioxidant, anti-inflammatory, anti-carcinogenic and anti-proliferative activities. It induce apoptosis in cancer cell lines including breast, prostate and colon cancer involving caspase-2, -3, and -9 [86, 87, 111, 151].

We have recently reported the cytotoxic effect of polyphenol extract from whole fruit pomegranate in breast cancer cells and nude mice containing breast cancer cell xenografts by targeting AKT/ PI3K/p85 pathway [50]. In the present study, we have demonstrated the anti-carcinogenic and anti-proliferative activities of a mango extract in breast cancer cells and in an animal model. Our preliminary data indicates the presence of gallic acid, mono-galloyl glucoside, tetra –galloyl glucoside, penta- gallyl glucoside,

OH-benzoic acid hexoside and unresolved gallotannins as the major polyphenols present in the mango pulp extract. The result of the present study showed that gallic acid and gallotannin rich mango pulp extract (Mg) inhibited cell proliferation of breast cancer cell line BT474 by activating cleaved product of the substrate Poly (ADP-ribose)-polymerase1 (PARP) and inhibited expression of survivin. Mg also suppressed tumor growth in athymic BALB/c nude mice with BT474 xenografts.

Several literature studies have indicated that NF-kB –activity, at least in part, is mediated through AKT, a kinase, that in part is modulated by PI3K, a key regulator in cell survival and cell function [69, 106]. In this study, mango polyphenols (Mg) suppressed phosphorylation of NF-κB (p65) which is accompanied by downregulation of PI3K-dependent phosphorylation of AKT (Ser473). Thus Mg was shown to modulate the expression of AKT/ PI3K/p85 pathway (Figure 42). In addition, Mg also reduced the expression of mammalian target of rapamycin (mTOR) and its downstream transcription factor hypoxia-inducible factors (HIF-1α). The PI3K/AKT/ mTOR are well known for the carcinogenesis progression in several types of cancer such as colon, lung and breast cancer [211, 212]. PI3K/AKT controls cell proliferation and initiates cell survival pathways through phosphorylation of several downstream targets such as NF-κB and mammalian target of rapamycin (mTOR) [213-215]. Mg also reduced expression of angiogenesis marker such as vascular endothelial growth factor (VEGF) and its receptor VEGFR. Similar results were observed in athymic BALB/c nude mice with BT474 xenografts.

Our preliminary microRNA profile screening indicated that Mg targets several microRNA that are important in cancer cell proliferation. Tumor suppressor microRNA miR494, miR 31, let7b, let7a, miR708 and miR126 are upregulated, whereas the expression of oncogenic miR221 was downregulated with the treatment of Mg. miR494 suppressed cell proliferation, miR 31 inhibited breast cancer metastasis and miRNA221 induce cell proliferation and angiogenesis. Let7b and let7a reduced tumor growth, miR708 induced cell proliferation and miR126 controlled cell angiogenesis. Thus preliminary data of miRNA screening indicate that Mg modulated the expression of different miRNA involved in carcinogenesis progression.

Conclusion

In summary, Mg exhibited antioxidant, anti-inflammatory, anti-carcinogenic and anti-proliferative activities in breast cancer cells in vitro and in vivo. In addition Mg inhibited cell proliferation of breast cancer cell line BT474 and suppressed tumor growth in athymic BALB/c nude mice with BT474 xenografts. Mg modulates the expression of miRNA regulated cell proliferation.

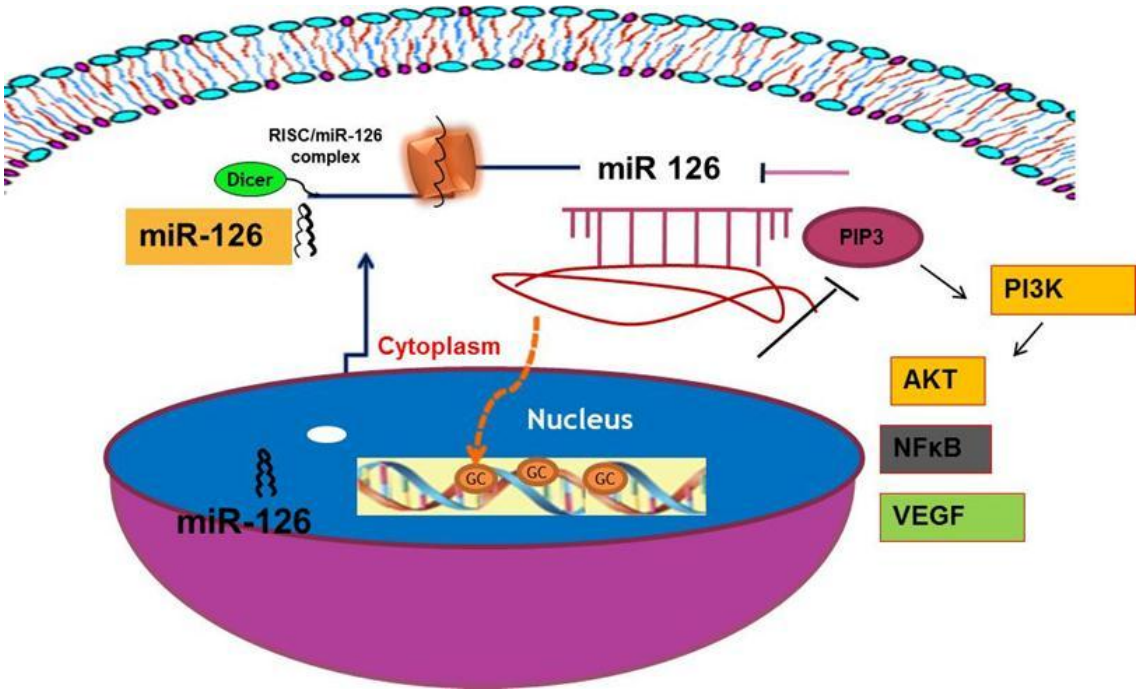

Figure 42 Schematic representation showing mango polyphenols targeted miR126 which modulated PI3K/AKT pathway and also NF-κB and VEGF expression

CHAPTER IV

POMEGRANATE EXTRACT CONTAINING ELLAGITANNINS AND ANTHOCYANINS SUPPRESSED AZOXYMETHANE-INDUCED COLORECTAL ABERRANT CRYPT FOCI (ACF) AND INFLAMMATION: POSSIBLE ROLE OF MIR126-VCAM-1 AND MIR126-PI3K/AKT-MTOR*

Summary

The antitumorigenic activities of polyphenols such as ellagitannins and anthocyanins in pomegranate (Punica granatum L.) have been previously studied where cytotoxic, anti-inflammatory and antioxidant effects were evident in various cancer models. The objective of this study was to investigate the role of miR-126/VCAM-1 and miR-126/PI3K/AKT/mTOR in pomegranate-mediated anti-inflammatory and anti-carcinogenic effects in vivo and in vitro. Sprague Dawley rats (n=10/group) received pomegranate juice (2504.74mg gallic acid equivalents/L) or a polyphenol-free control beverage ad libitum for 10 weeks and were injected with azoxymethane (AOM) subcutaneously (15 mg/kg) at weeks 2 and 3. Consumption of pomegranate juice suppressed the number of aberrant crypt foci (ACF) and dysplastic aberrant crypt foci by 29% and 53.5% (P=0.05 and 0.04) respectively, and significantly lowered proliferation

* Reprinted with permission from "Pomegranate Polyphenolics Suppressed Azoxymethane-Induced Colorectal Aberrant Crypt Foci (ACF) and Inflammation: Possible role of miR126-VCAM-1 and miR126-PI3K/AKT-mTOR", by Banerjee N, Hyemee K, Talcott S and Mertens –Talcott SU, 2013, Carcinogenesis. 34, 2814-2822, Copyright 2013 by Oxford

of mucosa cells. Pomegranate juice significantly downregulated pro-inflammatory enzymes iNOS and COX-2 mRNA and protein expression. In addition, it suppressed NF-kB and VCAM-1 mRNA and protein expression in AOM treated rats. Pomegranate also inhibited phosphorylation of PI3K/AKT and mTOR expression, and increased the expression of miR-126. The specific target and functions of miR-126 were investigated in HT-29 colon cancer cell lines. In vitro, the involvement of miR-126 was confirmed using the antagomir for miR-126, where pomegranate reversed the effects of the antagomir on the expression of miR-126, VCAM-1 and PI3K p85b. In summary, therapeutic potentials of pomegranate in colon tumorigenesis were due to in part to targeting miR-126-regulated pathways, which contributes in the underlying anti-inflammatory mechanisms.

Introduction

Colorectal cancer is the third leading cause of death in the US [3]. According to epidemiological and intervention studies, colon carcinogenesis is significantly influenced by dietary, genetic, and environmental factors. These factors may cause disruption of normal cell growth, uncontrolled proliferation and differentiation associated with inflammation and oxidative stress. Owing to the low survival rate of colon cancer, it is crucial to investigate nutritional prevention approaches and their underlying mechanisms of action [93, 215].

Epidemiological, preclinical and clinical studies suggest that a diet rich in natural polyphenolics may protect against colonic inflammation and colon cancer [44-46]. Clinical and preclinical studies have reported that polyphenolics from pomegranate

exert anti-carcinogenic, anti-inflammatory and antioxidant activities [47-50]. Polyphenols extracted from pomegranate peels and seed, have shown to induce cytotoxicity in several cancer cell lines such as lung, prostate and breast and also in chemically-induced colon cancer in animal studies [47, 49, 108, 109, 119, 165, 217]. Predominant polyphenolics in pomegranate include ellagic acid, ellagitannins, punicalagin, flavonoids, and 3-glucosides/3,5-diglucosides of the anthocyanins delphinidin, cyanidin, and pelargonidin [50, 70] and these exhibit antioxidant, anti-inflammatory, and anti-carcinogenic properties in vitro and vivo [113, 218, 219].

Previous studies have demonstrated the downregulation of a common colon cancer marker vascular cell adhesion molecule 1 (VCAM-1) [220, 221] by polyphenols [222]. In addition, pomegranate polyphenols also modulates the expression of phosphatidylinositol-3- kinase (PI3K)/AKT pathways. In the human lung carcinoma cells (A549) and lung tumors in mice, where inflammation was induced by the induction of NF-κB and PI3K/AKT signaling pathway, pomegranate polyphenols down regulated inflammation by suppressing PI3K and AKT phosphorylation and decreased the activation of NF-κB [69, 106]. Insulin growth factor (IGF) influences the regulation of the phosphorylation of PI3K/AKT which is crucial in the pathogenesis of colon cancer. PI3K/AKT pathway influences cell survival through phosphorylation of downstream targets such as NF-kB, and mammalian target of rapamycin (mTOR). NF-kB regulates inflammatory markers including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [223].

The anti-inflammatory miR-126 has target regions in the promotor regions of VCAM-1 and PI3K p85β subunit, and low expression of miR-126 has been associated with colon cancer [211, 224]. It also was previously shown that pomegranate increased the expression of miR-126 and that is involved down-regulating VCAM-1 and PI3K [211, 225].

In our preliminary studies in HT-29 colon cancer cells, Pg induced apoptosis and increased the expression of miR-126, suppressed VCAM-1, NFkB, iNOS, COX-2 and decreased the phosphorylation of AKT. Based on these preliminary in vitro findings, the objective of this study was to investigate the efficacy and underlying mechanisms of Pg in the prevention of AOM-induced aberrant foci in a rat model. The working hypothesis was that miR126-VCAM-1 and miR126-PI3K/AKT-mTOR are significantly involved in the anti-inflammatory, cytotoxic and cancer-preventive activities of Pg in AOM-treated rats and also in vitro in HT-29 colon cancer cell.

