POLYPHENOLICS FROM MANGO (*MANGIFERA INDICA* L.) AND

POMEGRANATE (*PUNICA GRANATUM* L.) SUPPRESS INFLAMMATION IN *IN*

VIVO AND *IN VITRO* MODELS FOR COLITIS

A Dissertation

by

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ABSTRACT

Ulcerative colitis is a chronic inflammation of the large intestine, and it may increase risk of human colorectal cancer. Polyphenolics from mango and pomegranate have been shown to have potent anti-inflammatory properties, thus they could be the potential therapeutic agents for colitis. However, the mechanism underlying these effects of polyphenolics has not yet been elucidated.

To determine the anti-inflammatory effects and possible mechanisms of polyphenolics from mango (gallic acid and gallotannins), and pomegranate (ellagic acid and ellagitannins) in dextran sodium sulfate (DSS)-induced colitis in rats, Sprague Dawley rats were administered control, mango, or pomegranate juice, and were exposed to three cycles of 3% DSS followed by 2-week recovery period. Colon inflammation and injury scores were assessed, and cell proliferation was evaluated by immunohistochemical detection of Ki-67. The mRNA and protein expressions involved in the inflammatory response and the mTOR pathway were analyzed by qRT-PCR, low density arrays, western blot analysis and multiplex bead assay. The involvement of miRNAs was additionally investigated with the antagomiR-126 and antagomiR-145 in lipopolysaccharide (LPS)-treated CCD-18Co, non-cancer colon fibroblasts cell lines.

Both mango and pomegranate showed anti-inflammatory properties *in vitro* and *in vivo*. Mango and pomegranate juice reduced DSS-induced colon inflammation score (41% and 50%) and cell proliferative index (38% and 36%) during chronic colitis in rats compared to control juice. Mango and pomegranate juice significantly attenuated the

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pro-inflammatory cytokines, CRP, TNF-α, IL-1β, GM-CSF, and IL-6 levels in colonic tissues. In addition, mango and pomegranate juice suppressed COX-2 and iNOS mRNA and protein expressions. Mango juice suppressed HIF-1α by decreasing the PI3K(p85β)/AKT-mTOR signaling axis via up-regulation of miR-126, while pomegranate decreased p70S6K and HIF-1α by up-regulating miR-145. The interactions of mango with miR-126/PI3K(p85β) and pomegranate with miR-145/p70S6K1 were additionally identified in CCD-18Co cells, where mango and pomegranate extract reversed the effect of the antagomiR.

In addition, the modulation of microbiota composition (*Blautia*, *Fusobacterium*, and *Ruminococcaceae*) by pomegranate and short-chain fatty acids (SCFA; Isovalerate and valerate) production by mango may be involved in at least in part the anti-inflammatory effects of polyphenolics.

These results suggest that both mango and pomegranate polyphenolics seem to have potential in the prevention and mitigation of colon inflammation.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Inflammatory bowel disease (IBD) is an inflammatory condition of the GI tract. There are two main subtypes of IBD: Crohn's disease (CD) and ulcerative colitis (UC). They have similar symptoms, but they may also have certain differences. The differences between CD and UC are the location, pathology, and radiology of the inflammatory response. UC is characterized by a chronic and relapsing inflammation of the mucosal layer of the large intestine, which includes the colon and rectum. In CD, inflammation extends deep into the bowel wall of any part of the gastrointestinal tract. In addition, UC is a symmetrical, continuous inflammation causing shallow ulcers, while CD is discontinuous and occurs in patches with deep ulcers with strictures and fistulae. UC has a higher incidence than CD (1, 2).

Ulcerative colitis

In UC, the inflammation causes loss of the lining of the colon, leading to rectal bleeding, diarrhea, and occasional abdominal pain, as well as fever and weight loss in severe cases, and it is associated with an increased risk for human colorectal cancer. The frequency of UC has increased since the mid-20th century. UC has an incidence of 1 to 20 cases per 100,000 people per year, and it has a prevalence of 8 to 246 per 100,000 people. The highest incidence and prevalence are shown in the populations of the United States, Canada, and the United Kingdom, while it is rare in Asia. 1.4 million people in

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the United States and 2.2 million people in Europe suffer from UC, and it usually first

occurs in people between 15 and 30 years of age, but there is a second peak between the

ages of 55 and 80. This disease appears equally in men and women (3).

Etiology

IBD is a multifactorial condition, and the development of this disease is affected

by four factors: genetic susceptibility, gut microbiota imbalance, immune dysregulation,

and environmental factors (e.g., smoking and diets high in fat and sugar) (Figure 1) (3-

5).
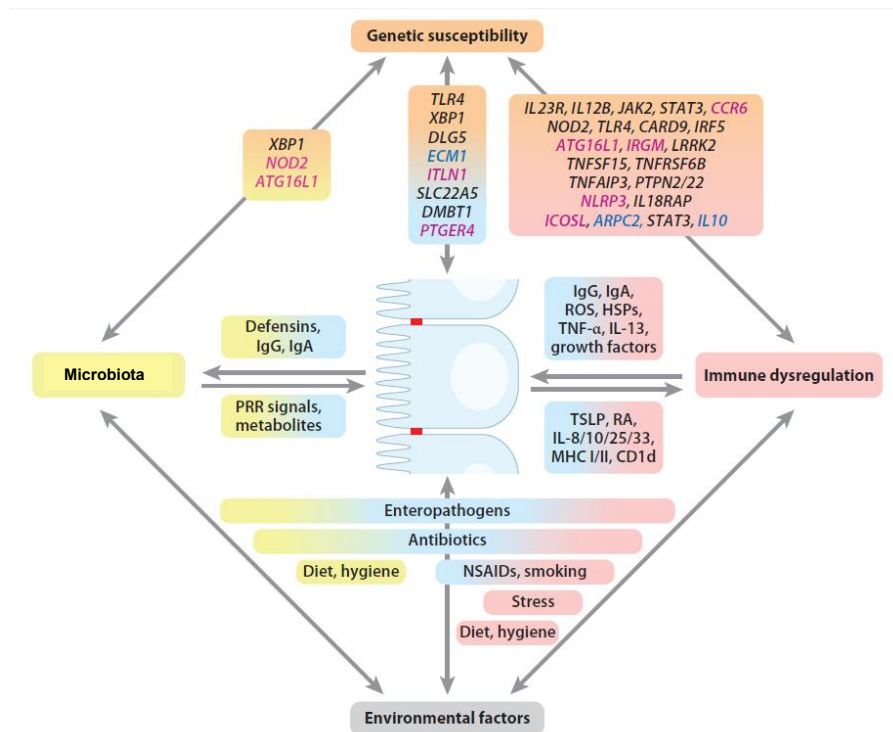
Figure 1. Etiology of inflammatory bowel disease (IBD) (Kaser A *et al*, 2010) (5).
Polymorphisms shared between CD and UC are shown in black, genes specific for CD
are shown in magenta, and genes specific for UC are shown in blue.

Genetic susceptibility

IBD is considered a polygenic disorder, and twin and family studies have indicated that the inheritable factor is stronger in CD than in UC (6). Genome-wide searches for IBD susceptibility loci identified genes (IBD1-9 locus) that contribute to IBD susceptibility. In initial screenings, the nucleotide binding oligomerization domain 2 (NOD2/CARD15; IBD1 locus) gene on chromosome 16 was identified as a CD susceptibility gene. It was reported that variants of the NOD1 and NOD2 elevated the NF-κB activation and the IL-1β and IL-18 secretion (7). In addition, autophagy genes (ATG16L1) and intelectins (ITLN1) were confirmed to be specific for CD, whereas immune regulators (IL-10 and ARPC2) and dysfunction of the intestinal epithelial cells (ECM1) were specific to UC (Figure 1) (8, 9). A sequence variant in IL-10 induced a risk of developing UC near 35% (10), and IL-10 knockout mice developed clinical and histologic features similar to human UC (11). In addition, genes involved in the IL-23 signaling (IL23R, IL12B, JAK2, STAT3) were related to both CD and UC (5).

Microbiota imbalance

An aberrant ratio of protective to aggressive microbiota in the large intestine results an inappropriate immune response, which can lead to IBD, irritable bowel syndrome, and colon cancer. The microbiota are integral in triggering and enhancing IBD. It was shown that germfree mice had no evidence of UC (12), and the use of antibiotics and prebiotics caused the remission of IBD (13). There are more than 10^{14} microbiota, from more than 1000 species, in the gastrointestinal tract, mostly in the

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colon. There will be 100-1000 fold more anaerobes than aerobes. Some of the microbiota are permanent residents in the gut, while others are altered by diet or environment. Microbiota are important for colon health, as they protect against pathogens and are important in the induction of immune regulatory functions and in processing nutrients into short chain fatty acids (SCFAs) (14).

Most microbiota are gram-negative *Bacteroidetes* (about 20%) and gram-positive *Firmicutes* (about 80%; mainly composed of *Clostridia*), while the remainder are *Actinobacteria* (3%), *Proteobacteria* (1%), *Verrucomicrobia* (0.1%), viruses, protists and fungi (Table 1) (15, 16). Among the levels of genus, *Bacteroides* is the most abundant genus in the gut microbiota, followed by *Faecalibacterium, Bifidobacterium*, and *Lactobacillus* (17).

Table 1. Microbiota of the GI-tract (Hakansson A *et al*, 2011) (16).

Phyla	Class	Family	Genus	Gram
Firmicutes	*Bacilli*	*Streptoccaceae*	*Streptococcus*	+
		Lactobacillaceae	*Lactobacillus*	+
		Turicibacteraceae	*Turicibacter*	+
	Clostridia	*Ruminococcaceae*	*Ruminococcus*	+
			Faecalibacterium	-
		Clostridiaceae	*Blautia*	+
Bacteroidetes	*Bacteroidia*	*Bacteroidaceae*	*Bacteroides*	-
Fusobacteria	*Fusobacteria*	*Fusobacteriaceae*	*Fusobacterium*	-
Proteobacteria	*Grammaproteobacteria*	*Enterobacteriaceae*	*Escherichia coli*	-
Actinobacteria	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*	+

Microbiota activate the immune system to produce reactive oxidative stress (ROS) or cytokines, and they are involved in the development of inflammatory disease and colon cancer, partly though the induction of the toll-like receptors (TLR)s/MyD88/NF-κB signaling pathway (18). Gram-negative bacteria (*Bacteroides, Fusobacterium*, and *Escherichia coli*) contain lipopolysaccharide (LPS) as the major component in the cell membrane. The TLR family is located on the cell surface and in endosomes, and the stimulation of TLRs by LPS triggers an inflammatory response through myeloid differentiation primary response 88 (MyD88) and NF-κB activation and overproduction of pro-inflammatory cytokines, such as TNF-α and IL-6 (19, 20). *Bifidobacterium* and *Lactobacillus* are considered to be probiotics that induce health benefits, and exert anti-inflammatory properties through inhibition of NF-κB activation and IL-8 secretion (21). Other gram-positive bacteria (*Ruminococcaceae, Blautia, Faecalibacterium,* and *Streptococcus*) were also shown to have anti-inflammatory effects, as the production of SCFAs blocked NF-κB activation and the induction of IL-10 (22).

SCFAs are a sub-group of fatty acids with 2 to 6 carbons, primarily acetate, propionate, butyrate, and valeric acid (Figure 2). The level of SCFAs in the gut depends on the amount of non-digestible oligosaccharides in the diet and the composition of the microbiota (23). SCFAs are metabolites produced from undigested carbohydrates, fibers, proteins and polyphenols by protective microbiota, and they seem to be beneficial in the suppression of inflammatory responses and UC (24).
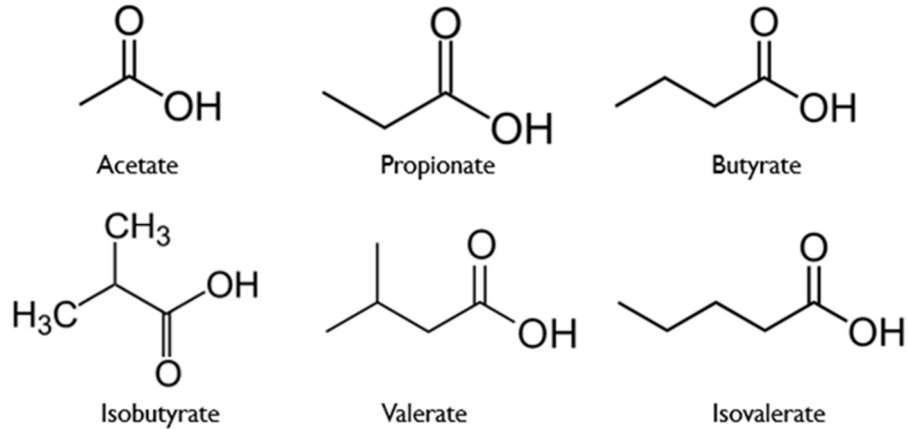
Figure 2. Short chain fatty acids produced in GI tract.

SCFAs are an energy source for colonic epithelial cells, and they play a role in the maintenance of colonic barrier integrity and function. Acetate inhibits enteropathogenic bacteria by reducing pH, while propionate reduces fatty acid and cholesterol biosynthesis in the liver (25). SCFAs, such as acetate, propionate, and especially butyrate bind the G-protein coupled receptor 43 (GPR43, FFAR2) or GRP41 (FFAR3). SCFA endogenous receptor interaction is necessary for the suppression of inflammatory responses, as it decreases the production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-12, IL-17, inducible protein (IP)-10), increases the production of anti-inflammatory cytokine IL-10, and suppresses the activation of various immune cells such as macrophages, eosinophil, T cells, B cells, and dendritic cells (24, 26).

Immune dysregulation

The gut consists of the epithelial barrier (enterocytes, goblet cells, enteroendocrine cells, paneth cells, and M cells), stromal/fibroblast cells, and the mucosal immune system (T and B lymphocytes, dendritic cells, macrophages, and neutrophils) which exchange signals via the production of mediators. The gut homeostasis maintains a controlled innate immune response to the microbiota, which is binding on the transmembrane TLRs and the nucleotide-binding oligomerization domain-like receptors (NLRs) on the epithelial and immune cells. Upon binding the pathogens, TLR signaling (mainly TLR4) induces MyD88, transforming growth factor – β activated kinase 1 (TAK1), and NF-κB expression for antimicrobial defense (27). However, when the immune system is damaged in the colonic mucosa, uncontrolled immune responses impair the gut's epithelial barrier. Glycolipids and bacteria induce the up-regulation of interleukin-13 receptor α2 (IL-13 α2) in mucosal natural killer T cells. The activation and feedback loop of IL-13 causes epithelial-barrier dysfunction, resulting in increased permeability. This leads to bacteria invading the mucosal barrier directly to generate antibacterial antibodies. Exposure of macrophages, neutrophils and dendritic cells to the microbiota triggers the activation of TLRs and NLRs, which induces the production of pro-inflammatory cytokines and chemokines (e.g., IL-1β, IL-6, IL-8, IL-17, IL-23, MIP-2α, MRP-14, MMP-1, and TNF-α) (Figure 3) (28, 29). Abnormal adaptive immunity occurs, such that IgM, IgA, and IgG levels are elevated, and the aberrant responses of naïve CD4+ T cells are divided into Type 1 helper T cells (Th1 cell-mediated response; CD) and Type 2 helper T cells (atypical Th2 response;

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UC). CD4+ T cells are essential regulators of the immune response. Induction of Th1 in CD increases the interferon-γ (IFNγ), TNFα, and lymphotoxin production that is critical for cell-mediated immunity, whereas the atypical Th2 response in UC produces abundant IL-4, IL-5, IL-10, and IL-13, which induces epithelial cell damage, apoptosis, and epithelial barrier dysfunction. The balance of Th1 and Th2 causes the differentiation between UC and CD (30).
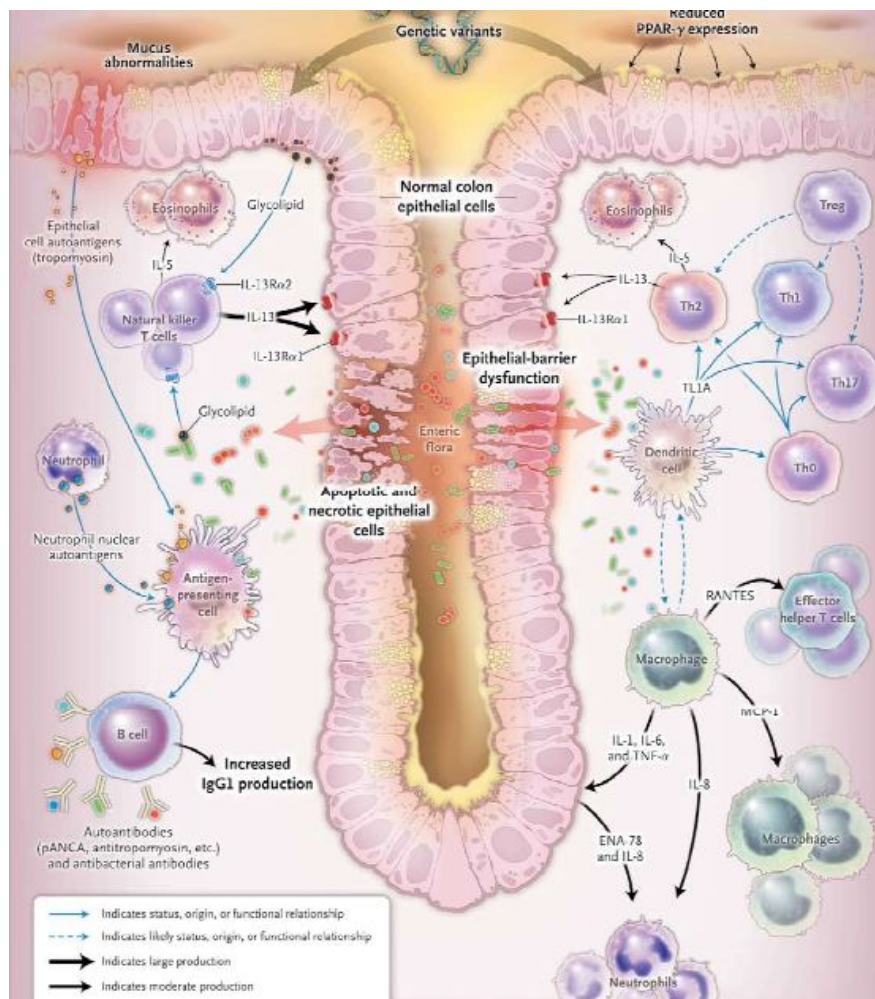
Figure 3. The immune dysregulation during ulcerative colitis (Danese S *et al*, 2011) (28).

Environmental factors

Environmental factors, such as an unhealthy diet, stress and smoking history, may trigger the development of UC by influencing the composition of the gut microbiota or disrupting the intestinal barrier. Among these factors, dietary factors may highly influence on colitis development and progression. Diets high in dietary fiber, vitamin D, and n-3 polyunsaturated fatty acids (PUFA) help decrease intestinal inflammation. It was reported that soluble fiber from fruits and vegetables was protective against colitis through fermentation into SCFAs by gut microbiota, whereas non-fermentable insoluble fiber from grain intake did not reduce colitis (31). Vitamin D is an immune system regulator, and its deficiency is related to UC (32). Vitamin D receptors have been identified on almost cells involved in immune system, and vitamin D affects inflammation through modulation of the Th1 and Th2 pathway (33). Arachidonic acid (AA) is the precursor in the eicosanoids production. Intake of n-3 PUFAs has been reported to decrease the production of inflammatory eicosanoids, cytokines, and reactive oxidative stress through replacing AA in inflamed cell membrane and inhibiting AA metabolisms (34). In addition, n-3 PUFAs alters the expression of inflammatory gene through the modulation of transcription factors during colitis (35). However, western-style diets characterized by a high fat, high protein, n-6 PUFAs, and low consumption of fruits and vegetables, are known to trigger colitis. High fat diet induced colonic inflammation through modulation of natural killer T cells and regulatory T cells in colitis (36). Cohort studies reported that high animal protein intake was associated with the induced risk of IBD (37). A high intake of n−6 PUFAs induced the eicosanoids

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production, which is an important mediator to produce pro-inflammatory cytokines (34). In addition, a high intake of alcohol increased the risk of developing UC (38). The relationship between UC and smoking is complex. Former smokers are associated with a high risk of UC; however, it has been shown that nicotine can be used as a therapeutic agent for UC treatment (39).

Increased risk of colorectal cancer

The most serious complication of UC is the high risk of developing human colorectal cancer (40). The risk of colorectal cancer increases with the duration of the disease, with a cancer risk of 2% after 10 years, 8% after 20 years, and 18% after 30 years, and it has high mortality (> 50%) (41, 42). The risk factors for developing colon cancer include the long duration of colitis (more than 8 years), a young age at onset, severe inflammation, and a family history of colorectal cancer (28).

The activated inflammatory cells (neutrophils and macrophages) in colitis generate large amounts of ROS and reactive nitrogen intermediates (RNI). The ROS induces DNA damage and mutation in adjacent epithelial cells and releases pro-inflammatory cytokines such as TNF-α, IL-6, and IFN-γ, and causes tissue injury and destruction in mucosal epithelial cells (43). Activation of the DNA damage results in epigenetic changes that induce oncogenes and suppress tumor suppressor genes, which leads to telomere shortening and the induction of senescence in cells. Senescent cells are also characterized by an increase of pro-inflammatory cytokines, which contribute to mutagenesis in the epithelial cells, and can lead to dysplasia and carcinogenesis (44).

Treatment

Conventional treatment of colitis

Since UC is a major risk factor for colorectal cancer, interventions for UC can potentially decrease the morbidity and mortality associated with human colorectal cancer. The medical or surgical treatment of UC depends on the extent of the disease. Mild to moderate UC responds to drugs, such as anti-inflammatory drugs (sulfasalazine, 5-aminosalicylic acid (5-ASA; Mesalamine, Olsalazine, and Balsalazide) or corticosteroids (Prednisone)), antibiotics (Metronidazole, Ciprofloxacin, and Rifaximin), and/or immunosuppressive drugs (Azathioprine, Methotrexate, Cyclosporine, or Infliximab) (45).

5-ASA and corticosteroids, as anti-inflammatory agents, are first line therapies to decrease inflammation in mild to moderate UC, and they have a remission rate of about 50%. 5-ASA is available in oral forms and in enemas, and it is prescribed in a combination of these two forms (up to 4.8g per day). Mesalamine shows the best clinical response in patients with mild UC. Corticosteroids are generally used in people who did not respond to 5-ASA, as it has a number of side effects, such as weight gain, mood swings, high blood pressure, and diabetes.

Antibiotics and immunosuppressive drugs (Cyclosporine, Infliximab) are used in patients who do not respond to 5-ASA or after remission is achieved in severe UC. These drugs not only treat inflammation but also target the immune system. However, these agents still have serious side effects and clinical limitations for in terms of use. Among these drugs, Infliximab is increasingly used due to its effectiveness and better

short-term safety compared with other therapies. Infliximab is a monoclonal antibody against TNF-α, and it works by removing TNF-α in patients with moderate to severe UC. If these medications do not work, surgery to remove part of the colon is needed (colectomy) (46).

Alternative nutritional treatments

The use of nutritional components has become an attractive approach for treating UC, particularly with those who fail to respond to medical therapies or have concerns about the side effects. Similar to medical therapies, natural compounds are used as anti-inflammatory agents and immunomodulators, and they include prebiotics, essential fatty acids, and polyphenolics (47). The benefits of prebiotics are that they are safe for the host, genetically stable, capable of surviving passage through the gastrointestinal tract, and they produce SCFAs from bacterial fermentation in the colon. Unlike CD, UC may respond to probiotics, and the most common probiotics used in UC are *Bifidobacteria*, *Lactobacillis,* the *E. coli* strain Nissle 1917, and VSL#3 which contains several species of *Bifidobacteria, Lactobacillus*, and *Streptococcus* (13). These species can prevent colonization of pathogenic bacteria by producing SCFAs and antimicrobial compounds (Bacteriocins), and by inducing immune regulatory responses (48). n-3 polyunsaturated fatty acids result in potent anti-inflammatory agents by inhibiting arachidonic acid metabolism and altering the expression of inflammatory genes (34).