Materials and methods

Experimental juice and extract

Rats were divided into two groups Control juice or Pomegranate juice. Pomegranate juice was obtained from the Stiebs (Kirkland, WA). For calorie adjustment, 15.7g sugar and 0.05g citric acid were added in 100ml of Control juice. Total phenolic content in the Pomegranate juice was meassured spectrophotometrically by the Folin-Ciocalteu assay against an external standard of gallic acid and expressed as gallic acid equivalents. The extract was prepared following the procedure described in our previous literature [50].

Reagents

Standards for HPLC-MS analysis were obtained from Sigma-Aldrich (St Louis, MI) and Chromadex (Irvine, CA). Antibodies against NF-κB (p65), phosphorylated NF-κB (p65), COX-2, iNOS, IGF, pmTOR were purchased from Cell Signaling Technology (Beverly, MA). All other antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Western lighting chemiluminescence reagent was purchased from Perkin-Elmer Life Sciences (Waltham, MA). All primers were purchased from Integrated DNA Technologies (San Diego, CA). mirVana TM extraction kit, reverse transcription (RT) and real-time PCR amplification kits were purchased from Applied Biosciences (Foster City, CA). Antagomers of miR-126 (inhibitor) as well as scrambled miRNA were from Dharmacon, Inc. (Lafayette, CO) [50].

Polyphenolic composition of pomegranate juice determined by HPLC-MS

According to our previous study, pomegranate polyphenolics predominantly include ellagic acid, ellagitannins, and anthocyanins [50]. The chromatographic profiles showed flavonoids with peak absorption at 360nm (Fig. 1A) and ellagitannins with peak absorption at 280nm (Fig. 1B).

Cell culture

Human colon carcinoma cell lines HT-29, were obtained from American Type Tissue Collection (ATCC) and maintained according to the supplier guidelines (ATCC, Manassas, VA).

Cell proliferation

The cell proliferation was assessed with an electronic cell counter at 48 h (Z2™ Series, Beckman Coulter, Inc, Fullerton, CA), as previously described [33] and were showed as net growth.

Animal treatments and tissue collection

Twenty male Sprague-Dawley rats (3 week-old) supplied from Harlan Teklad (Houston, TX) were randomly distributed by weight into Control juice and Pomegranate juice groups. Animals were kept in suspended cages in a room controlled at a temperature of 23±2°C, humidity of 55±5% and 12 h light/dark cycle, and they had free access to regular food pellets and experimental juices. All rats were given intra-peritoneal injection of azoxymethane (AOM; 15 mg/kg body weight; Sigma Chemical Co., St Louis, MO) twice, at 2 and 3 week, after starting the experimental diets. Rats were sacrificed 6 weeks after the second AOM injection, and colon tissues were collected. One centimeter sections were cut from the distal end of each rat colon, fixed in 4% paraformaldehyde (PFA), and then embedded in paraffin. Half of the colon was fixed at 70% EtOH for aberrant crypt foci (ACF) determination and the other half was gently scraped for collecting protein and RNA [212, 226]. The animal use protocol was approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Assessment of aberrant crypt foci

After 24 h of fixation in 70% EtOH, tissue was stained with 0.5% methylene blue for 1 min, and the total number of high multiplicity ACF (HMACF; foci containing

4 or more aberrant crypts) was counted using a light microscope at 40x magnification [212, 226].

Cell proliferation assay

For Ki-67 immunohistochemistry analysis, sections were treated with primary antibody against Ki-67 (Dilution 1:50, BD Pharmingen, San Jose, CA) and incubated with biotinylated anti-mouse IgG, Vectastain ABC Elite kit (Vector Lab, Burlingame, CA). Ki-67-containing nuclei, indicative of proliferating cells, were visible as brown spots within colonic crypt columns. Twenty-five crypt columns per rat were selected for analysis.

Quantitative RT-PCR

Total RNA from the colon mucosal scraping was isolated using the mirVana™ miRNA Isolation Kit. HT-29 cells were seeded (3×10^5 cells onto a 6-well plate) and incubated for 24h. Cells were treated with Pg extract and mRNA was extracted after 24h [50]. Equal amount (1ug) of mRNAs was converted to cDNA using a reverse transcription kit (Invitrogen Corp., Grand Island, NY) [212, 226]. Real-time PCR reactions were performed using 2uL of cDNA using a Reverse Transcription Kit (Invitrogen). SYBR Green PCR master Mix (Applied Biosystems Inc., Foster City, CA) was used for the qPCR analyses on Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems Inc., Foster City, CA). The sequence of primers was designed using Primer3Plus® (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi) and were obtained from Integrated DNA Technologies

(Coralville, IA). GAPDH was used as the endogenous loading control [50].

Western blotting

The mucosal scrapings were homogenized in a protein buffer (500mM Tris-HCL, 1M Sucrose, 200mM EDTA, 100mM EGTA, 0.4M NaF, 10% Triton X-100, 10mM Sodium Orthovanadate, and Protease Inhibitor Cocktail), were centrifuged at 15,000 g for 20 min at 4°C, and the supernatant was stored in a 80°C freezer [212, 226]. HT-29 cells were seeded (1×10^5 onto 6-well plates) and incubated for 24h to allow cell attachment. They were treated with Pg extract (5-25 μg/ml) for 24h and protein was extracted after 24h [50]. 60ug of protein was loaded onto 10% electrophoresis gel, followed by electrotransfer onto PVDF membranes. The blots were probed with the primary antibodies against VCAM (Santa Cruz biotechnology, Santa Cruz, CA), Cox-2 (Cell signaling, Danvers, MA) and β-actin (Sigma, St Louise, MO) [50]. Transfection with Ant. of 126

Cells seeded (1×10^5 onto 12-well plates) were incubated for 24h to allow cell attachment. Transfections with 20nM Ant. (inhibitors) of miR-126 were performed as previously described [227].

Statistical analysis

Quantitative data represent mean values with standard error. Data were analyzed by one way ANOVA using Tukey's post hoc test ($p<0.05$) using SAS version 9 (SAS Institute Inc., Cary, NC)[50].

Results

Chemical analysis of pomegranate (Pg) juice

The polyphenolics profile of Pg juice is representative of 100% pomegranate juice [228]. The chromatographic profiles show flavonoids with peak absorption at 360nm (Figure 43a) and ellagitannins with peak absorption at 280nm (Figure 43b).

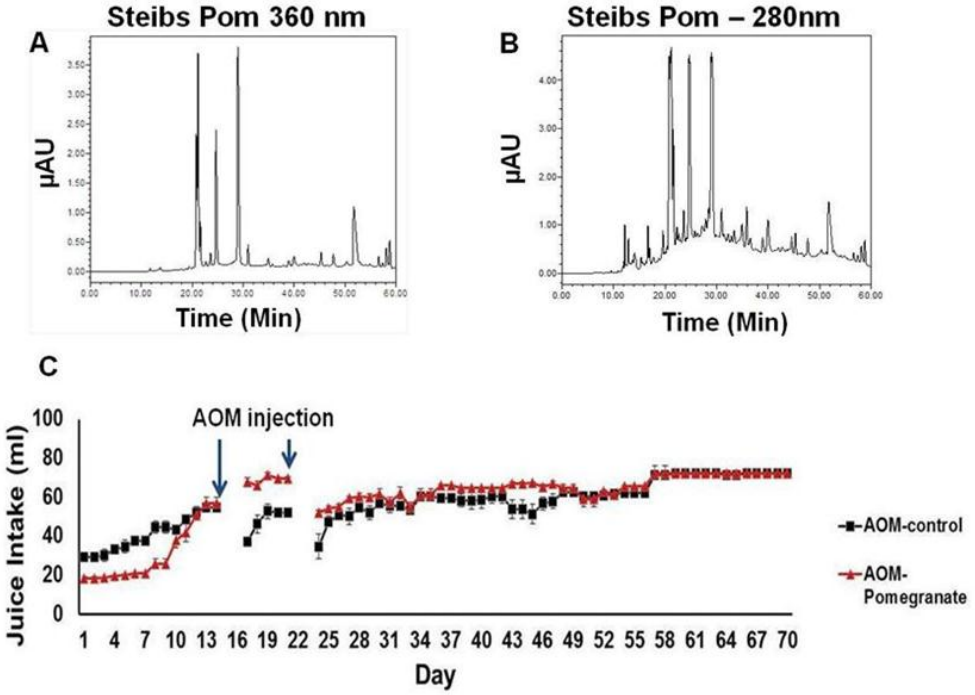

Figure 43 Representative chromatogram of polyphenolic compounds in pomegranate juice and the daily juice consumption by rats (a) flavonoids at 360nm, tentative peak assignments (b) ellagitannins at 280nm, tentative peak assignments1 punicalins, 2 punicalagin A, 3 punicalagin B, 4 ellagic acid. (c) the daily juice consumption by rats was measured every day. Values are mean ± SE, n=20

Table 5 Effects of pomegranate juice on final body weight, food and juice intake

Group	Final Body weight (g)	Food intake (g/day)	Juice intake (ml/day)
Control	355.975 ± 30.007	10.460 ± 1.804	53.186 ± 11.31^{ns}
Pomegranate	326.557 ± 20.034*	16.956 ± 1.430*	57.211 ±16.75

Each value is a mean ± SD (n=20). Values are statistically significant at *$p<0.05$. ns means not significantly different. Food intake was measured as the mean (± SD) weight (g) of food intake per 48 h period at 9 weeks after the second AOM injection. Juice intake was calculated as the mean (± SD) volume (ml) of juice consumed for whole study

Pg intake, food intake and body weight of AOM treated rats

The concentration of total soluble phenolics of Pg juice used for the animal study was 2504.74 mg/L GAE. The intake of Pg and control beverage was determined every alternative day (Figure 43c). For the initial 9 days, the intake of Pg was slightly but significantly lower compared to the control group by around 10% on average, likely because animals had to get used to the astringency of contained ellagitannins, however, the overall intake of liquids was not significantly different over the entire study duration. For the initial 48h after each AOM injection (week 2 and 3), the intake of liquids was significantly lower in the control group compared to the Pg-treated group, by 3ml and 30ml, respectively, potentially due to ameliorating effects of Pg on appetite-suppressing effects of AOM. The intake of solid food was significantly higher in the Pg group by 6 g/day compared to the control group. In contrast, the Pg group gained less weight

compared to the control group, where the final average body weight of the control group was 30g above the Pg group (Table 5).

Pg juice inhibits high multiplicity ACF (HMACF > 4 aberrant crypts/focus) in AOM-treated rats

High multiplicity ACF (HMACF) is used as a marker in the assessment of the initial stage of carcinogenesis in rat colon of azoxymethane (AOM) treated animals. Developmento of preneoplastic lesions, especially HMACF is found in human and animal colon cancer models and is known to be predictive of colon carcinogenesis [212, 226]. The number of HMACF was assessed over the entire length of the colon mucosa. The total number of HMACF was significantly decreased by the pomegranate treatment by 53.5% (Figure 44aand b) compared to the control group (p= 0.04).

Pg juice suppresses cell proliferation

Previous study show that pomegranate polyphenolics reduced cell proliferation in colon carcinogenesis [95]. Cell proliferation of mucosal tissue harvested from the colon of AOM-induced rats were determined by immunohistochemistry using the Ki67 antibody as an indicator for the proliferative index (Figure 44c). The colon mucosa of Pg-treated rats showed a significant reduction of Ki67-positive nuclear staining compared to the control groups. In the control group, Ki67-positive nuclei staining in the colon mucosa were 34.24% of cells, and it was only 23.41% of the cells in Pg treated animals, which represents an 11% reduction compared to the control group (P=0. 0127) (Figure 44d).