Polyphenols are known for their antioxidant properties, which depend on chemical structures that allow them to trap, quench or scavenge ROS, therefore

preventing oxidative DNA damage (49). Some studies show that polyphenolics, such as quercetin, quercitrin, genistein, theaflavin-3,3'digallate, thearubigin, curcumin, 4-coumaric acid, ellagic acid, resveratrol, and piceatannol, lowered UC severity *in vivo* via suppression of the NF-κB signaling cascade and reduction of the pro-inflammatory mediators (50). For example, quercetin suppressed UC in DSS-treated rats through inhibition of NF-κB activation and reduction of iNOS (51, 52). Curcumin also lowered UC through suppression of NF-κB signaling and the down-regulation of iNOS and COX-2 expression in TNBS-treated rats. IL-10 expression was increased by curcumin (53, 54). The consumption of fruits and vegetables rich in polyphenolics should be a therapeutic strategy for preventing and managing UC.

mTOR pathway

The mammalian target of rapamycin (mTOR) pathway plays a central role in the regulation of cell proliferation, anti-apoptosis and inflammation that lead to the induction of carcinogenesis. The name is derived from the inhibitor rapamycin, which was isolated from a soil bacterium (55). In the immune system, it is known that the stimulation of T and B cell receptors, cytokine receptors, and TLRs leads to the activation of the mTOR pathway. During the early stages of UC, LPS from bacteria and IFNs from activated mucosal T cells may trigger the signal transduction of NF-κB and the mTOR pathway (56). Once phosphatidylinositol 3-kinase (PI3K) is activated by insulin, IGF-1, or TLR4, it starts a signal transduction cascade via the protein SER/Thr-kinase (AKT) and the mTOR. mTOR exists in two complexes within the cell: mTOR

complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Activation of mTORC1 regulates protein synthesis (HIF1α), cell growth, and cell proliferation via downstream transduction of proliferative signals, such as 4E-BP1 and p70S6K, which phosphorylates the ribosomal protein S6 (Figure 4) (57).
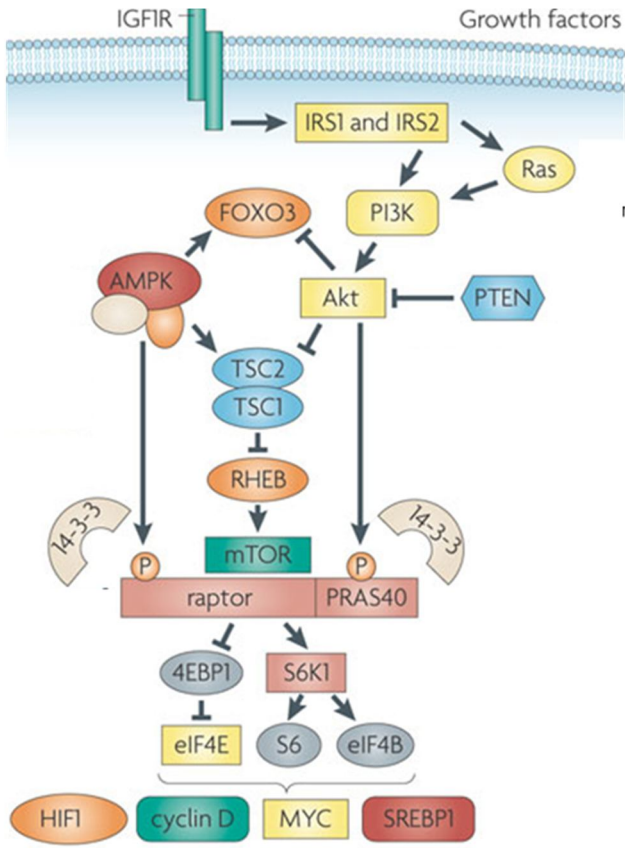
Figure 4. The mTOR signaling pathway (Zhang YJ *et al*, 2009) (57).

It is well documented that the mTOR pathway plays an important role during tumorigenesis (58). Currently, there is increasing evidence of the link between mTOR activity and inflammation. Cytokines, growth factors, insulin, and LPS stimulation cause the activation of p70S6K1 and 4EBP1, which are down-stream targets of the mTOR pathway (56). Moreover, LPS treatment induced the mTOR pathway and NO production, and it activated STAT1 dependent transcription in macrophages (59). Several studies showed that mTOR inhibitors suppressed experimental murine colitis by inhibiting T cell function and blocking IFN-γ release (60-62). Targeted suppression of these activated pathways during colitis using natural compounds provides an important strategy for colitis and colon cancer prevention.

miRNAs targeting the mTOR pathway

microRNAs (miRNAs) are emerging as potential post-transcriptional regulators for the regulation of inflammatory and immune responses (63). miRNAs are a class of small, non-coding RNA molecules in length of 5-20 nucleotides (nt) that bind to sequences located in the 3′-untranslated region of target mRNAs and regulate mRNA degradation or repress their translation (64). Inflammatory cytokines and microbiota components induce expression of miRNAs such as miR-146α and miR-155, via the NF-κB pathway in myeloid cells (65, 66). It has been shown that miR-146α modulates TLRs and TNF-α signaling pathways by reducing the expression of Interleukin-1 receptor-associated kinase 1 (IRAK1), TNF receptor-associated factor 6 (TRAF6), and COX-2

(65). miR-155 is shown to regulate adaptive immune responses, such as antigen presentation and T cell receptor signaling (67).

The mTOR pathway is a promising target of miRNAs for anti-colitis and anti-cancer therapeutics (68). miR-126 has a target site in the promoter region of the phosphatidylinositol 3-kinase regulatory subunit beta (PI3Kp85β), an upstream component of the mTOR pathway (64). Accordingly, miR-126 may be a potential chemopreventive and anti-inflammatory target via the inhibition of angiogenesis, vascular integrity, inflammation, and proliferation (69). miR-145 also has a chemopreventive effect through the target of the promoter regions of p70S6K1, a downstream target of the mTOR pathway, which regulate cell proliferation, inflammation, and angiogenesis (70). Previous studies indicated that the miR-126 and miR-145 could provide a rationale for the suppression of the mTOR pathway, and the mTOR pathway is a promising target for miRNAs in colitis therapy.

Recent studies have shown that dietary factors influence miRNA expression. Moreover, polyphenolics are known to modulate post-transcriptional regulation by regulating the expression of miRNAs (71).

Polyphenolics

Polyphenolics are secondary metabolites of plants and are characterized structurally by the presence of phenol rings. Polyphenolics are produced by the natural defense of plants against ultraviolet radiation and insets. The Shikimic acid pathway is the main metabolic pathway to synthesize the aromatic compounds, such as tryptophan,

phenylalanine, and tyrosine, which serve as the precursors to many phenolic compounds (72). More than 8000 phenolic compounds have been identified, and they can be classified into four different classes: phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (anthocyanins, flavanols, flavonols, flavones, flavanones, and isoflavones), stilbenes, and lignins (73). Polyphenolics are proposed to have anti-oxidant, anti-aging, anti-carcinogenic, and anti-inflammatory effects and to exert their beneficial effects by reducing the oxidative stress and inhibiting pro-inflammatory cytokines through the modulation of NF-κB, COX-2, and iNOS activity in an inflamed colon (74, 75). However, even though the anti-inflammatory activity of individual polyphenolics has been shown, the combination of a variety of polyphenolics in fruits and vegetables might have a synergistic effect with regard to UC prevention (76).

Mango (Mangifera indica L.)

Mango (*Mangifera indica* L.) is one of the most cultivated tropical fruits consumed worldwide. It has mainly been cultivated in South Asia, but currently mango is commercially grown in almost 87 countries ranking fifth in total production among major fruit crops. Its chemical composition varies according to the location of cultivation, variety, and stage of maturity. Mango is a good source of dietary fiber (pectin and cellulose), vitamin C, *ß*-carotene, calcium, iron, and phenolic compounds (77). In this study, mango was peeled to remove skin and seed, and only the pulp was

homogenized and centrifuged for removing fiber and producing the experimental mango juice. The mango juice contained sugar, vitamin C, and polyphenolics.

Among over 30 different varieties of mango, Alphonso is the most popular variety due to its strong aroma, thin skin, delicious taste, and high nutritive value (78). In the US, Ataulfo, Haden, Keitt, Kent, and Tommy Atkins are the major mango cultivars (79). In this study, the Keitt variety has been used.

Polyphenolics in mango

Mango is rich in polyphenols, such as gallic acid, gallotannins, flavonoids (quercetin and kaempferol), benzophenone derivatives, and mangiferin. These polyphenolics are distributed in the different parts of the mango (pulp, seed kernel, peel, leaves and bark), and the concentration of bioactive compounds varies according to variety or cultivar, environmental factors, harvest conditions, and processing (77). Gallic acid is a tribydroxibenzoic acid found in both free form and as part of gallotannins, and it is the most predominant polyphenolic in mango. Gallotannins are compounds formed when gallic acid binds with the hydroxyl group of a polyol carbohydrate such as glucose (Figure 5) (80).
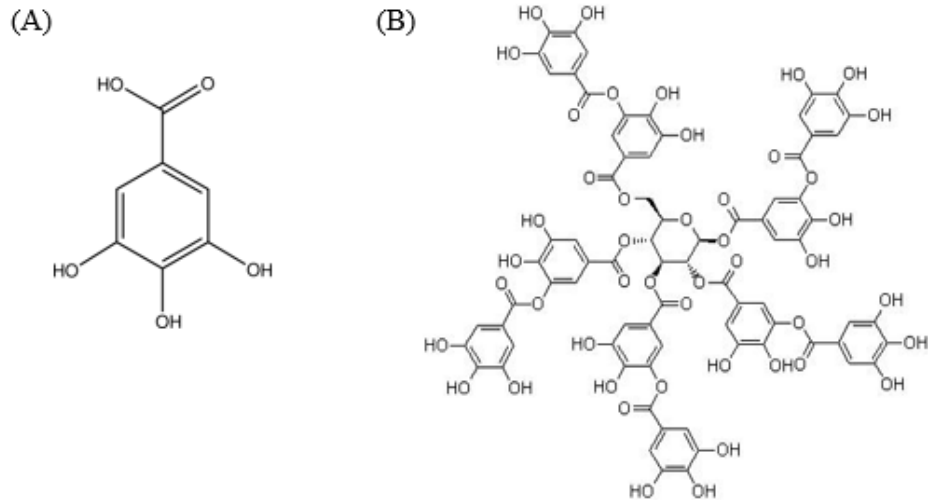
Figure 5. Chemical structure of (A) gallic acid and (B) gallotannin.

Mango polyphenolics in inflammation

Mango polyphenolics may protect against UC by exhibiting anti-inflammatory properties. Gallic acid is known to have antioxidant, anti-inflammatory, and anti-cancer effects (77, 81), and gallotannins are also considered to be the major antioxidant polyphenols found in mango (80). Vimang, a stem bark extract of the mango that is used in Cuba, showed anti-inflammatory activity through inhibition of PGE2 (82), TNFα and NO in *in vivo* and *in vitro* experiments (83). It was reported that mango extract has anti-inflammatory properties via the reduction of iNOS, COX-2, TNFα, and TNFR-2 in a DSS-induced rat colitis model (84).

The PI3K/AKT pathway is well-established upstream regulators of mTOR. Gallic acid has been shown to have anti-inflammatory and chemopreventive effects through the down-regulation of PI3K/AKT pathway and NF-κB activity in gastric cancer

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cells (85). Lupeol is a triterpene found in mango, and it showed chemopreventive effects through the down-regulation of PI3K/AKT pathway and NF-κB activity in skin carcinogenesis in mice (86). Thus, mango may modulate the mTOR pathway through suppression of the PI3K/AKT pathway.

For the reasons described above, mango is a potential source of polyphenolics with anti-inflammatory properties that could be used to reduce and/or prevent the incidence of UC.

Pomegranate (Punica granatum L.)

Pomegranate (*Punica granatum* L.) is oriented in the Mediterranean region and India, and it has been used for medicinal purposes. Currently, it is mainly cultivated in India, Spain, China, and some parts of the US. Pomegranate is consumed fresh or in processed form as juice, wines, and extracts. It is a good source for dietary fiber, vitamin C, vitamin K, and polyphenols (87, 88). In this study, the pomegranate juice mainly contained sugar and polyphenolics.

Pomegranate polyphenolics in inflammation

Pomegranate is rich in polyphenols, such as ellagic acid, ellagitannins (punicalagins and punicalins), flavonoids (quercetin, kaempferol, and luteolin glycosides), and anthocyanins (cyanidin, delphinidin, and pelargonidin glycosides) (89). About 124 phytochemicals have been identified in the pomegranate (90).