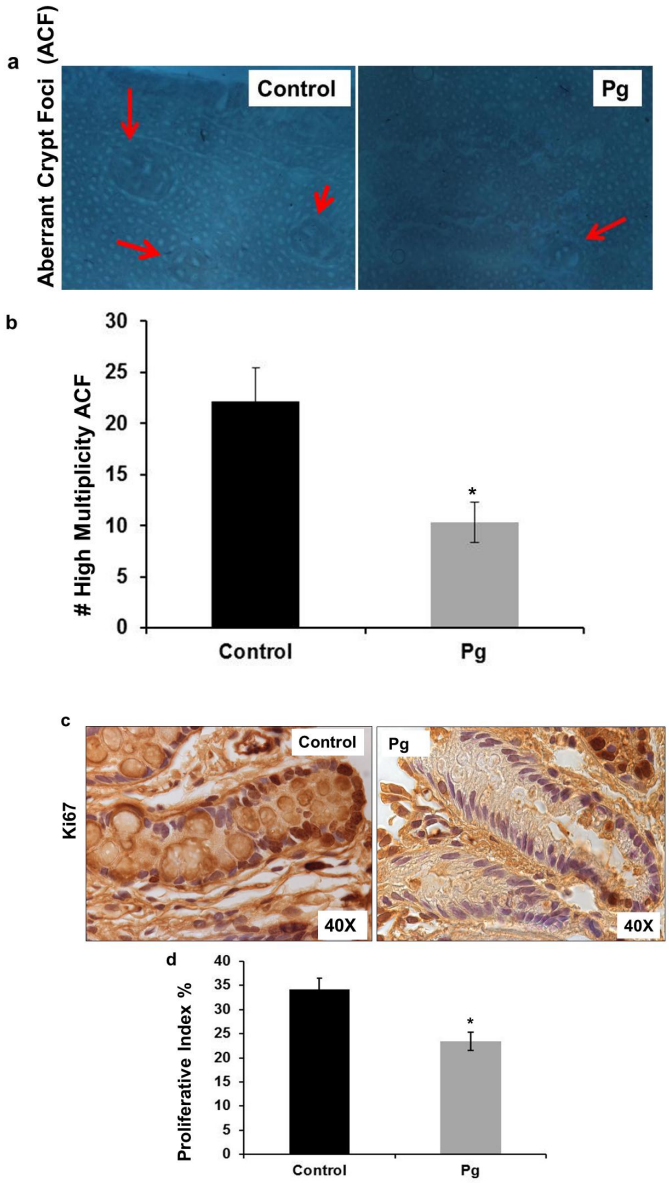

Figure 44 Effects of pomegranate juice on lesion formation and colonocyte proliferation (a) the number of HMACF image under light microscope. (b) the number of HMACF was significantly decreased by the pomegranate treatment by 53.5% compared to the control group (p=0.04). (c) Ki-67 immunohistochemistry staining (d) pomegranate juice inhibited cell proliferation compared with the control juice group. Values are mean ± SE, n=20. *P < 0.05

Pg juice suppresses the expression of inflammatory enzymes Cox-2, iNOS, NF-κB (p65) and VCAM-1 in rats treated with AOM

In colon cancer, the overexpression of inflammatory marker plays a significant role. Previously, we demonstrated the anti-inflammatory activities of polyphenols from pomegranate against the inflammation in breast cancer [50]. In this study, the anti-inflammatory activities of Pg juice in rats treated with AOM were investigated. Treatment of AOM-injected rats with Pg decreased the expression of COX-2, iNOS, and VCAM-1 mRNA and protein (Figure 45 a,b, c and d). Interactions between pomegranate polyphenolics and NF-κB signaling have been previously studied in several studies [69, 106, 229]. In our previous study, Pg significantly reduced the expression of constitutive expression and phosphorylation of NF-κB p65 in breast cancer cells BT474 and MDA-MB-231 [50] and revealed the anti-inflammatory properties of pomegranate polyphenolics. Similarly, we have found that Pg also suppressed the constitutive expression and phosphorylation of NF-κB p65 in colon cancer cells (Figure 45 c and d).

Pg juice modulates the PI3K/AKTmTOR pathway in AOM treated rats

The PI3K/AKT signaling pathway plays an important role in carcinogenesis for several types of cancer including colon cancer [211] and influences cell survival pathways through phosphorylation of downstream targets such as NF-κB and mammalian target of rapamycin (mTOR) [213-215]. Previously we have shown that the anti-inflammatory effects of polyphenols from pomegranate were in part mediated through the suppression of the PI3K/AKT pathway in breast cancer cells in vitro and vivo [50]. Insulin growth factor (IGF) regulates the phosphorylation of PI3K/AKT and

pmTOR. In this study, pomegranate juice decreased in the expression of insulin growth factor (IGF) and pPI3K accompanied by decreased expression of pAKT and pmTOR mRNA and protein level (Figure 45e and f).

Pg juice increases the expression of miR-126 in AOM-treated rats

Small non-coding microRNAs (miRNAs) are known to influence several biological processes such as cell development, differentiation and maintenance, and also to controll the process of carcinogenesis [211]. In previous studies, it was reported that the expression of miR-126 is significantly reduced in colorectal cancer compared to non-tumor tissue, where miR-126 is involved in suppressing excessive proliferative activity [211, 230]. miR-126 targets several mRNAs, that have a complementary sequence within their 3'UTR region such as PI3K/AKT and VCAM-1 [211, 225]. Previously we have demonstrated the ability of polyphenolics to increase the expression of miR-126 in LPS-treated human colon-derived CCD-18Co myofibroblast cells [222]. Pomegranate extract increased the expression of miR-126 (Figure 45 gFig. 3G) and correspondingly, this was accompanied by reduction of PI3K, AKT and VCAM-1 mRNA and protein expression in AOM-treated rats (Figure 45 c,d e and f).

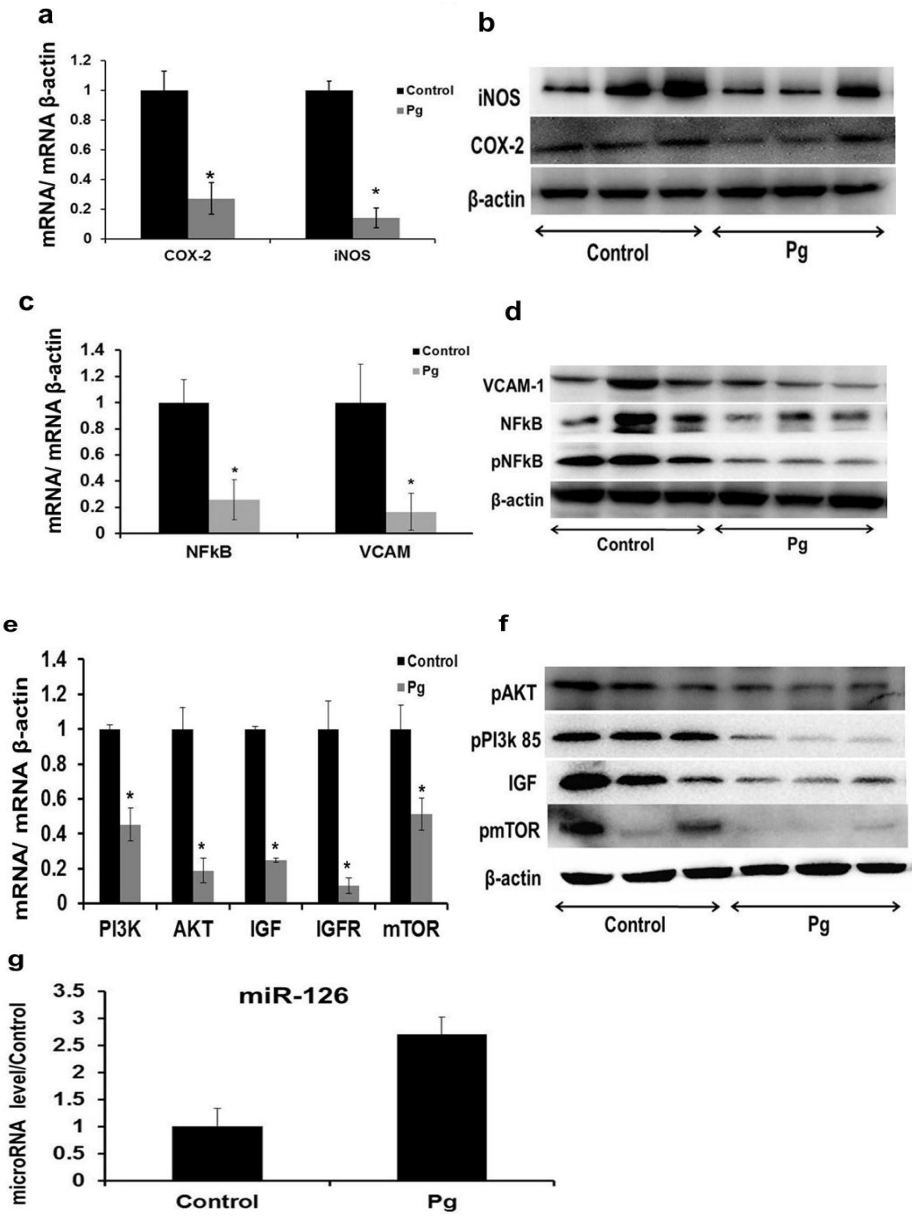

Figure 45 Effects of pomegranate juice on the inflammatory and the mTOR signalling pathways in AOM treated rats (a) Pg juice decreased the expression of COX-2 and iNOS mRNA and (b) protein in AOM treated rats. (c) Pg juice suppressed the expression of VCAM-1 and NF-kB mRNA and (d) protein in AOM treated rats. (e) Pg juice decreased in the expression of IGF and pPI3K accompanied by decreased expression of pAKT and pmTOR mRNA and (f) protein levels in AOM treated rats. (g) Pg juice increased the expression of miR-126 in AOM treated rats. All experiments were performed as described in the material and method section, and result were expressed as mean ± SE.* indicates significant changes at $p<0.05$

Pg reduces cell viability and increases markers for apoptopsis in HT-29 colon cancer cell lines

The cytotoxic activities of Pg were confirmed in HT-29 human colon cancer cells. The result showed a concentration-dependent decrease in cell viability in HT-29 cells after treatment with Pg (5-25 μg/ml) after 48 and 72h (Figure 46a). Reduced cell viability was accompanied by an increase of the activated form of capase-3, a primary apoptosis-executing enzyme, and the cleaved form of its substrate Poly (ADP-ribose)-polymerase1 (PARP) (Figure 46b).

Pg decreases inflammation and angiogenesis in HT-29 colon cancer cell lines

Previously, we have shown the anti-inflammatory activities of polyphenols from pomegranate in breast cancer [50]. In AOM-injected rats, Pg inhibited the expression of inflammatory markers, such as VCAM-1. Similarly, in colon cancer cells, Pg decreased the expression of inflammatory markers, pAKT, NF-κB p65, VCAM-1, ICAM-1, COX-2 and angiogenesis marker vascular endothelial growth factor (VEGF) mRNA and protein (Figure 46c and d). However, there was no changes of VEGF expression by Pg in vivo study.