Ellagic acid is a polyphenolic found in fruits and nuts such as pomegranates, raspberries, strawberries, and walnuts, and it is found either in a free form or bound as ellagitannins. Punicalagin is a unique form in pomegranates, and it is a type of ellagitannin (Figure 6) (91). Flavonoids are classified into flavonols (quercetin and kaempferol), flavones (apigenin), flavanols (epicatechin and epigallocatechin-3-gallate), flavanones (naringenin), and anthocyanidines (cyanidine, delphinidin, and pelargonidin) (92).
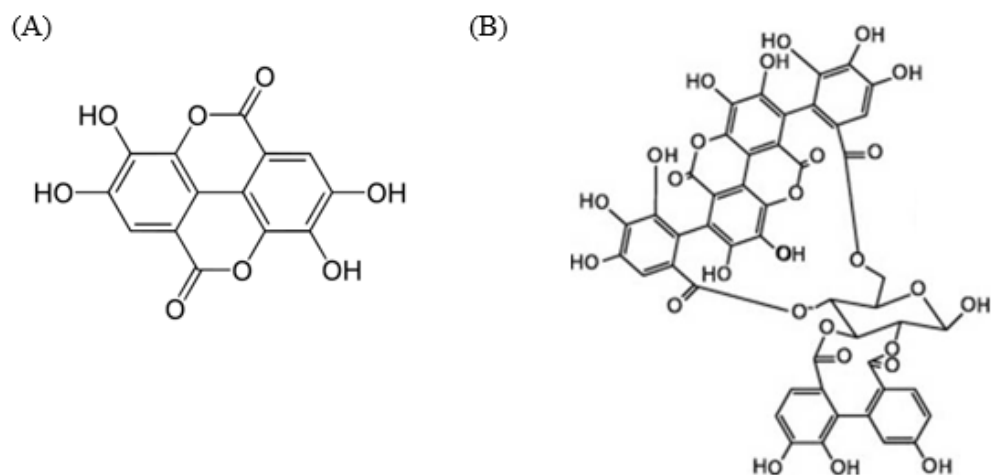
Figure 6. Chemical structure of (A) ellagic acid and (B) punicalagin (ellagitannins)

Pomegranate and inflammation

Pomegranate polyphenolics may also protect against UC by exhibiting anti-inflammatory properties. Ellagic acid inhibited NF-κB activity through induction of IkB phosphorylation and suppression of reduced IL-8 secretion in Caco-2 cell lines (93), and it reduced colitis severity via reduction of MPO and TBARS activity in DSS-treated rats

(94). Previously, the anti-inflammatory effects of pomegranate extracts have been reported in DSS or trinitrobenzene sulfonic acid (TNBS)-induced colitis rat models (Table 2). Dietary pomegranate showed anti-inflammatory activities through inhibition of MPO activity and TNFα levels. In addition, the diet decreased COX-2 and iNOS expression and reduced MAPK pathway (p38MAPK, JNK, and ERK1/2) and NF-κB activity in chronic TNBS-induced colitis (95, 96). In DSS-induced colitis models, pomegranate extract reduced colitis severity via modulation of the microbiota in the gut (*Bifidobacterium*, *Lactobacillus*, and *Clostridium*) and the decrease of COX-2, iNOS, PTGES, and PGE$_2$ (97).

The mTOR pathway could be the target of pomegranate polyphenolics. In human lung carcinoma cells and lung tumors in mice, pomegranate polyphenols down-regulated inflammation by suppressing the PI3K/AKT pathway and decreasing the activation of NF-κB (98, 99). In addition, pomegranate extracts have been shown to induce cytotoxicity and anti-inflammatory properties in colon cancer *in vitro* and *in vivo* by inhibiting the PI3K/AKT pathway and the activation of NF-κB (100-102). The inhibition of the PI3K/AKT pathway by pomegranate polyphenolics may modulate the expression of mTOR.

Although pomegranate polyphenolics suppressed inflammatory responses by suppressing the PI3K/AKT pathway, the underlying mechanisms and *in vivo* confirmation of their effects, have yet to be elucidated. This study, with respect to understanding the abilities and mechanisms of these compounds to suppress

inflammation, may provide valuable information about their potential application in colitis prevention and therapy.

Table 2. Effects of pomegranate on chemically-induced inflammation in intestinal *in vivo* models.

Type	Dose	Inflammatory effects	Ref.
Rats; TNBS	400mg/d	Lowered colitis, inhibit TNFα-induced priming of NADPH oxidase by targeting the p38MAPK and MPO release	(95)
Rats; TNBS	250-500mg/kg/d	Lowered colitis, reduced TNFα and MPO activity, decreased iNOS, COX2, MAPK pathway and NF-κB	(96)
Rats; DSS	200mg/kg/d	Lowered colitis, reduced MPO activity	(103)
Rats; DSS	250mg/kg/d	Lowered colitis, reduced iNOS, COX-2, PTGES and PGE$_2$	(97)

Absorption and metabolism of polyphenolics

Pharmacokinetics is the study of the absorption, distribution, metabolism, and excretion (ADME) of compounds. The bioavailability of polyphenolics can vary, and the absorption and metabolism depend on the parent structure (gallic acid or ellagic acid), degree of chemical modification, molecular size, degree of polymerization, and solubility. Small molecules, such as gallic acid, are easily absorbed through the gut, but larger polyphenolics are very poorly absorbed (104). Polyphenolics have been considered to mainly be absorbed in the small intestine due to the high surface area. However, it has been reported that a large number of polyphenols pass from the small

intestine to the large intestine, which indicates the importance of the colon in polyphenol metabolism (105). Polyphenolics that are not absorbed such as gallotannins and ellagitannins enter into the colon, where they could be hydrolyzed by gut microbiota to low molecular weight polyphenols via esterase, glucosidase, demethylation, dehydroxylation and decarboxylation activities (106).

The metabolism of the gut microbiota on polyphenolics may modify the immune response and the potential health effects. Polyphenolics are metabolized by gut microbiota in colon, and produce a number of metabolites. Polyphenolics and their metabolites can influence the composition and function of the gut microbiota (107). The effect of polyphenolics on bacterial growth and metabolism depends on strain, polyphenols structure, and dosage. Gram-negative microbiota are more resistant to polyphenolics than Gram-positive due to the differences of cell wall composition (108). The gut microbiota are considered to be a critical factor in UC. Understanding the interactions between the gut microbiota and polyphenolics would be important for preventing UC.

Pharmacokinetics of mango polyphenolics

The predominant polyphenols in mango are gallic acid and gallotannins. In the colon, the biodegradation of gallotannins by microbiota leads to the production of gallic acid, which is followed by the decarboxylated product, pyrogallol, and the dehydro product, resorcinol, as well as a host of other smaller molecular weight compounds (Figure 7) (109). Increased levels of *Clostridium* and *Bacteroides* are a cause of colitis,

and the growth of pathogenic *E. col*i and *Clostridium perfringens* was strongly inhibited by gallic acid (110). Gallotannins showed anti-microbiota activity against gram-positive bacteria except *E. coli, Pseudomonas,* and *lactic acid* (111).

Pharmacokinetics of pomegranate polyphenolics

Pomegranate is rich in ellagic acid and ellagitannins. The biodegradation of ellagitannins in the colon produces ellagic acid followed by gallic acid (Figure 7) (109). The main metabolite of pomegranate polyphenolics is urolithin-A. After pomegranate ellagitannins intake, the level of urolithin-A was increased, and it also facilitated the colonization of beneficial bacteria, such as *Lactobacilli, Bifidobacterium,* and *Clostridium,* which contributed to colonic health (97). The metabolites of polyphenolics may have anti-inflammatory effects on the intestinal environment through the modulation of the gut microbiota populations.
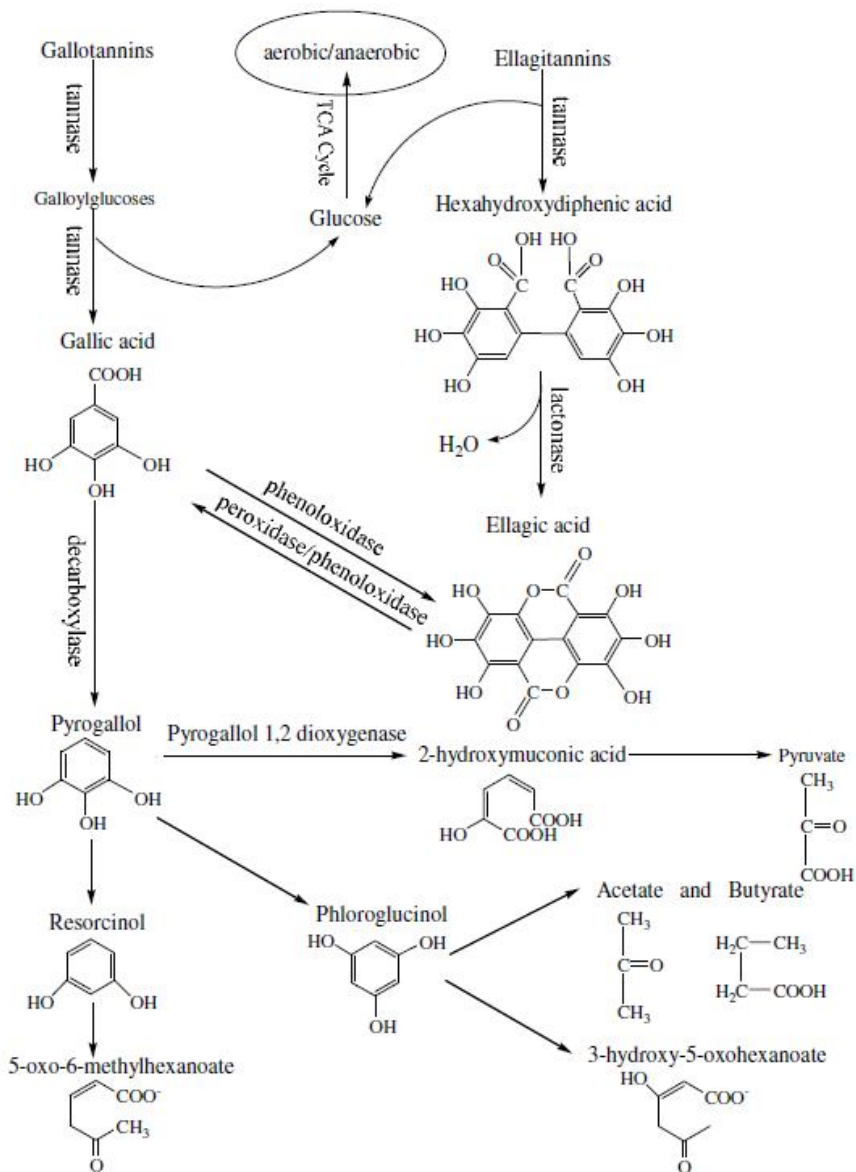
Figure 7. Biodegradation pathway for gallotannins and ellagitannins (Li M *et al*, 2006) (109).

Advances in disease-specific methodologies and models

Currently, at least 66 models of experimental IBD have been developed to

investigate the etiology of this disease. They have been classified into genetic,

immunological, bacterial, and chemical models (112). Genetic models, including gene

knockout (KO) and transgenic models, are useful for identifying components of the immune system that are involved in colitis development (113). Immunological models perform the adoptive transfer of host T cells into immunodeficient recipient mice (112). Bacterial models use various bacterial strains that are known to induce colitis in immunodeficient mice or mice housed under special environmental conditions (germ free, specific pathogen free, and gnotobiotic) (114). In chemical models, intestinal inflammation was increased by a single intrarectal administration of Oxazolone (4-ethoxoymethylene-2-phenyloxazol-5-one), TNBS, or dinitrobenzene sulfonic acid (DNBS). In addition, an oral administration of DSS, or an intraperitoneal injection of LPS also chemically increase inflammation (115, 116). TNBS-induced colitis resembles CD and DSS-induced colitis is similar to UC (117). Although these colitis murine models have a limited capability to fully recapitulate human colitis, there is still an opportunity to test various therapeutic strategies and gain insight into the etiology of the disease.

Genetic models

Most of these models in which targeted genes encoding specific immune factors (IL-2, IL-10, TCRα, TGFβ, WASP, SHIP1, TLR5, and MUS2), have been knocked out, and they are useful for identifying components of the immune response that are involved in IBD development (118). In addition, overexpressing IBD susceptibility genes (TNFSF15 and IL-7) have been confirmed to develop IBD (119). IL-10 is a known IBD susceptibility gene. IL-10 knockout mice have the colitis caused by enteric microbiota,

and many studies with probiotics were performed with this model (116). IL-2 is necessary for T cell homeostasis. IL-2 knockout mice develop an autoimmune disease characterized by colitis with a massive infiltration of T cells (120). While these genetic models recapitulate the histopathological and clinical features associated with human IBD, there are many key features in the development of inflammation. These genetic models can demonstrate the important interaction between environmental factors and genetic predisposition and the crosstalk between intestinal epithelium and mucosal immune response systems, which are necessary for maintaining normal homeostasis and which can contribute to the development of inflammatory bowel disease.

DSS-induced colitis model

The DSS-induced colitis model is the most effective in producing the clinical and histological features of UC characteristics in experimental animals. DSS is a sulfated polysaccharide of dextran that is synthesized by certain bacteria, such as *Leuconostoc spp* and *Strptococcus spp* from sucrose (121). The molecular weight of DSS ranges from 5kDa to 1,400kDa. The severity of colitis depends on the different molecular weights. DSS of 40kDa can cause the most severe colitis, while DSS of 5kDa or 500kDa developed relatively milder colitis (122). Depending on the concentration, the duration, and frequency of administration of DSS in the drinking water, acute or chronic colitis developed in colon. Clinical manifestation of DSS colitis is bleeding, diarrhea, ulcerations, intestinal inflammation, shortening of colon length, and weight loss (123).

Histological changes by DSS treatment mainly occur on the mucosa, and can be classified as acute and chronic. Acute colitis causes mucin depletion, epithelial degeneration, and necrosis of epitherial cells. Chronic colitis induces infiltration of lymphocytes and neutrophil, cryptitis, and crypt abscesses (124). Cryptitis is the migration of neutrophils to the epithelial lining of mucosa, while crypt abscesses is related to the migration of neutrophils into lumen of crypts. Cryptitis and crypt abscesses are the main feature of human IBD (121).

The epithelial barrier injury caused by DSS may lead the colonic mucosal permeability, and allows permeation of DSS through loss of tight junction components. It was shown that loss and redistribution of the tight junction such as occludin, ZO-1, and claudin proteins causes the impairment of epithelial barrier system in acute colitis by DSS (125). The response of inflammatory mediators by DSS have been implicated in the pathogenesis of human IBD. The expression of inflammatory mediators such as TNF-α, IFN-γ, IL-1β, and IL-6 are up-regulated, whereas synthesis of anti-inflammatory cytokines such as IL-10 are down-regulated in DSS-induced colitis. In addition, DSS − induced colitis is linked with the induction of nitric oxide and iNOS (126). In particular, epithelial homeostasis, regeneration and wound healing in DSS-treated rats is similar to human IBD (127). Although the DSS-induced colitis model does not represent the complexity of the human disease, it is still a valuable tool for investigating the involvement of dietary factors into in pathogenesis of IBD and evaluating different therapeutic options (128).

AOM/DSS murine model

Intestinal inflammation is a risk factor of human colorectal cancer, but the underlying mechanisms are still unclear. Colon cancer is a multistep process, and the combination of azoxymethane (AOM) and DSS induce a similar aberrant crypt foci-adenoma-carcinoma sequence to human colon cancer. AOM/DSS-induced tumors showed several molecular features, such as mutations in the β-catenin, K-Ras, p53, c-Myc and NF-κB, and epigenetic modification similar to human colon cancer. This model is simple and has high reproducibility and potency, and it is an outstanding model for chemopreventive intervention studies (129).

LPS-treated CCD-18Co myofibroblast cells

Intestinal myofibroblasts CCD-18Co are non-transformed colon cells, and they spontaneously become immortalized without transfection. Myofibroblasts are located subjacent to the epithelium, and define the structure of tissue microenvironments. In addition, they play crucial roles in intestinal inflammation and wound healing through the secretion of pro-inflammatory cytokines, prostaglandins, and growth factors (130). Intestinal inflammation damages the epithelial barrier systems, allows the penetration of pro-inflammatory agents into the lamina propria, and exacerbates the loss of the surface epithelial cells. It increases the accumulation of immune cells, such as CD4+ T cells and the activation of non-immune cells, such fibroblasts, which then become directly involved in immune responses, in the lamina propria (131). In chronic inflammation, myofibroblasts respond to the surrounding stimulus, exhibit a stable pro-inflammatory

phenotype, which may contribute to chronicity of the inflammation. Myofibroblasts are also involved in the repair of epithelial cell monolayers during intestinal inflammation (132).

Myofibroblast cell lines express TLR-4, which is activated by LPS, a component of gram-negative bacteria, which subsequently leads to the secretion of pro-inflammatory mediators (133) and the induction of COX-2, iNOS and prostaglandin synthesis (134). It can also secrete large amounts of IL-6, IL-8, M-CSF, and GM-CSF upon stimulation. The cytokine profile and the amount of cytokines released suggest that colonic myofibroblasts may play a role during mucosal inflammation. Therefore, this LPS-treated CCD-18Co myofibroblast cells model can be used to investigate mechanisms of inflammation (135).

Summary and project overview

UC is the most common form of IBD, and it is characterized by a chronic inflammation of the mucosal layer of the large intestine (1, 2). Although colitis is rarely lethal on its own, people with colitis are at high risk for developing colon cancer (40). Hence, there is a need to develop natural therapeutic agents for the prevention and treatment of colitis.

The anti-inflammatory effects of polyphenolics from mango (*Mangifera indica* L.) and pomegranate (*Punica granatum* L.) have been studied. However, their underlying molecular mechanisms are still unclear. The major objective of this research is to determine the anti-inflammatory effect of mango and pomegranate polyphenolics

on ulcerative colitis. In addition, we investigate the specific mechanisms of these natural compounds in the inflammatory responses for prevention of intestinal inflammation *in vivo* and *in vitro* models.

The mTOR pathway plays a central role in the regulation of the cell growth, cell proliferation, and inflammation that leads to the induction of colitis (136). The suppression of the mTOR pathway may play a key role in the anti-inflammatory strategy for treating colitis. miRNAs are important post-transcriptional regulators because they induce mRNA degradation or block translation, and it may be involved by modulating the target mRNA expressions (64). The mTOR pathway is a promising target for miRNAs in colitis treatment (68). In addition, microbiota and their metabolite SCFAs are beneficial in the suppression of the inflammatory response in the colon. They can activate the immune system to suppress ROS or pro-inflammatory cytokines, and the TLR4/mTOR signaling pathway can be a target of microbiota and SCFAs to reduce the inflammatory response (18).

Polyphenolics derived from dietary sources are considered sources of natural chemopreventive and chemotherapeutic compounds through their antioxidant defense systems and anti-inflammatory effect from the suppression of NF-κB, COX-2, and iNOS activation (74, 75). Previously, we demonstrated that mango polyphenolics inhibited the growth of several cancer cells, particular colon cancer cells, by inducing apoptosis and reducing ROS generation (81). In addition, gallic acid, the most abundant polyphenol in mango, has been shown to have an anti-inflammatory effect by the down-regulation of the PI3K/AKT pathway and NF-κB activity in colon and gastric cancer cells (85, 93). A

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previous study demonstrated that pomegranate inhibited cancer growth by inhibiting the PI3K/AKT pathway and NF-κB activity in colon cancer cell lines (102). Although these compounds exhibited anti-inflammatory effects by suppressing the PI3K/AKT pathway which is the upstream regulators of the mTOR pathway, the underlying mechanisms and *in vivo* confirmation of their effects, have yet to be elucidated.

Gene expression analysis can be used to compare the gene expression profiles altered by the intake of different polyphenolics in *in vivo* colitis models. This analysis allows us to figure out the potential therapeutic target of the natural compounds for the prevention of inflammatory disease and to elucidate the underlying mechanisms by which the polyphenolics ameliorate the inflammatory response.

The development of natural anti-inflammatory products, either as part of a diet or as supplements, may be important due to their efficacy, safety, wide availability, and good stability. Targeted suppression of activated mTOR pathways during inflammation responses by these natural compounds may provide an important strategy for the prevention of ulcerative colitis.

Hypothesis

The overall hypothesis is that polyphenolics from mango and pomegranate may show anti-inflammatory effects in colitis *in vivo* and *in vitro*. Furthermore, polyphenolics may suppress cell proliferation and colon inflammation at least in part by modulating the AKT/mTOR/HIF1-α axis and associated factors.

Objectives

1) Investigate the mechanisms underlying suppression of the mTOR pathway by polyphenolics from mango on colitis - Potential Involvement of the miR-126/PI3K/AKT/mTOR pathway

2) Investigate the mechanisms underlying suppression of the mTOR pathway by polyphenolics from pomegranate on colitis - Potential Involvement of the miR-145/p70S6K/HIF1-α pathway

3) Compare the gene expression profiles altered by mango and pomegranate polyphenolics on colitis

4) Determine the role of intestinal microbiota and metabolites changed by mango and pomegranate polyphenolics

CHAPTER II

MANGO POLYPHENOLICS REDUCE INFLAMMATION IN INTESTINAL

COLITIS - POTENTIAL INVOLVEMENT OF THE MIR-126/PI3K/AKT/MTOR

PATHWAY IN VITRO AND IN VIVO

Summary

Ulcerative colitis is a chronic inflammation of the large intestine, and it may increase risk of human colorectal cancer. Mango polyphenolics such as gallic acid and gallotannins have shown antioxidant and anti-inflammatory properties in several studies. However, the mechanism underlying these effects is not yet clear.

The objective of this study was to investigate the potential role of the miR-126/PI3K/AKT/mTOR signaling pathway in the anti-inflammatory effects of mango juice in dextran sodium sulfate (DSS)-induced colitis in Sprague Dawley rats. Lipopolysaccharide (LPS)-treated human CCD-18Co colon-myofibroblastic cell lines were also used.

Animals were administered control juice (15.7g sugar and 0.05g citric acid/100ml) or mango juice (total phenolic content of 475.80mg/L gallic acid equivalents (GAE)), and exposed to three cycles of 3% DSS followed by 2-week recovery period. The mRNA and protein levels of pro-inflammatory cytokines and the PI3K/AKT/mTOR signaling pathway were measured by RT-PCR, western blot analysis and multiplex bead assay *in vivo* and *in vitro*.

Results showed that mango juice protected against DSS-induced colon inflammation and suppressed cell proliferation as measured by Ki-67 staining in rats during chronic colitis compared to control juice. The mango juice significantly attenuated the expressions of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α, IL-1β, COX-2 and iNOS at mRNA and protein levels. Moreover, the expressions of PI3K, AKT, and mTOR were reduced while miR-126 targeting PI3K (p85β) was up-regulated by the mango group compared to the control. Similar results were observed in LPS and mango extract treated CCD-18Co cell lines. The relationship between miR-126 and its target gene PI3K (p85β) was confirmed with the further *in vitro* treatment with miR-126 antagomiR.

These results suggest that mango polyphenols attenuated inflammatory response by modulating the PI3K/AKT/mTOR pathway in part though up-regulation of miRNA-126 expression both *in vitro* and *in vivo*, and mango may be a potential therapeutic for colitis.

Introduction

Ulcerative colitis (UC) is a kind of inflammatory bowel disease (IBD) and has a close association with human colorectal cancer (43). Long-term chronic inflammation produces high levels of pro-inflammatory cytokines and inflammatory molecules, which increases the risk of cancer (40). It is suggested that more than 20% of IBD patients develop colon cancer within 30 years of disease onset, and have a 50% increased risk for mortality (41). Accordingly, anti-inflammatory drugs play an important role in the

prevention and treatment of colitis and colorectal cancer (137). Several studies have

focused on developing natural anti-inflammatory products as part of the diet or from

supplements due to its efficacy, safety, wide availability and good stability (138).

Polyphenolics derived from fruits and vegetables are highly considered to be

sources of natural anti-inflammatory compounds through inhibition of NF-κB activation

and induction of antioxidant defense systems (139). Mango is rich in polyphenols,

including gallic acid, galloyl derivatives, flavonol glycosides and benzophenone

derivatives, that exhibit antioxidant and anti-inflammatory properties in master cells and

lung cancer cells (140-142). Gallic acid, the most abundant polyphenols in mango, has

been shown the chemopreventive effects by reducing colon carcinogenesis through

inhibition of oxidative stress and NF-κB activation (143, 144). In addition, mango

extracts were reported to have anti-oxidant (145) and anti-inflammatory activities (82,

84), and we previously demonstrated that mango extract inhibited the growth of several

cancer cells, particular colon cancer cells through induction of apoptosis and reduction

of reactive oxygen species (ROS) generation (81). However, the underlying mechanisms

relevant to the preventive effects of mango polyphenolics on colitis have not been well

investigated.