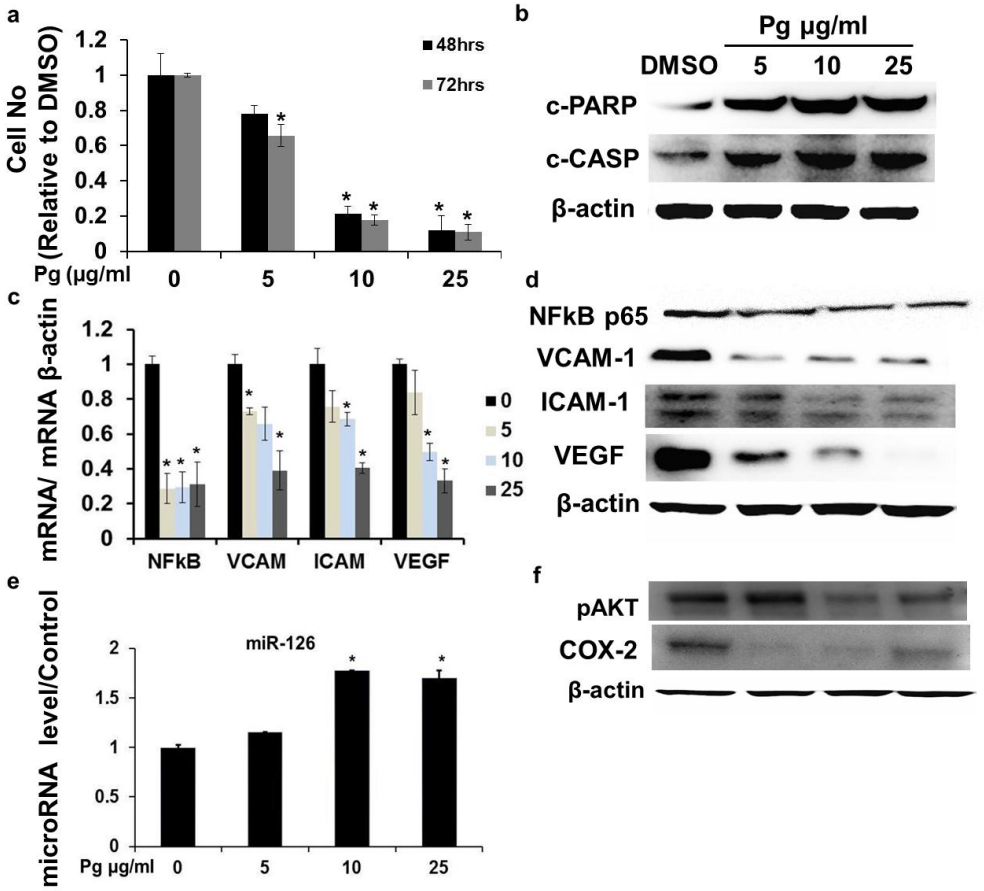

Figure 46 Effects of Pomegranate extract on the inflammatory and the mTOR signalling pathways in HT-29 human colon cancer cell lines (a) Pg extract inhibited proliferation in human colon cancer cells. (b) Pg extract induced the protein expression of apoptosis-associated proteins (Cleaved caspase-3 and cleaved PARP). (c) Pg decreased the expression of inflammatory markers, NF-κB p65, VCAM-1, ICAM-1and angiogenesis marker vascular endothelial growth factor (VEGF) mRNA and (d) protein in HT-29 human colon cancer cells. (e) pomegranate extract increased the expression of miR-126 In HT-29 cells. (f) pAKT, and Cox-2 protein expression. Cells were treated with solvent DMSO (control) or different concentration of Pg (5-25 µg/ml) for 24 h. All experiments were performed as described in the material and method section and were performed at least three times, and result were expressed as mean ± SE.* indicates significant changes at $p<0.05$.

Pg increases the expression of miR-126 in HT-29 cells

In HT-29 cells, pomegranate extract increased the expression of miR-126 (Fig. 4E) and this was accompanied by reduction of PI3K/AKT and VCAM-1 mRNA and protein expression (Figure 46c, d and f).To further understand whether the underlying mechanisms of Pg with PI3K and VCAM-1 are based on an increase in miR-126, HT-29 cells were transfected with the miR-126 antagomir (inhibitor). When transfected cells were treated with 10 μg/ml pomegranate polyphenols extract, this repression of miR-126 by the antigomer treatment was reversed by Pg (Figure 47a) and this was accompanied by a decreased expression of VCAM-1 mRNA and protein in transfected cells that were treated with Pg (Figure 47 and c). Additionally, when cells were transfected with the miR-126 antagomir, the mRNA expression of PI3K was increased, and the expression of mRNA of PI3K were partially reversed by the treatment of 10μg/ml pomegranate polyphenols (Figure 47d). These findings indicate that Pg increases miR-126 and it is involved in the reduction of VCAM-1 and the suppression of PI3K/AKT in HT-29 colon cancer cells.

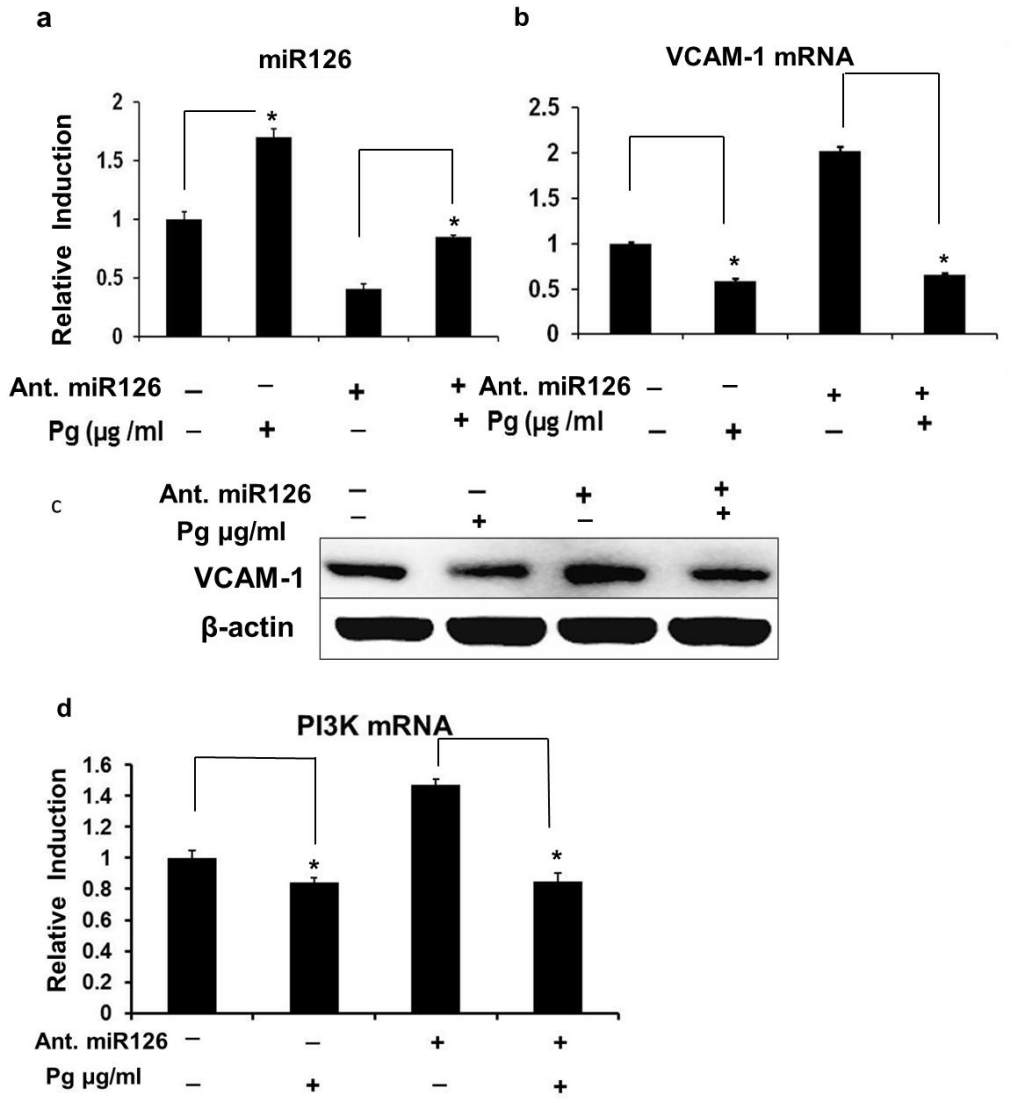

Figure 47 Effects of pomegranate extract on miR-126 levels and its target genes in HT-29 cells transfected with Ant.miR-126 (a) Pg extract reversed this repression of miR-126 by the antigomer treatment. Cells were transfected with 20nM Ant. miR-126, and were treated with 10 µg/ml Pg extract (b) the reversed expression of miR-126 was accompanied by a decreased expression of VCAM-1 mRNA and (c) protein in HT-29 colon cancer cells were transfected with 20nM Ant. miR-126. (d) induced mRNA expression of PI3K was partially reversed by the treatment of 10 µg/ml pomegranate polyphenols in transfected cells. All experiments were performed as described in the material and method section and were performed at least three times, and results were expressed as mean ± SE.* indicates significant changes at $p<0.05$

Discussion

In this study, we demonstrated the cancer-preventive and therapeutic activities of polyphenolics in pomegranate through the suppression of cell proliferation and inflammation. The number of HMACF was significantly decreased and the percentage of proliferative cells by Pg treatment in azoxymethane (AOM)-treated rats. In order to confirm in vivo findings, the proapoptotic and anti-inflammatory effects of pomegranate polyphenolics were also investigated in vitro, in HT-29 colon cancer cells, where Pg induced apoptosis and inhibited cell proliferation. Moreover, similar results were observed in HT-29 colon cancer cell lines where Pg upregulated the expressions of capase-3, a primary apoptosis-executing enzyme, and poly (ADP-ribose)-polymerase1 (PARP1) in HT-29 cells .

Polyphenols extracted from pomegranate induced antioxidant, anti-inflammatory, and anti-tumorigenic effects in colon cancer and other cancer models [49, 50, 68, 109, 119]. In our previous studies, we demonstrated the cytotoxic effect of pomegranate polyphenols in breast cancer BT474 cells and the inhibition of tumor growth by Pg in anthymic nude mice bearing BT474 cells as xenografts [50]. Previously, pomegranate polyphenols induced apoptosis modulating caspase-3 and targeted multiple pathways in several cancer cell lines including breast, prostrate, liver, lung and skin cancer [47, 49, 50, 106, 109, 217]. In vitro data in this study demonstrated similar results.

Common colon cancer and inflammatory markers such as VCAM-1 and NF-κB mRNA and protein expression were decreased both in vitro and vivo model by Pg.

Previous studies demonstrate that pomegranate polyphenols decreased inflammation and lung-tumor growth in mice by decreasing the activation of NF-κB [69, 106]. NF-κB plays a central role in inflammatory pathways, and controls oncogenes and tumor suppressor genes, as well as adhesion molecules VCAM-1 [231]. Cell adhesion molecules such as VCAM-1 disrupt normal cell differentiation, which in turn leads to neoplastic transformation, progression, angiogenesis and metastasis of cancer cells. Thus increased expression of VCAM-1 possibly influences colon cancer progression [232]. In this present study, Pg suppressed the expression of VCAM-1 in AOM treated rats. Similar results were observed in HT-29 colon cancer cells which were accompanied by reducing angiogenesis marker vascular endothelial growth factor (VEGF).