The mTOR pathway plays a central role in the regulation of cell growth and cell

proliferation through downstream transduction of proliferative signals, such as 4E-BP1

and p70S6K1 (57). Activation of mTOR signaling occurred in bacterial induced colitis

in mice, and made it crucial (146). mTOR inhibitors have shown to be effective as anti-

inflammatory drugs in colitis by inhibiting NF-κB activation and T cell function (60, 62,

147). Targeted suppression of these activated pathways during colitis by natural compounds may provide an important strategy for preventing colitis.

Polyphenolics are known to modulate the post-transcriptional regulation of miRNAs (71). The mTOR pathway is a promising target by miRNAs for anti-cancer therapeutics (68). miR-126 has target site in the promoter regions of phosphatidylinositol 3-kinase regulatory subunit beta (PI3Kp85β), an upstream component of the mTOR pathway (64, 69). Accordingly, miR-126 may be a potential chemopreventive and anti-inflammatory target via the inhibition of angiogenesis, vascular integrity, inflammation, and proliferation (69). The induction of miR-126 could be a rationale for prevention and treatment of colitis.

The objective of this study was to understand the molecular targets involved in anti-inflammatory effects of mango juice on dextran sodium sulfate (DSS)-induced colitis in Sprague Dawley rats and in lipopolysaccharide (LPS)-treated human CCD-18Co colon-myofibroblastic cell lines. We hypothesized that mango juice has anti-inflammatory properties in part due to the modulation of the miR-126/PI3K/AKT/mTOR pathway, which plays an important role in the inflammatory response in ulcerative colitis. In this study we are reporting for the first time that mango juice may modulate the post-transcriptional regulation of inflammation by regulate the expression of miR-126.

Materials and methods

Chemicals, antibodies, and reagents

DSS was purchased from MP biomedicals (Solon, OH). Antibody against Ki-67 was purchased from BD Pharmingen (San Jose, CA). Antibodies against NF-κB, pNF-κB, COX-2 were purchased from Cell signaling technology (Beverly, MA). The antibody for iNOS was purchased from Cayman chemical (Ann Arbor, MI). The antibodies for PI3K (p85β) and HIF-1α were purchased from Abcam (Cambridge, MA). Lipofectamine 2000 was purchased from Invitrogen (Grand Island, NY). mirVana™ miRNA Isolation Kit, reverse transcription and real-time PCR amplification kits were purchased from Applied Biosciences (Foster City, CA). All primers were purchased from Integrated DNA Technologies (San Diego, CA). AntagomiR of miR-126 was purchased from Dharmacon (Lafayette, CO) (89).

Mango chemistry

Mango (Keitt, Mexico) was received as a kind gift from the National Mango Board (Orlando, FL). For obtaining the experimental mango juice, full ripe mango was eled to remove skin and seed, and only the pulp was homogenized in a blender, and heated with cellulase and pectinase to break down the fiber. After heating at 55°C for 1h, the puree was centrifuged, and the supernatant was stored at -20°C. The mango extract for the *in vitro* study was prepared as described in our previous literature (81). Mango is a good source of dietary fiber (pectin and cellulose), vitamin C, ß-carotene, calcium, iron, and phenolic compounds (77). In this study, mango juice contained most of the

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nutrients from fruit such as sugar, vitamin C, and polyphenolics except fiber, while mango extract had only total polyphenolics from mango. Total phenolic contents in the mango juice and extract were measured spectrophotometrically by the Folin-Ciocalteu assay against an external standard of gallic acid. Individual polyphenolics in the juice were analyzed on a Waters Alliance 2690 HPLC-MS system (Milford, MA). In addition, identification by mass spectrometric analyses was performed on a Thermo Finnigan LCQ Deca XP Max mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA) (81).

Animal treatment and tissue sampling

The animal use protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M University. The DSS model is an excellent preclinical model of colitis that exhibits many phenotypic characteristics relevant to the human disease (148). The DSS treatment causes injury to the colonic epithelial barriers resulting in an increase in colonic mucosal permeability, causes destruction of the crypts, and produces pro-inflammatory cytokines (121). Ten week-old Sprague-Dawley rats supplied from Harlan Teklad (Houston, TX) were acclimated for a week, and were randomly distributed by weight into control and mango juice groups. For calorie and pH adjustment, 15.7g sugar and 0.05g citric acid was added in 100ml of control juice. The intake of liquid was determined every day. After 3 weeks of rat chow pellets and experimental juices *ad libitum*, all rats were administered 3% (w/v) DSS (3 cycles, 14 d separation, n=10/diet) in the experimental juices for 48 h. Rats were sacrificed 2 weeks

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after three times of DSS administration, and colon tissues were collected (Figure 8). One centimeter sections were cut from the distal end of each rat colon, and fixed in 4% paraformaldehyde (PFA) for embedding in paraffin. The remained colon was gently scraped for collecting protein and RNA samples (149).
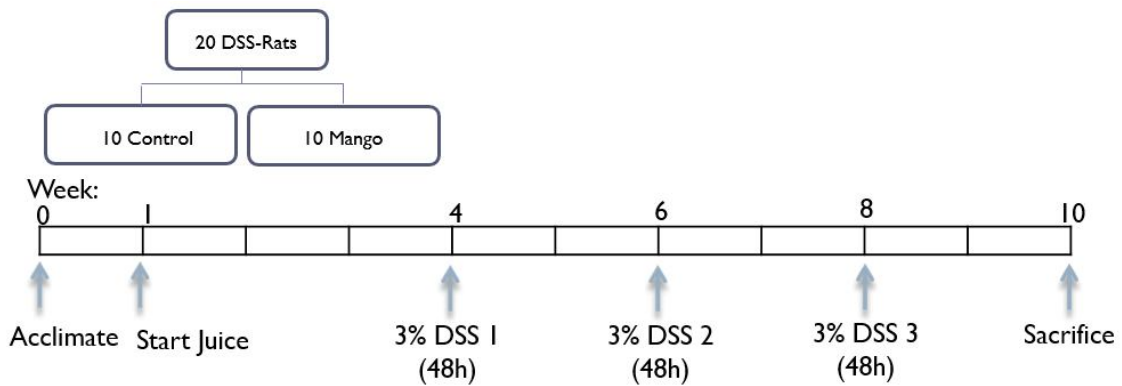
Figure 8. Experimental design.

Quantifying inflammation and cell proliferation assay

For histopathology and immunohistochemistry analysis, colon tissues were dehydrated, embedded in paraffin and serially sectioned into 4 μm thick. Hematoxylin and eosin (H&E) stained slides were then examined by a board-certified pathologist (C.P.) in blinded manner for accessing the histological colitis level. Scores were assessed on a scale of 0-3, where 0 is none observed and 3 is severe inflammation (150). For Ki-67 immunohistochemical staining, the primary antibody against Ki-67 (Dilution 1:50) 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

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columns. Twenty-five crypt columns per rat were selected for quantitative analysis. The number and position of labeled cells were recorded. Proliferative index was calculated as 100 times the number of labeled cells divided by the total number of cells per crypt column.

Cell culture

Human colon CCD-18Co myofibroblastic cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA), and cultured as recommended by the ATCC. Cells were seeded for cell proliferation assay onto a 24-well plate (15,000 cells/well), for RNA extractions onto a 12-well plate (150, 000 cells/well), and for the protein analysis onto a 6-well plates (300,000 cells/well). Cells were pre-incubated for 24h before exposing the treatment of the mango extract (0-10mg GAE/L). The level of cell viability was quantified with a cell counter after 48h (Z2™ Beckman coulter, Fullerton, CA). RNA was extracted for gene expression analysis after 3h, and protein was extracted after 24h.

Quantitative RT-PCR

For mRNA and miRNA analysis, total and micro RNA from the colon mucosal scraping was isolated using the mirVana™ miRNA Isolation Kit according to the manufacturer's protocol. Equal amount (1ug) of mRNAs was converted to cDNA using a reverse transcription kit (Invitrogen, Grand Island, NY). Real-time PCR reactions were performed using 2uL of cDNA using a Reverse Transcription Kit (Invitrogen, Grand

Island, NY). SYBR Green PCR master Mix (Applied biosystems, Foster City, CA) was used for the qPCR analyses on Applied Biosystems 7900HT Fast Real-Time PCR system (Applied biosystems, 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. Quantification of miR-126 was measured using the Taqman MicroRNA reverse Transcription kit (Applied Biosystems, Foster City, CA) and qRT-PCR reaction using Taqman 2X Universal PCR master mix (No AmpErase UNG) (Applied Biosystems, Foster City, CA). The miR-4.5S and miR-NU6B were used as endogenous control for micro RNA expression (89).

Multiplex bead assay

The protein extract (20ug) from each tissue was used to determine the relative abundance of total protein and the phosphorylation status in the AKT/mTOR signaling (AKT, mTOR, p70 S6 kinase, and RPS6) using the multiplex kits (Millipore, Billerica, MA) according to the manufacturer's protocol. Multiplex analysis was performed by the Luminex L200 instrument (Luminex, Austin, TX), and data was analyzed by Luminex xPONENT software.

Western blotting

The mucosal scrapings were homogenized in 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. After 24h mango extract treatment, cells were lysed in RIPA buffer added 1% protease and proteinase inhibitor cocktail (Pierce/Thermo Scientific, Rockford, IL), and centrifuged (151). 60ug of protein was loaded onto acrylamide gel, followed by transfer onto PVDF membranes. The membranes were incubated with primary antibodies against NF-κB, p-NF-κB, COX-2, iNOS, PI3K (p85β), HIF-1α and β-actin.

Transfection with antagomiR of miR-126

Cells seeded into 12-well plates were transfected with 20nM antagomiR of miR-126 with using Lipofectamine 2000 according the manufacturer's protocol. For the control, a nonspecific oligonucleotide was used. After transfection for 4h, the transfection mix was replaced with medium with 10mg GAE/L of mango extract, and after 1h, cells were treated with medium with mango extract and 1μg/ml LPS for 24h as previously described (152).

Statistical analysis

Quantitative data represent mean values with standard error. Data were analyzed by student t-test and one way ANOVA using Tukey's post hoc test ($p < 0.05$) using SAS version 9 (SAS Institute Inc., Cary, NC).