In colorectal cancer patients and rodents with chemically induced colon cancer, chronic inflammatory markers such Cox-2 and iNOS are up-regulated [212, 226, 233]. Excessive nitric oxide (NO) induced the expression of Cox-2, which in turn controls cell proliferation, inflammation and may inhibit apoptosis in colon cancer cells [233, 234]. Thus upregulation of Cox-2 can increase cell proliferation [223, 235]. Previously it was shown that pomegranate polyphenols suppressed the expression of Cox2and iNOS, and inhibited cell proliferation by inducing cell cycle arrest and apoptosis in HT-29 colon cancer cells [119]. In this study, Pg decreased mRNA and protein expression of Cox-2 and iNOS in AOM treated rat model (Fig. 3A and B) and Cox-2 protein level in HT-29 human colon cancer cell. Pg inhibited cell proliferation in HT-29 colon cancer cells and also in AOM rat model showed by Ki-67 staining and this was accompanied by the

decrease in the number of HMACF, a colon cancer biomarker in rats treated with Pg juice .

Previous studies have suggested the potential role of small non-coding microRNAs (miRNAs) in the pathogenesis of cancer and its control of diverse biological processes including cell proliferation, development, differentiations, maintenance and carcinogenesis [224]. miRNAs are known to function as tumors suppressors or oncogenes. In order to investigate the underlying mechanisms of the down-regulation of VCAM-1 and the PI3K/AKT pathway, the potential role of tumor suppressor miR-126, a colonic prognostic marker, involved in the anti-inflammatory activity by targeting VCAM-1 [225] was investigated. Pg decreased expression of VCAM-1 which was accompanied by up-regulation of miR-126 in vivo and vitro. VCAM-1 is regulated by miR-126 via a target-binding site in the 3' UTR region of the VCAM-1 mRNA [225]. To confirm the involvement of miR-126, a specific antagomir for miR-126 (as-miR-126) was used to decrease miR-126 expression and to increase the expression of VCAM-1, where Pg partially but significantly reversed the effects of the antagomir.

Additionally, findings show that Pg down-regulates the expression of IGF, PI3K and AKT. Pg reduced the activation of NF-kB and the phosphorylation of PI3K p85b, and AKT, and this is consistent with our previous finding where pomegranate extract decreased the expression of and phosphorylation of NF-kB and the PI3K/AKT pathways [50]. Also it was shown that Pg modulated expression of Insulin growth factor (IGF) and PI3K/AKT which is crucial in the pathogenesis of colon cancer. The PI3K/AKT pathway controls cell survival mechanism through phosphorylation of

downstream targets such as NF-kB and mammalian target of rapamycin (mTOR). The regulatory p85b subunit of PI3K has been shown to be the direct target for miR-126 in colon cancer cancer [211]. The decreased expression of PI3K p85b was accompanied by an upregulation of miR-126 as well as decreased phosphorylation of AKT and reduced phosphorylation of mTOR.In order to confirm the involvement of miR-126 in the regulation of PI3K p85β, a specific antagomir for miR-126 (as-miR-126) was used. The antagomir for miR-126 decreased the expression of miR-126 in vitro and increased the expression of the PI3K p85b subunit. Pg reversed the effects of the antagomir significantly. As found in vivo in rats, CCD-18Co colon myofibroblast cells showed significant reduction of miR-126 and increased expression of PI3K p85b subunit [211].

It has to be noted that animals in this study treated with Pg gain less body weight compared to control animals, even though their caloric intake was increased. Previous studies with polyphenolics indicated that the consumption of polyphenolics caused a reduction of weight gain in animals [236, 237]. It has previously been reported that weight loss had beneficial effects on biomarkers of inflammation in rats [236-238]. Therefore, in this study, it is not conclusively clear, whether the observed anti-inflammatory effects of Pg polyphenolics are derived from a direct interaction of polyphenolics with inflammatory pathways or whether at least part of the benefits is derived from the anti-inflammatory effects of reduced weight-gain in animals treated with Pg. Overall, there was no correlation between weight gain and the number of HMACF (R^2=0.0956, p=0.2439), for this reason, the effects of reduced weight-gain

caused by Pg may not have significantly contributed to the reduction of HMACF caused by Pg.

Conclusion

In summary, pomegranate polyphenols (Pg) exerted cytotoxic and anti-inflammatory effects in AOM-treated rats and colon cancer cells. Interactions of Pg with miR126/VCAM-1 and miR126/PI3K/AKT/mTOR axes Figure 48) were identified as mechanisms that at least in part appear to be involved in the anti-inflammatory and anti-proliferative activities of pomegranate polyphenolics.

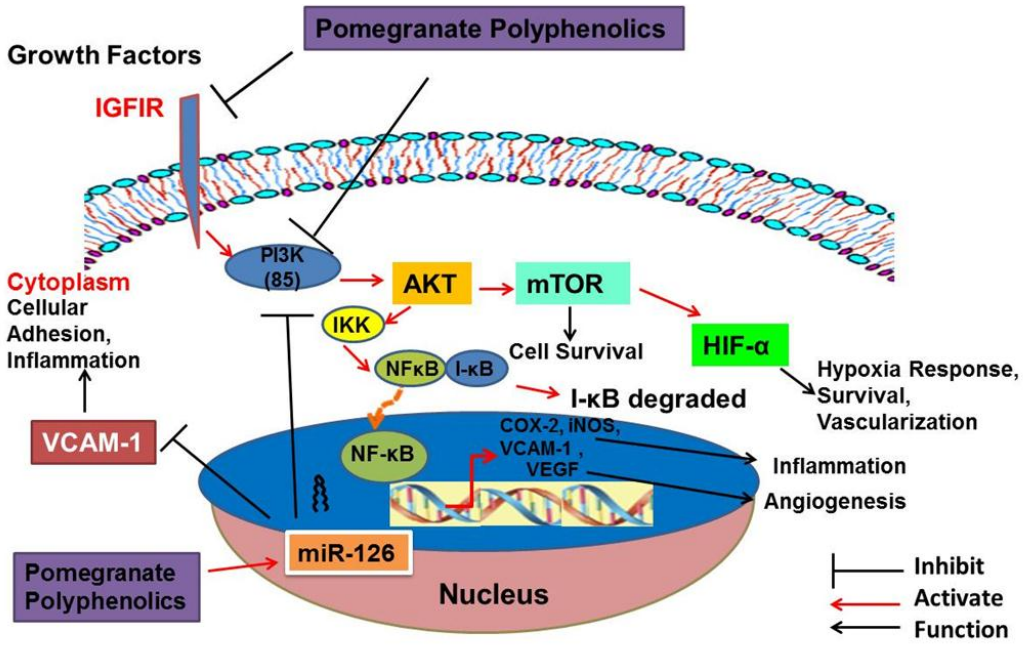

Figure 48 Schematic representation showing molecular mechanisms involved in colon carcinogenesis treated with Pg polyphenols. Pg targeted miR126 by modulating both miR126/VCAM-1 and miR126/PI3K/AKT/mTOR axe

CHAPTER V

CHOLOROGENIC ACID RICH PLUM JUICE INHIBITED AOM TREATED COLORECTAL ABERRANT CRYPT FOCI (ACF): POTENTIAL ROLE OF MIR143/ MTOR PATHWAY

Summary

The antitumorigenic cytotoxic, anti-inflammatory and antioxidant activities of polyphenols, chlorogenic acid and neo-chlorogenic acid in plum juice (PL) have been previously studied and were evident in various cancer models. Our preliminary result exhibited anti-inflammatory property of PL which decreased the expression of VCAM-1, a marker for colon cancer, and the activation of PI3K/AKT, that is crucial in the regulation of cell survival and proliferation in AOM treated in vivo study. In addition, PL also increased microRNA143, which targets mTOR.

Hence, the objective of this study was to investigate the underlying cytotoxicity mechanisms involving miR143/PI3K/AKT/mTOR in PL-mediated anti-inflammatory and anti-carcinogenic effects in vivo and in vitro.

Sprague Dawley rats (n=10/group) received PL juice (1345.68ppm GAE) or a polyphenol-free control beverage ad libitum for 10 weeks and were injected with azoxymethane (AOM) subcutaneously (15 mg/kg) at weeks 2 and 3.

Consumption of PL juice suppressed the number of high multiplicity aberrant crypts foci (HMACF>4 ACF) by 47.98% ($P<0.05$) and significantly lowered

proliferation of mucosa cells. The results were accompanied by a downregulation of nuclear factor kappa B (NF-κB), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), vascular adhesion molecule-1 (VCAM-1) and mRNA and protein. PL also inhibited phosphorylation of PI3K/AKT, and mTOR/HIF-1α pathways. PL increased the expression of miR143 expression.

In summary, PL suppressed number of HMACF formation, colon tumorgenesis where microRNA-143-regulated pathways significantly contributed in the underlying cytotoxicity mechanisms.

Introduction

According to the American cancer society colorectal cancer is the third leading cause of death in the US with an estimated of 102,480 new cases of colon cancer and 40,340 new cases of rectal cancer diagnosed in 2013 and record showed 50,830 deaths from colorectal cancer [2, 3]. The Incidence of colorectal cancer and the associated mortality rates were increasing worldwide and the lifetime risk of developing colorectal cancer is about 1 in 20 [2, 3]. In addition to available therapies there is a need to develop novel therapeutic agents in the prevention and treatment of colorectal cancer [91-93]. Epidemiological, preclinical and clinical studies suggest that a diet rich in natural polyphenolics plays an important role in the protection against colonic inflammation and colon cancer [45, 46, 93, 95]. Plant polyphenols have drawn immense attention due to potential antioxidant, anti-inflammatory, and anti-tumorigenic effects in colon cancer and other cancer models and have shown potential as a cancer-suppressing agents [49,

50, 74, 119, 239]. Polyphenols are known to target different molecular pathways in cancer cell lines with less toxic effects in normal cell lines [50, 100, 133].

Plums (Prunus domestica) belonging to the family Rosaceae, contains a mixture of polyphenols that exerts anti-cancer and antioxidant properties [155, 240]. In commercial available variety of plums, the most predominant and therapeutically relevant compounds are flavonols, (the flavan 3-ols catechin, and epicatechin; the flavonols quercetin 3-glucoside), anthocyanins (cyanidin 3- galactoside, 3- glucoside, 3-rutinoside, and 3-acetylglucoside), procyanidins (dimers and trimmers), phenolic acids (chlorogenic acid and neo-chlorogenic acid) and carotenoids [73-75, 240].

Literature showed chlorogenic acid and neo-chlorogenic acid present in fruits are known to function as chemoprevention and therapeutic agent in several cancer cell lines such as breast cancer, U937 leukemia cells, and also in AOM treated in Fisher 344 male rats [74, 75, 155, 241]. Recent studies have shown that several other polyphenols such as flavonoid quercetin and stilbene and resveratrol prevent the formation of colorectal aberrant crypt foci (ACF) and inhibit the occurrence of colon tumors in rats injected with azoxymethane (AOM). Polyphenolic constituents of grapefruit and blueberry suppressed HMACF formation in animal studies [45, 233]. Similarly plum also function as a cancer blocking agent and cancer suppressing agents by targeting several cellular pathways at the cancer initiation stage. Plum also protect cells from oxidative stress by scavenging ROS and other oxidative species, and known to detoxify carcinogen activity and initiate DNA repair [155, 242]. In addition, Plum also suppress

cancer promotion and progression stages by down regulating activation of transcription factor NF-kB and initiates tumor cell apoptosis [242].

In our preliminary studies, we have demonstrated the effect of plum juice (PL) in carcinogen, AOM treated rats. PL was effective in suppressing the high multiplicity of ACF (HMACF〉4 aberrant crypts/focus) and decreased cell proliferation in the carcinogen, AOM treated rats. PL inhibited the expression of colon marker VCAM-1 expression and also suppressed the phosphorylation of PI3K/mTOR /HIF1α signaling pathways in AOM treated rats. In addition PL also targeted inflammatory marker such as iNOS and COX-2 and decreased expression of iNOS and COX-2 through inhibition of NF-kB activation.

Hence, the objective of this study was to understand the anti-inflammatory, anti tumorogenic and antiproliferative properties of polyphenolic mixture present in PL juice which exerted chemoprevention and chemotherapeutic activities in cancer model with low toxicity in control groups.

Materials and methods

Experimental juice and extract

Control juice or Plum juice groups rats. Commercial varieties of red-fleshed plum ‘Black Splendor’ (BS) were collected at a mature firm stage from California. Upon arrival at the Texas A&M University, fruits were used for the extraction of phenolic compounds. Plum juice (PL) was obtained from a total of 100 g of plum fruit with flesh and skin was chopped, enzyme pectinase was added to it and then boiled at water bath for 1 h until the temperature reached at 70 degree centigrade. Extracts were

filtered through whatman #1 filter paper. The remaining aqueous extracted plum juice was saved at 4 degree centigrade. For calorie adjustment, 15.7g sugar and 0.05g citric acid were added in 100ml of Control juice. Total phenolic content in the plum juice was measured spectrophotometrically by the Folin-Ciocalteu assay against an external standard of gallic acid. Individual pholyphenolics (1345.68 mg/L GAE; gallic acid equivalent) in the plum juice were characterized using a Waters Alliance 2690 (Milford, MA) HPLC system [50].

Measurement of antioxidant enzyme activity

Activities of SOD (Superoxide Dismutase), CAT (Catalase), and GPx (Glutathione peroxidase) in colonocytes were measured spectrophotometrically using assay kits from Cayman Chemical(Ann Arbor, MI) according to the manufacturer's protocol. The protein extract was obtained as described in the Western blot procedure. Samples were assayed in duplicate in 96 well microplates with standards. Microplates were read on a FLUOstar Omega plate reader (BMG Labtech Inc. Durham, NC). Briefly, SOD activity was evaluated by measuring the rate of generation of a chromophore at 525 nm. CAT activity was calculated at 520 nm by measuring absorbance of quinoneimenedye. GPx was evaluated at 340 nm indirectly by oxidation of NADPH to $NADP^{+}$. Activity was normalized to protein concentration as determined by Bradford reagent (Bio-rad, Hercules, CA).

Reagent

All primers were purchased from Integrated DNA Technologies (San Diego, CA). mirVana TM extraction kit, reverse transcription (RT) and real-time PCR

amplification kits were purchased from Applied Biosciences (Foster City, CA as well as scrambled miRNA were from Dharmacon, Inc. (Lafayette, CO).) [50]. Antibodies against COX-2, iNOS, HIF-1alpha, mTOR, pPI3K85 were purchased from Cell Signaling Technology (Beverly, MA). All other antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Western lighting chemiluminescence reagent was purchased from Perkin-Elmer Life Sciences (Waltham, MA).

Animal treatment and tissue sampling

The animal use protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M University. Twenty male Sprague-Dawley rats (3 week-old) were purchased from Harlan Teklad (Houston, TX) and were distributed randomly by weight into control juice and plum juice groups. Rat groups were kept in suspended cages in a room controlled at a temperature of 23±2°C, humidity of 55±5% and 12 h light/dark cycle, and had free access to regular food pellets, rat chow and experimental juices. All animals received intra-peritoneal injection of azoxymethane (AOM; 15 mg/kg body weight; Sigma Chemical Co., St Louis, MO) twice at 2 and 3 week after starting experimental diets. Rats were sacrificed 6 weeks after the second AOM treatment, and colon tissues were collected. From the distal end of each rat colon one centimeter sections were cut, and fixed in 4% paraformaldehyde (PFA). Half of the colon was fixed at 70% EtOH for aberrant crypt foci (ACF) assay and the other half was gently scraped for collecting protein and RNA samples [212, 226].

ACF counting

Half of the colon was fixed at 70% EtOH for aberrant crypt foci (ACF) assay.

After 24 h of fixation in 70% EtOH, tissue was stained with 0.5% methylene blue for 1 min, and the total number of high multiplicity ACF (HMACF; foci containing 4 or more aberrant crypts) was counted under a light microscope using 40x magnification [212, 226]. HMACF and ACF were assessed over the entire length of the colon mucosa and also within segments. The mucosa was divided from the distal through the proximal end.

Cell proliferation assay

For cell proliferation, analysis Ki-67 immunohistochemistry was performed. Colon tissues were dehydrated, embedded in paraffin and cut into 4 μm thick sections. The distal colon section (1cm) was fixed in 70% ethanol and then embedded in paraffin. Paraffin embedded sections were stained with Ki67 [243]. The primary antibody against Ki-67 (Dilution 1:50, BD Pharmingen, San Jose, CA) was treated on the sections, and sections were incubated with biotinylated anti-mouse IgG from the Vectastain ABC Elite kit (Vector Lab, Burlingame, CA). Ki-67-containing nuclei, indicative of proliferating cells, showed up as brown spots within colonic crypt columns. Twenty-five crypt columns per rat were identified for analysis.

Quantitative RT-PCR

Total RNA from the colon mucosal scraping was isolated using the mirVana™ miRNA Isolation Kit. Equal amount (1ug) of mRNAs was converted to cDNA using a reverse transcription kit (Invitrogen Corp., Grand Island, NY) [212, 226]. Real-time PCR reactions were performed using 2uL of cDNA using a Reverse Transcription Kit (Invitrogen). SYBR Green PCR Master Mix (Applied Biosystems Inc., Foster City, CA)

was used for the qPCR analyses on Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems Inc., Foster City, CA). The sequence of primers was designed using Primer3, online primer design tool and were obtained from Integrated DNA Technologies (Coralville, IA). GAPDH was used as the endogenous loading control[50].

Western blotting

The mucosal scrapings were homogenized in a protein buffer (500mM Tris-HCL, 1M Sucrose, 200mM EDTA, 100mM EGTA, 0.4M NaF, 10% Triton X-100, 10mM Sodium Orthovanadate, and Protease Inhibitor Cocktail), then centrifuged at 15,000 g for 20 min at 4°C, and the supernatant was stored in a 80°C freezer[212, 226]. 60ug of protein was loaded onto 10% electrophoresis gel, followed by electrotransfer onto PVDF membranes. The blots were probed with the primary antibodies against VCAM-1 (Santa Cruz biotechnology, Santa Cruz, CA), Cox-2, , iNOS, HIF-1alpha, mTOR, pPI3K85 (Cell signaling, Danvers, MA) and b-actin (Sigma, St Louise, MO) [50]. We performed western blotting as previously mentioned [50, 139, 140]. Proteins were visualized with a Kodak Molecular Imaging System (Carestream Health, Rochester, NY) [50].

Multiplex bead assay

The protein extract (20ug) from each tissue was used to determine the relative abundance of the phosphorylation status and total protein in the Akt/mTOR signaling (kit contained Akt, GSK3β, GSK3α, IGF1R, IR, IRS1, mTOR, p70 S6 kinase, PTEN, RPS6, and TSC2) using the multiplex kits (#48-611, #48-612, Millipore) according to

the manufacturer's protocol. Multiplex analysis was performed by the Luminex L200 instrument (Luminex, Austin, TX), and data were analyzed by Luminex xPONENT software [210].

SCFA GC/MS assays

SCFA analysis was performed using a gas chromatograph (HP 5890, Hewlett-Packard, Palo Alto, CA, USA) coupled to a quadrupole mass spectrometer (HP-5989A). Sample preparation for short chain fatty acids with GC/MS assay is as follows. Grinded feces (0.5g) were vortexed in 2N HCL for 30 minutes followed by centrifugation at 3000rpm for 20 min. The upper phase was transferred to C18 cartridge after adding 200mM internal standard (d7-butyric acid). The sample was then eluted with diethyl ether. Removing the top layer was repeated after adding diethyl ether and vortexing for 15min. After adding MTBSTFA (N-tert-butyldimethylsilyl-N methytrifluoracetamide), samples were transferred to vials for the GC/MS injection.

Statistical analysis

Quantitative data represent mean values with standard error. Data were analyzed by one way ANOVA using Tukey's post hoc test ($p<0.05$) using SAS version 9 (SAS Institute Inc., Cary, NC)[50].

Results

Chemical analysis of plum (PL) juice

The polyphenolics profile of Plum (PL) juice is representative of 100%.[73-75, 240]. The chromatographic profiles show presence of phenolic acids (chlorogenic acid and neo-chlorogenic acid) (Figure 49a).

Plum juice intake, food intake and weight in AOM treated rats

In the rat study the concentration of total soluble phenolics of plum (PL) juice used was 1345.68mg/L GAE. Every alternative day the intake of PL and control beverage was measured. Overall, no significant differences were observed between the intake of PL and control group (Figure 49 b). For the first 9 days, the intake of PL was significantly lower compared to the control group, likely because of the change of taste. For the initial 48h after the AOM injection (week 2 and 3), the overall intake of liquid was significantly lower in the control group compared to the PL-treated group by 30ml and 15ml, respectively, potentially due to ameliorating effects of PL on the adverse effects of AOM. The control group gained more weight compared to the PL group, where the final average body weight of the control group was 43g above the PL group (Table 6). Similarly the intake of solid food was significantly lower by 6 g/day in the control group compared to the PL group.

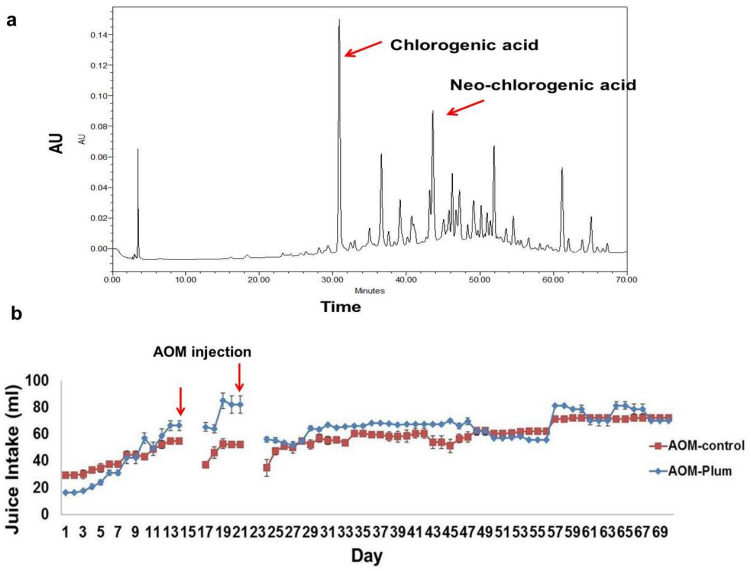

Figure 49 Representative chromatogram of polyphenolic compounds in plum juice and the daily juice consumption by rats (a) effects of plum juice on final body weight, food intake, and juice intake (b) the daily juice consumption by rats was measured every day for 10 weeks. Values are mean ± SE, n=20

Table 6 Effects of plum juice on final body weight, food intake, and juice intake

Group	Final Body weight (g)	Food intake (g/day)	Juice intake (ml/day)
Control	355.975 ± 30.007	10.460 ± 1.804	53.186 ± 11.31^{ns}
Plum	313.296 ± 23.367*	16.886 ± 1.271*	58.494 ±16.48

Each value is a mean ± SD (n=20). Values are statistically significant at *$p<0.05$. ns means not significantly different. Food intake was measured as the mean (± SD) weight (g) of food intake per 48 h period at 9 weeks after the second AOM injection. Juice intake was calculated as the mean (± SD) volume (ml) of juice consumed for whole study

Antioxidant enzyme activity

Antioxidant enzyme activities for GPx, SOD and CAT were greater in the control group as compared to the plum juice group (Figure 50). In the colon mucosa tissue GPx, SOD and CAT enzyme activities were significantly suppressed 70%, 45% and 80% respectively in the PL group than control (Figure 50a) but in plasma only SOD expression were significantly reduced 50% in PL group (Figure 50b). Thus antioxidant activity of plum induced the expression of GPx, SOD and CAT [244].