Results

Polyphenolics composition of mango juice and the juice consumption of DSS-treated

rats

The concentration of total soluble phenolics of mango juice used for this study

was 475.80mg gallic acid equivalents (GAE)/L. HPLC-MS analysis of mango juice

showed Monogalloyl Glucoside (Peak 1, 12.19mg GAE/L), Gallic acid (Peak 2, 6.41mg

GAE/L), OH-Benzoic Acid hexoside (Peak 3, 2.97mg GAE/L), and Dihydrophaseic acid

(Peak 4, 12.43mg GAE/L) with peak absorption at 280nm (Figure 9A). However, the

overall the mango juice consumption and final body weight in the mango group were

significantly lower compared to those in the control group (Table 3). The reason might

be that it took time for rats to become used to the taste of tannins in the early stage of

this study (Figure 9B). The total caloric intakes were not significantly different, because

the food intake in the mango group was higher than the intake in the control group

(Table 3). In addition, during the DSS administration, the intake of mango juice

containing 3% DSS was significantly higher than the control juice, potentially due to the

improvement of the bitter taste of DSS (Table 3). When a rat (445.39g body weight)

drank 84.174ml of mango juice (475.80mg GAE/L), the dose was equivalent to 89.74

mg GAE/kg/day in rats. The human equivalent amount of mango juice consumed by rats

was calculated with the body surface area normalization method (human equivalent dose

(mg/Kg)=rat dose (mg/Kg)×rat K_m/human K_m (6/37)) (153, 154). The 70 kg human

equivalent dose of mango juice was around 1g GAE/day of mango polyphenolics or

2.1L/day of mango juice. There were no symptoms (for example, severe diarrhea or

bloody stool) of severe inflammation after DSS treatment.
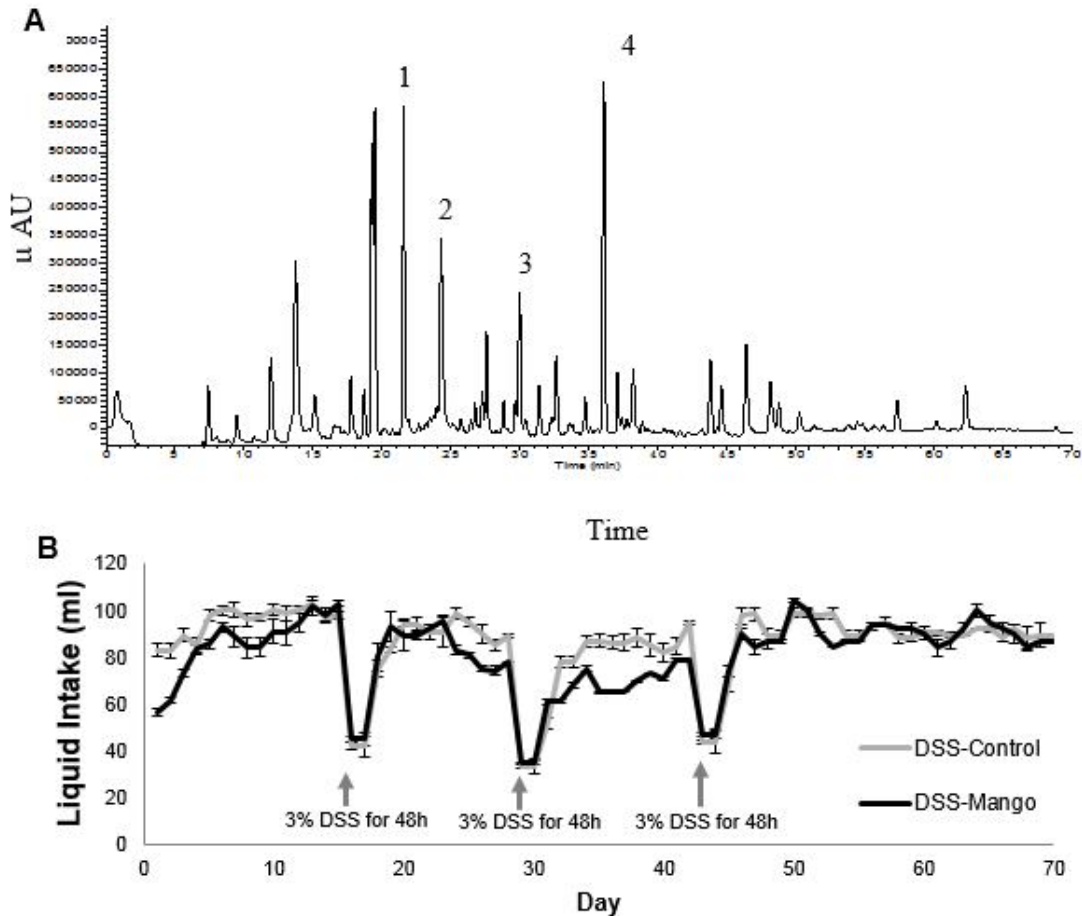
Figure 9. Representative chromatographic profile at 280nm of phenolic compounds in mango juice (Keitt) and the daily liquid consumption by rats.
(A) Total phenolic content of the mango juice was 475.80mg GAE/L. Tentative peak assignments: 1 Monogalloyl Glucoside (12.19mg GAE/L); 2 Gallic acid (6.41mg GAE/L); 3 OH-Benzoic Acid hexoside (2.97mg GAE/L); 4 Dihydrophaseic acid (12.43mg GAE/L). (B) The daily liquid consumption by rats was measured every day. Values are mean ± SEM (n=10 per group).

Table 3. Effects of mango juice on final body weight, food intake, juice intake, and caloric intake.

Group	Final Body weight (g)	Food intake (g/day)	Juice intake (ml/day)	DSS containing Juice intake (ml/day)	Caloric intake (kcal/day)
Control	475.61 ± 7.18	8.725 ± 0.281	91.242 ± 0.993	38.667 ± 1.545	82.193
Mango	445.39 ± 7.28*	10.888 ± 0.92*	84.174 ± 1.343*	42.333 ± 1.753*	84.713

Each value is a mean ± SEM (n=10 per group). Values are statistically significant at *p<0.05. Food intake measured as the mean (± SEM) weight (g) of food intake per 48 hour period at 8 weeks. Juice intake was calculated as the mean (± SEM) volume (ml) of juice consumed for whole study. Caloric intake was calculated on the basis of 3 kcal/g of pellet and 0.612 kcal/ml of juice.

Mango reduced the inflammation score and decreased cell proliferation in DSS-treated rats

The DSS-induced colitis murine model, which resembles human ulcerative colitis pathology, causes epithelial cell permeability and acute inflammation in colon. It is commonly used to test the efficacy of therapies such as food or drug on IBD (155). The 3 cycles of 3% DSS administration for 48h was sufficient to induce inflammation but not severe pathological ulceration (149, 154, 156). Histologic evaluation was done to access colonic inflammation injury. Mango juice intake reduced morphological signs of cell inflammation, and the inflammation score in the mango group was by 41% compared to the control juice (p=0.062, Figure 10A). Ki-67 proliferative index, an indicator for the level of cell proliferation, was primarily localized to the bottom and middle third of the crypts in both control and DSS groups (Figure 10B, C). The significant reduction in Ki-67 labeling in the mango juice group was detected compared to the control group (control 23.41% vs. mango 15.39%; P <0.05), which represented an 8% reduction compared to the control group (Figure 10B).
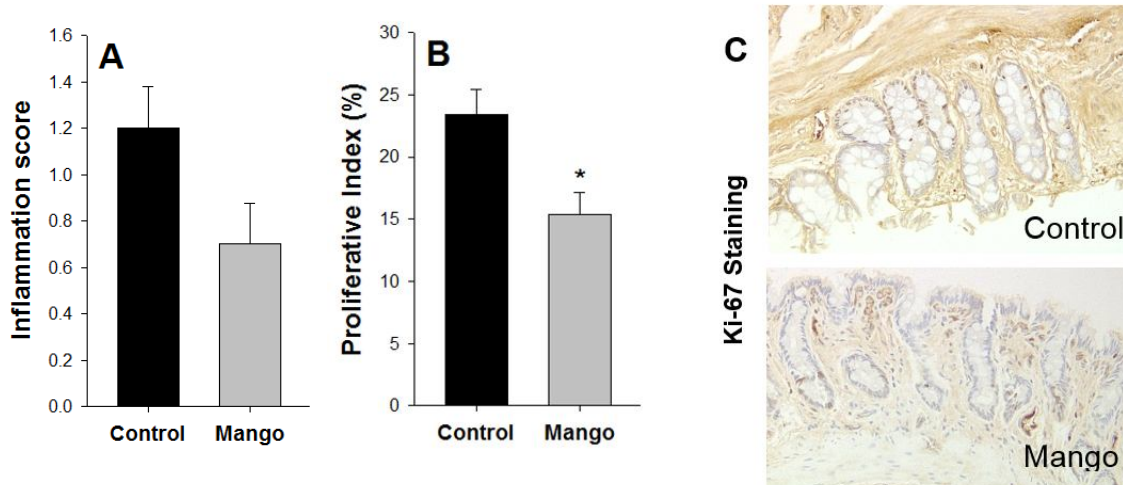
Figure 10. Effects of mango juice on DSS-induced colonic inflammation (A) and colonocyte proliferation (B, C) in DSS-treated rats.
Rats were fed a control juice or a mango juice, and were exposed to three cycles of DSS. (A) The inflammation score was decreased by the mango juice compared to the control juice intake in DSS-treated rats (p=0.062). Colonic inflammation was assessed by a board-certified pathologist (C.P.) in a blinded manner. Scores were assessed on a scale of 0-3. Values are mean ± SEM (n=10 per group). (B) Mango juice significantly suppressed colonocyte proliferation compared to control juice in DSS-treated rats. The measurement of Ki-67 was used to determine cell proliferation. Values are mean ± SEM (n=10 per group). *p < 0.05. (C) Ki-67 immunohistochemistry in rat colon mucosa in control and mango groups.

Mango suppressed markers of inflammation in DSS-treated rats

There is increasing evidence that gallic acid, a dominant polyphenolics in mango, targets key players in inflammation (141). Based on this information, we supposed that mango intake may reduce inflammation in colitis. To further quantify the effect of mango juice on inflammatory markers *in vivo*, we examined the inflammatory cytokines, NF-κB, COX-2 and iNOS expression. Mango juice inhibits mucosal levels of the inflammatory cytokines TNF-α and IL-1β mRNA (Figure 11A). The expression of

COX-2 and iNOS mRNA and NF-κB p65, p- NF-κB p65, COX-2 and iNOS protein expressions were reduced in DSS-treated rats consuming mango juice compared to the control group in colon epithelial cells (Figure 11B and C).
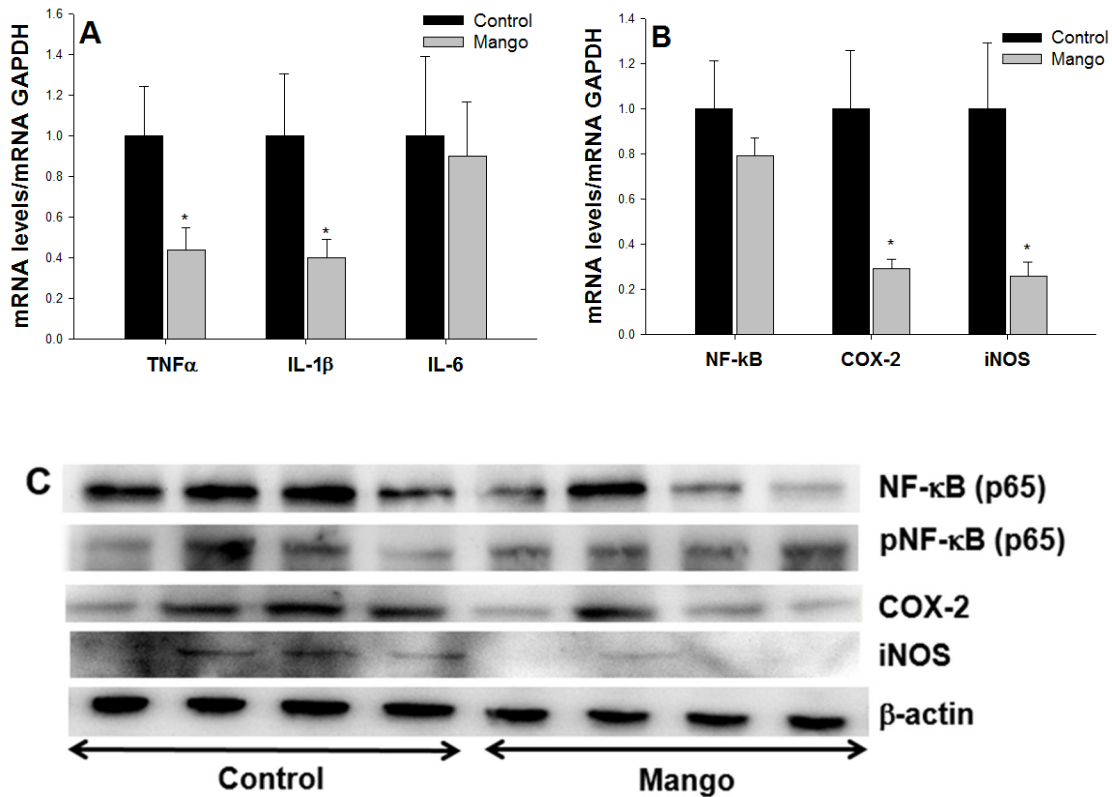
Figure 11. Effects of mango juice on pro-inflammatory cytokines, NF-κB, COX-2 and iNOS in DSS-treated rats.
Rats were fed a control juice or a mango juice, and were exposed to three cycles of DSS. Mango juice decreased the expressions of (A) TNFα and IL-1β mRNA and (B) COX-2 and iNOS mRNA in DSS-treated rats. Values are mean ± SEM (n=10 per group). *$p < 0.05$. (C) Mango juice suppressed the expression of NF-κB (p65), p-NF-κB (p65), COX-2 and iNOS protein in DSS-treated rats.