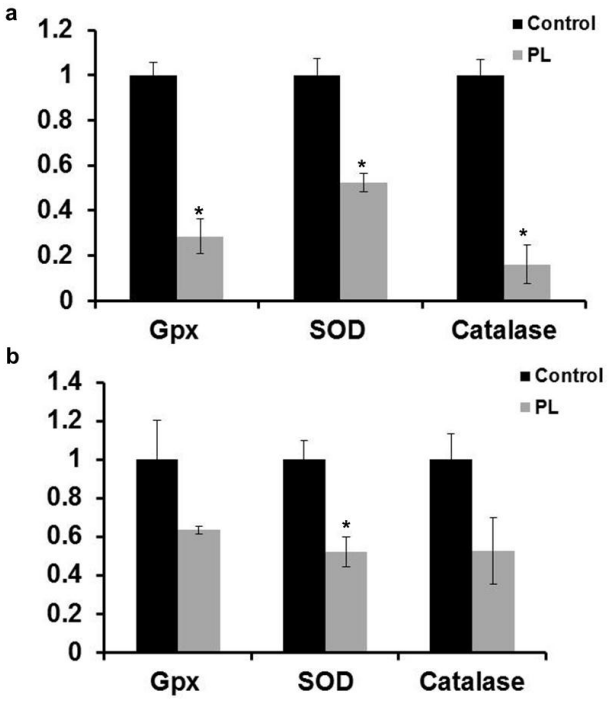

Figure 50 Effects of plum juice (PL) on antioxidant enzyme activity in AOM treated rats (a) antioxidant enzyme activities for GPx, SOD and CAT were significantly higher in the control group as compared to the plum juice group (in tissue). (b) antioxidant enzyme activities for SOD were significantly higher in the control group as compared to the plum juice group (in plasma). All experiments were performed as described in the material and method section, and result were expressed as mean ± SE.* indicates significant changes at $p<0.05$

Colorectal aberrant crypt foci (ACF) and high multiplicity ACF (HMACF > 4 aberrant crypts/focus) in AOM-treated rats

Both aberrant crypt foci (ACF) and high multiplicity ACF (HMACF), markers for the initial stages of colon cancer and these preneoplastic lesions are found in human and animal colon cancer models [212, 245, 246]. In the more distal segments, the number of HMACF was significantly reduced in rats treated with plum juice by 47.98% ($P<0.05$) to the control group (Figure 51a).

Ki67 staining for cell proliferation index

Cell proliferation index was calculated by using Ki67 immunohistochemistry staining of mucosal tissue harvested from the colon of AOM-treated rats and control groups. (Figure 2A). The colon mucosa of control group rats showed a significant induction of Ki67-positive nuclear staining compared to the PL-treated group. In the control group Ki-positive nuclei staining in the colon mucosa were 34.24% of the cells and in PL treated group showed 25.82% of cells. Thus PL group showed a significant reduction of 8.42% compared to the control group ($P\leq0.0127$) (Figure 51b).

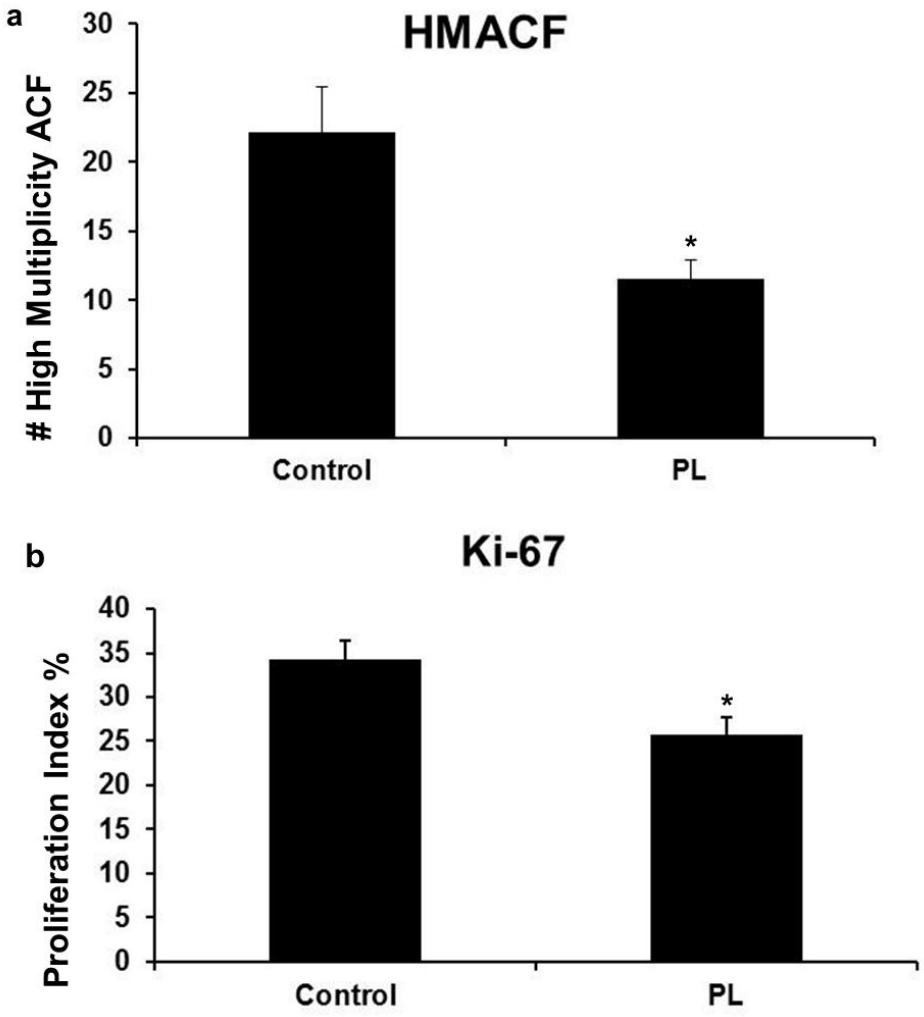

Figure 51 Effects of plum uice on lesion formation and colonocyte proliferation (a) the number of HMACF was significantly decreased by the plum treatment by 47.98% compared to the control group (p=0.04) (b) plum juice inhibited cell proliferation compared with the control juice group. Values are mean ± SE, n=20. *P < 0.05

PL juice suppressed the expression of inflammatory enzyme Cox-2, iNOS, NF-κB (p65) VCAM-1 in rats treated with AOM

In this study we investigated the anti-inflammatory and anticarcinogenic activities of PL juice in rats treated with AOM. PL juice polyphenols decreased

expression of COX-2, iNOS, NF-κB and VCAM-1, mRNA (Figure 52a and b). PL juice also suppressed protein expression of VCAM-1 (Figure 52d).

PL juice modulated AKT/PI3K/mTOR in AOM-induced rats

In this study, plum juice polyphenols decreased pPI3K and pAKT mRNA and PI3K protein expression (Figure 52c and d). This was accompanied by decreased expression of mTOR and its downstream HIF-1α, protein (Figure 52d). Multiplex bead assay also showed similar PI3K and mTOR protein result. As previous literature showed PI3K/AKT/ mTOR signaling pathway play a key role in carcinogenesis for several types of cancer such as colon cancer [211, 212]. PI3K/AKT regulates cell growth, proliferation and glucose metabolism and influences cell survival pathways through phosphorylation of several downstream targets such as mammalian target of rapamycin (mTOR) and which in turn controls HIF-1α, [213-215].

PL increases miRNA -143 in AOM-treated rats

Small non-coding microRNAs (miRNAs) are known to influence cancer progression by controlling several biological processes such as cell development, differentiation and maintenance. In our previous study we have shown the effect of polyphenols which target different miRNA that play important role in the progression of breast cancer [50]. In this study miRNA screening was performed. PL induced the expression of tumor suppressor miR143. miRNA 143 targets several mRNAs, that have a complementary sequence within their 3'UTR region such as of rapamycin (mTOR). PL extract induced the expression of miR-143 (Figure 52e) and correspondingly, this was accompanied by reduction of mTOR expression in AOM-treated rats (Figure 52c and d).

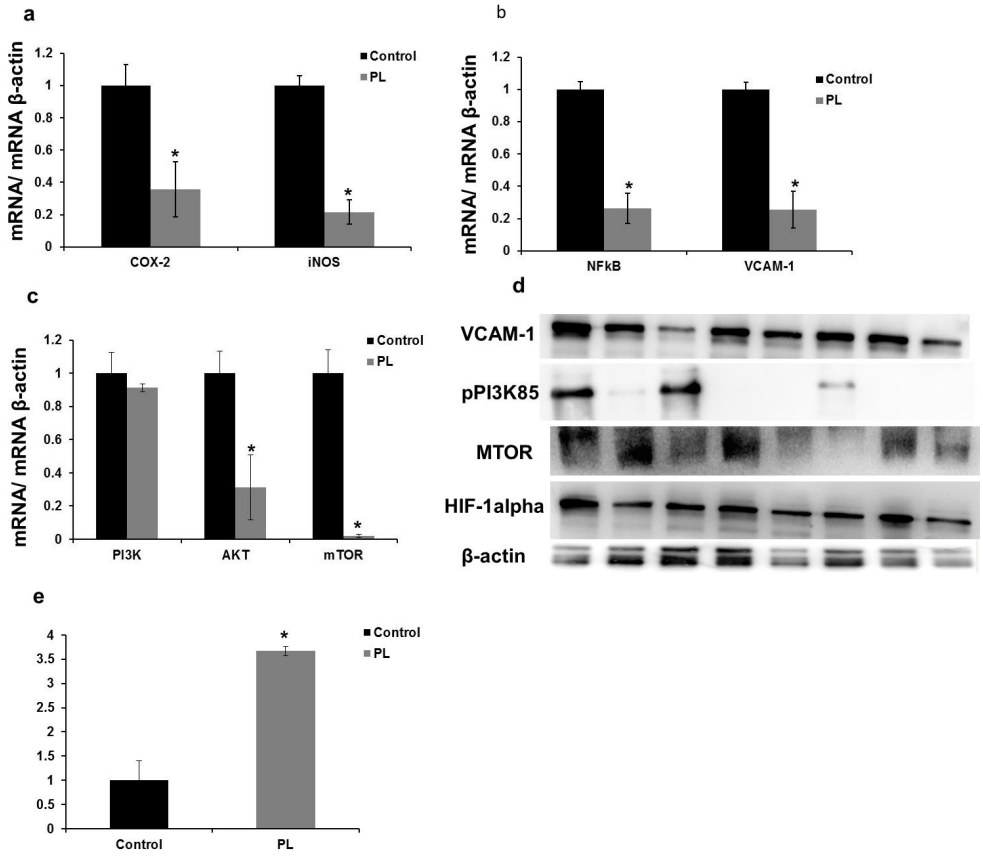

Figure 52 Effects of plum juice (PL) on the inflammatory and the mTOR signalling pathways in AOM treated rats (a) PL juice decreased the expression of COX-2 and iNOS mRNA and (b) VCAM-1 and NF-κB mRNA (c) PI3K, AKT and mTOR mRNA (d) PL juice suppressed the expression of and (D) VCAM-1, PI3K p85, mTOR protein in AOM treated rats (E) PL juice decreased in the expression of IGF and pPI3K accompanied by decreased expression of pAKT and pmTOR mRNA and (F) protein levels in AOM treated rats. (e) PL juice increased the expression of miR-143 in AOM treated rats. All experiments were performed as described in the material and method section, and result were expressed as mean ± SE.* indicates significant changes at $p<0.05$

SCFA GC/MS assays

Acetic, Propionic, Isobutyric, Butyric, Isovaleric and Valeric acids were measured in the feces of control and PL group. Acetic acid and Butyric acid were reduced in PL group but no significant differences were observed (Figure 53).

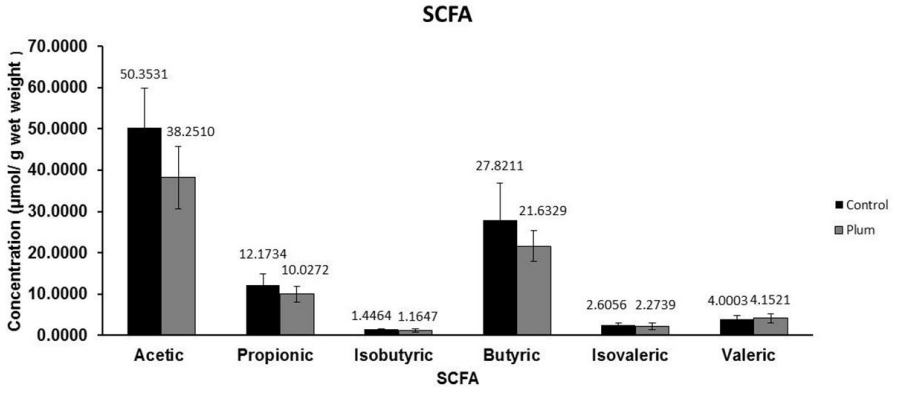

Figure 53 Effects of plum juice (PL) on the SCFA (Short Chain Fatty Acid) GC/MS assaysin AOM treated rats Acetic acid and Butyric acid were reduced in PL group but no significant differences were observed. No significant changes observed in propionic, isobutyric, butyric, isovaleric and valeric acids. All experiments were performed as described in the material and method section, and result were expressed as mean ± SE.* indicates significant changes at $p<0.05$

Discussion

The Incidence of colorectal cancer and its associated mortality rate are increasing worldwide. In addition to available therapies, there is a need to develop novel therapeutic agents in the prevention and treatment of colorectal cancer [91-93]. Several previous studies have shown the cancer-preventive and cytotoxic effects of polyphenols from green tea, blueberries, grapefruit, and pomegranate, as well as the flavonoids quercetin, stibene and resveratrol in suppressing the development of high multiplicity aberrant colorectal crypt foci (HMACF) [46, 131, 233, 239, 247, 248]. Plum juice with chlorogenic acid and neo-chlorogenic acid plays a key role in chemoprevention and as a therapeutic agents in several cancer cells lines such as breast cancer, U937 leukemia cells and also in AOM treated in Fisher 344 male rats [74, 75, 155, 241].

In this study we have investigated the cytotoxic and anti-tumorigenic effect of polyphenolics present in plum juice through the suppression of cell proliferation and inflammation. The number of HMACF was significantly decreased as well as the percentage of proliferative cells in azoxymethane (AOM)-treated rats. Plum juice rich in chlorogenic acid induced antioxidant, anti-inflammatory, and anti-tumorigenic effect in AOM treated rats. PL suppressed the expression of common colon cancer and inflammatory marker VCAM-1 and NF-κB in AOM treated rats [58]. NF-κB plays a central role in inflammatory pathways and the control of oncogenes and tumor suppressor genes, as well as adhesion molecules VCAM-1 [58]. Cell adhesion molecules such as VCAM-1 disrupt normal cell differentiation, which in turn leads to neoplastic transformation, progression, angiogenesis and metastasis of cancer cells [58]. Our preliminary data showed the effect of plum juice in the suppression of NF-κB and VCAM-1 expression in AOM treated rats.

In addition other chronic inflammatory markers such as Cox-2 and iNOS are up-regulated [212, 226, 233, 249] in colorectal cancer patients and rodents with chemically induced colon cancer. Excessive nitric oxide (NO) induced the expression of Cox-2, which in turn controlled cell proliferation, inflammation and might inhibited apoptosis in colon cancer cells [233]. Our data showed PL inhibited expression of Cox-2 and iNOS mRNA in AOM treated rat model. PL also inhibited cell proliferation in the AOM rat model showed by Ki67 staining and this was accompanied by the lower number of HMACF.

PL also targeted AKT/PI3K/mTOR pathway. Here, PL suppressed the activation of NF-kB by inhibiting the phosphorylation of PI3K/p85 and AKT and this was consistent with our previous finding where polyphenolic extract decreased the expression of and phosphorylation of NF-kB and PI3K/ AKT pathways [50]. The suppressed expression of PI3K/p85 was accompanied by decreased phosphorylation of AKT and mTOR and its downstream HIF-1 α and RPS6.

Additionally, our findings investigated the underlying mechanisms of down-regulation of mTOR, possible role of tumor suppressor miRNA 143. Decreased expression of mTOR was accompanied by increased expression of tumor suppressor miRNA 143 by plum juice. mTOR expression is controlled by miR-143 via a target-binding site in the 3' UTR region of the mTOR mRNA [203, 217, 224, 241].

Conclusion

In summary, plum juice polyphenols (PL) showed cytotoxic and anti-tumorigenic effects in AOM treated rats. Interactions of PL with miR143/PI3K/AKT/mTOR pathway were identified as the possible underlying mechanisms.

CHAPTER VI

SUMMARY AND CONCLUSIONS

In the presented work the anti-inflammatory and cytotoxic effects of polyphenolics relevant to carcinogenesis and tumor-growth were investigated. It was shown that ellagitannins and anthocyanin-rich pomegranate extract (Pg) decreased breast cancer cell growth and tumor volume through activation of cleaved caspase-3 and Poly (ADP-ribose)-polymerase1 (PARP) [186]. We have also investigated the potential role of mirna-27a and mirna-155 in cell survival and inflammation, while studying cytotoxicity and anti-inflammatory activities of Pg in breast cancer cells and in xenograft mice model.

It was shown that the miR-27a-ZBTB10-Sp axis was involved in Pg-induced downregulation of NF-kB and genes involved in survival and angiogenesis. Pg modulated miR-155-SHIP-1-PI3K axis in breast cancer model [185, 191]. Micro-RNA-mimic and -inhibitor studies demonstrated that both miR-27a and miR-155 are targets of Pg and their downregulation plays a significant role in the anti-inflammatory and cytotoxic efficacy of pomegranate extract. Overall we have demonstrated that pomegranate extract exhibited cytotoxic and anti-inflammatory activities in breast cancer cells in vitro and in vivo. In addition there is evidence that these activities are mediated in part through the effects of pomegranate extract on miR-27a-ZBTB10-Sp and miR-155-SHIP1- PIP3-AKT-NF-kB.

We further investigated the possible role of miR126-VCAM-1 and miR126-PI3K/AKT-mTOR in azoxymethane-induced colorectal aberrant crypt foci (ACF) and inflammation that was decreased by Pg. It was found that Pg decreased the expression of VCAM-1, this and was accompanied by up-regulation of miRNA-126 in vivo and vitro. We confirmed the involvement of miR-126 by showing decreased miR-126 and increased expression of VCAM-1 by a specific antagomir for miR-126 (ant. miR-126), where Pg partially but significantly reversed the effects of the antagomir. The study however, could not conclude whether the observed anti-inflammatory effects of Pg polyphenolics are derived from a direct interaction of polyphenolics with inflammatory pathways or whether at least part of the benefits is derived from the anti-inflammatory effects of reduced weight-gain observed in animals treated with Pg. Overall the results indicated that Pg polyphenols exerted cytotoxic and anti-inflammatory effects in AOM-treated rats and colon cancer cells. Interactions of Pg with miR126/VCAM-1 and miR126/PI3K/AKT/mTOR axes were identified as mechanisms that at least in part appear to be involved in the anti-inflammatory and anti-proliferative activities of pomegranate polyphenolics.

Mango polyphenolics including gallotannins and gallic acid were found to inhibit cell proliferation of the breast cancer cell line BT474 through inducing apoptosis as demonstrated by an increase in the cleaved product of Poly (ADP-ribose)-polymerase1 (PARP). The expression of survivin and tumor growth in athymic BALB/c nude mice with BT474 xenografts was decreased. In our preliminary data we demonstrated that Mg modulates the expression of different miRNA involved in

carcinogenesis. Mg modulates the AKT/ PI3K/p85 pathway and reduced the expression of mammalian target of rapamycin (mTOR) and its downstream transcription factor hypoxia-inducible factor (HIF-1α). The PI3K/AKT/ mTOR axis is well known for its involvement in the carcinogenesis progression in several types of cancer such as colon, lung and breast cancer [211, 212]. Mg also reduced expression of angiogenesis marker such as vascular endothelial growth factor (VEGF) and its receptor VEGF-R. In summary, Mg exhibited antioxidant, anti-inflammatory, anti-carcinogenic and anti-proliferative activities in breast cancer cells in vitro and in vivo.

The potential role of the miR143/mTOR pathway was investigated AOM-induced colorectal aberrant crypt foci (ACF) in rats that were treated with plum polyphenolics. Specifically, we have investigated the cytotoxic and anti-tumorigenic effect of polyphenolics present in plum juice in the suppression of cell proliferation and inflammation. Number of HMACF was significantly decreased as well as the percentage of proliferative cells in azoxymethane (AOM)-treated rats that were treated with plum. Additionally, plum suppressed the expression of the colon cancer and inflammatory markers VCAM-1, Cox-2 and iNOS in AOM treated rats.

In addition we observed that PL suppressed the activation of NF-κB, by inhibiting the phosphorylation of PI3K/p85 and AKT and this is consistent with our previous finding where polyphenolic extract decreased the expression of and phosphorylation within the NF-kB and PI3K/ AKT pathways [50]. The suppressed expression of PI3K/p85 was accompanied by decreased phosphorylation of AKT and mTOR and its downstream targets HIF-1 α and RPS6. In conclusion, results indicate that

Plum juice polyphenols (PL) exert cytotoxic and anti-tumorigenic effects in AOM treated rats. Interactions of PL with miR143/PI3K/AKT/mTOR pathway were identified as the possible underlying mechanisms

In summary, we have investigated polyphenol-induced anti-cancer effects and potential role of miRNA's in cell survival and inflammation. Pg exerted cytotoxic and anti-inflammatory activities in breast cancer cells and these are significantly mediated through the effects of pomegranate extract in the miR-27a-ZBTB10-Sp and miR-155-SHIP1- PIP3-AKT-NF-kB axes. Pg and plum juice also exhibited cytotoxic and anti-inflammatory effects in AOM-treated rats. Interactions of Pg with miR126/VCAM-1 and miR126/PI3K/AKT/mTOR axes and plum with miR143/PI3K/AKT/mTOR were identified as mechanisms that at least in part appeared to be involved in the anti-inflammatory and anti-proliferative activities of pomegranate polyphenolics. In addition Mg exhibited antioxidant, anti-inflammatory, anti-carcinogenic and anti-proliferative activities in breast cancer cells in vitro and in vivo by targeting miR126/PI3K/AKT/mTOR. Overall, this work will contribute significantly to the growing knowledge of how polyphenolics exert their anti-inflammatory, anti-carcinogenic and cytotoxic activities in breast and colon cancer.

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