Mango modulated the mTOR pathway and increased the expression of miR-126 in DSS-treated rats

The mammalian target of rapamycin (mTOR) pathway by activation of the PI3K/AKT pathway plays a central role in the regulation of cell growth, cell proliferation and inflammation through downstream transduction of proliferative signals, such as p70S6K, RPS6 and HIF-1α (57). The suppression of the PI3K/AKT/mTOR pathway may represent a key role in the anti-inflammatory against colitis (60). Mango juice decreased the expression of PI3K, mTOR, p70S6K, and HIF-1α mRNA in DSS-treated rats (Figure 12A). In addition, mango juice suppressed the expression of PI3K (p85β) protein levels (Figure 12B) accompanied by decreased expression of AKT and mTOR total and phosphorylated protein, p70S6K phosphorylated protein, RPS6 total protein (Figure 12C and D), and HIF-1α protein levels in DSS-treated rats (Figure 12B). miRNAs may regulate the inflammatory response, and the mTOR pathway is a promising target by miRNAs (68). miR-126 is known to target PI3K (p85β), which in turn regulates the PI3K/AKT signaling (64). Previously, we have shown that pomegranate polyphenolics reduced inflammation in AOM-treated rats via the modulation of the miR-126/PI3K pathway (152). Mango juice increased the expression of miR-126 compared to control juice (Figure 12E), which may be correspondingly accompanied by suppression of PI3K mRNA and protein expression in DSS-treated rats (Figure 12A and B).
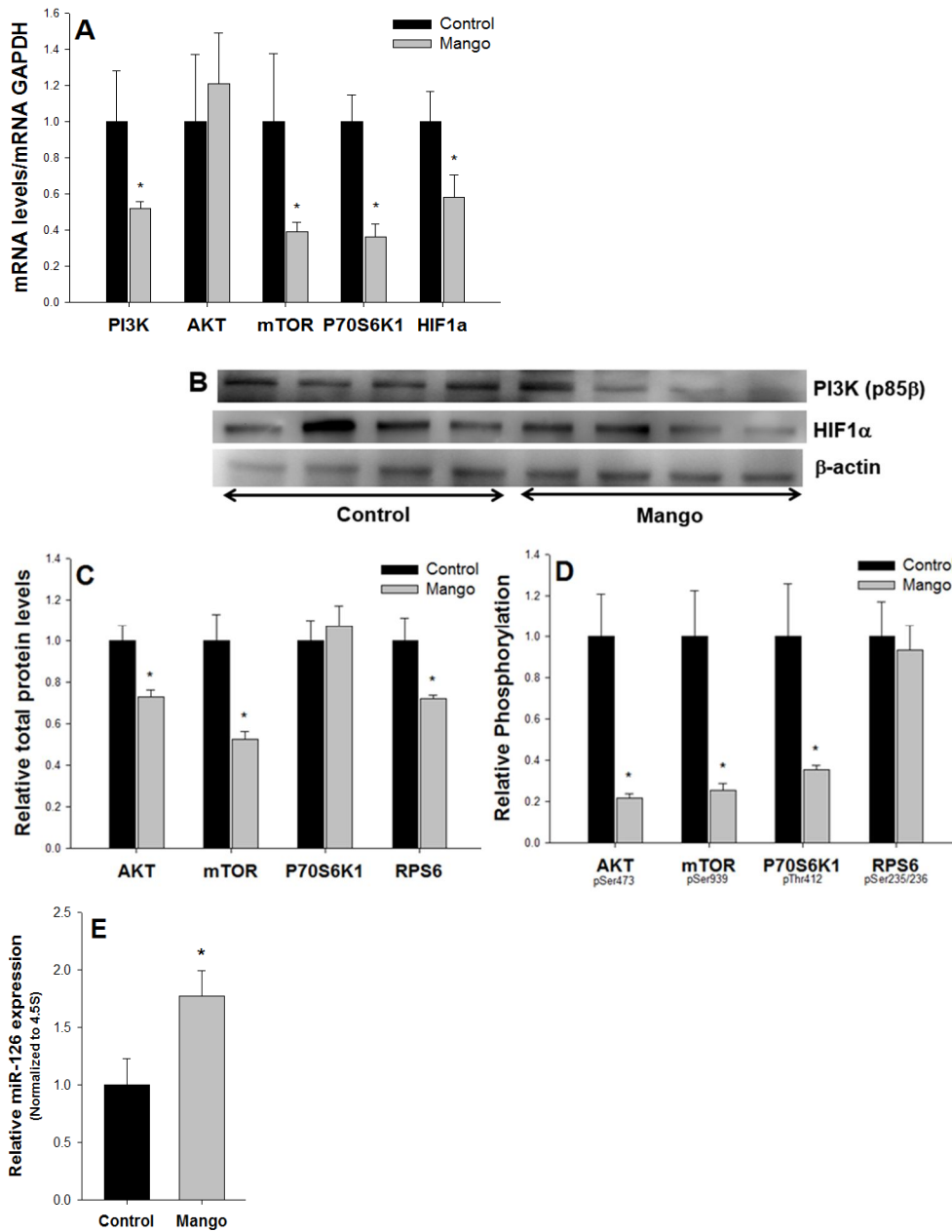
Figure 12. Effects of mango juice on the mTOR signaling pathway in DSS-treated rats.
Rats were fed a control juice or a mango juice, and were exposed to three cycles of DSS. (A) Mango juice decreased the expression of PI3K, mTOR, p70S6K1 and HIF-1α mRNA in DSS-treated rats. (B) Mango juice suppressed the expression of PI3K (p85β) and HIF-1α protein, (C) the total protein of AKT, mTOR and RPS6, and (D) the phosphorylated protein of AKT, mTOR and p70S6K1 in DSS-treated rats. (E) Mango juice increased the expression of miR-126 compared to control juice in DSS-treated rats. Values are mean ± SEM (n=10 per group). *$p < 0.05$.

Mango decreased markers in the inflammatory and the mTOR signaling pathway in vitro

Previously, we have shown the anticancer effects on mango extract *in vitro*, especially CCD-18Co cell lines (81). However, the specific mechanisms of the anti-inflammatory effects of mango were unclear. Mango extract did not affect cell viability in CCD-18Co cells (Figure 13A). Similar results of *in vivo* study were obtained *in vitro*, LPS-treated CCD-18Co cell lines. Mango extract suppressed the expression of inflammatory markers, TNF-α and IL-6 mRNA at 10mg GAE/L (Figure 13B), and the expression of NF-κB, iNOS mRNA (Figure 13C) and NF-κB, pNF-κB protein (Figure 13E) in LPS-treated CCD-18Co cells. In addition, mango extract also modulated the mTOR pathway in LPS-treated CCD-18Co cells. Mango extract suppressed the expression of PI3K (p85β) and mTOR mRNA at 10mg GAE/L (Figure 13D), PI3K (p85β) protein (Figure 13E), the total protein of p70S6K1 and RPS6 (Figure 13F), and the phosphorylated protein of mTOR and p70S6K1 (Figure 13G) in LPS-treated CCD-18Co cells. Mango extract (0~10mg GAE/L) also increased the expression of miR-126 in a dose-dependent manner in LPS-treated CCD-18Co cells (Figure 13H).
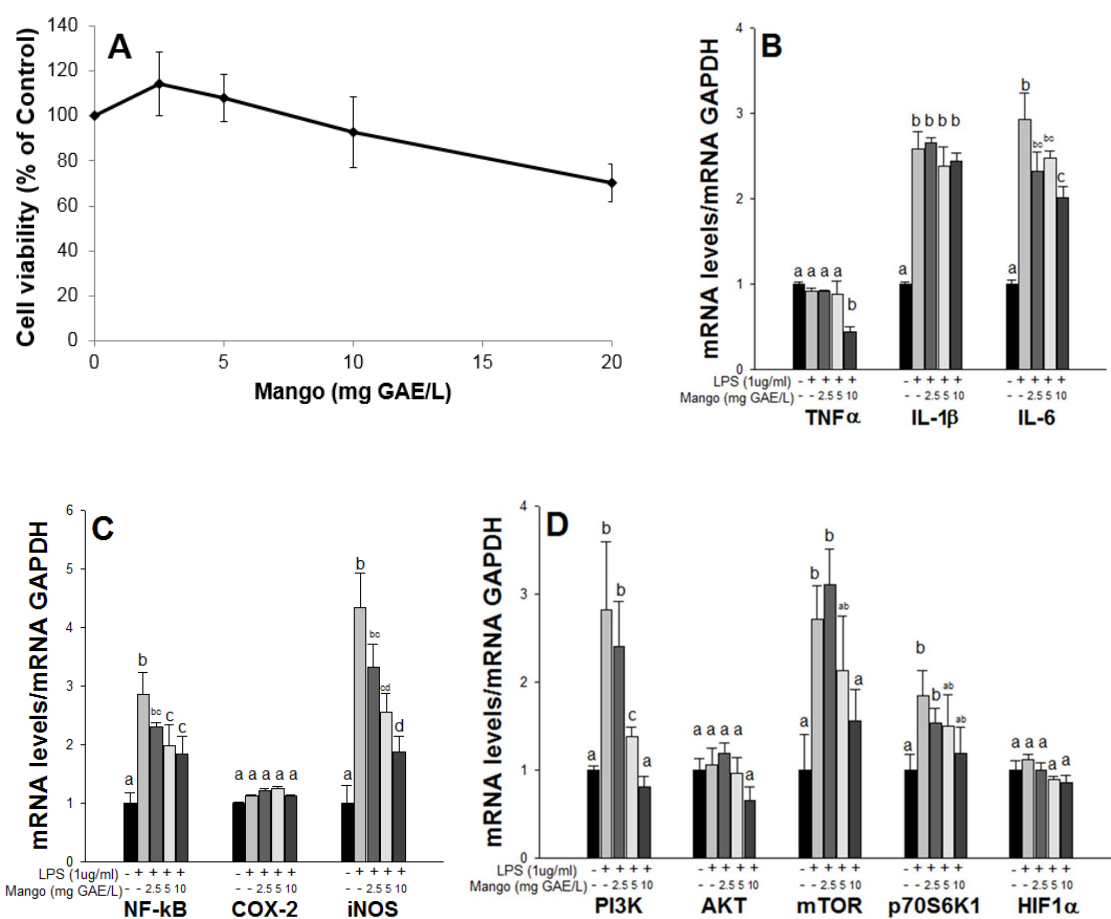
Figure 13. Effects of mango extract on the inflammatory and the mTOR signaling pathway in LPS-treated CCD-18Co cells.
(A) Mango extract did not affect cell viability in CCD-18Co cells. (B) Mango extract suppressed the expression of inflammatory markers, TNFα, IL-6, (C) NF-κB, iNOS mRNA and the expression of in LPS-treated CCD-18Co cells. (D) Mango extract suppressed the expression of PI3K (p85β) and mTOR mRNA in LPS-treated CCD-18Co cells. (E) Mango extract suppressed the expression of NF-κB, pNF-κB, PI3K (p85β) and HIF1-α proteins, (F) the total protein of p70S6K1 and RPS6, (G) the phosphorylated protein of mTOR and p70S6K1 in LPS treated CCD-18Co cells. (H) Mango extract increased the expression of miR-126 in LPS-treated CCD-18Co cells in dose dependent manners. Cells were treated different concentration of Mango extract (0-10 mg GAE/L) and LPS (1ug/ml) for 3h (RNA extraction) or 24h (Protein extraction). All experiments were performed at least three times, and result were expressed as mean ± SEM (n=3). Different letters indicate significance at p<0.05.

E

LPS (1ng/ml)   -   +   +   +   +
Mango (mg/L)   -   -   2.5   5   10

NF-κB (p65)
p-NF-κB (p65)
PI3K (p85β)
HIF1α
β-actin

F

Relative total protein levels

LPS (1ug/ml)   - + + + +
Mango (mg GAE/L)   - - 2.5 5 10

AKT   mTOR   p70S6K1   RPS6

G

Relative phosphorylation

LPS (1ug/ml)   - + + + +
Mango (mg GAE/L)   - - 2.5 5 10

AKT   mTOR   p70S6K1   RPS6
pSer473   pSer939   pThr412   pSer235/236

H

Relative miRNA levels/U6B

LPS (1ug/ml)   -   +   +   +   +
Mango (mg GAE/L)   -   -   2.5   5   10

Figure 13 Continued.

The involvement of miR-126 in the mango-induced modulation of PI3K (p85β)

In order to determine the involvement of miR-126 and its target gene, PI3K (p85β) in the anti-inflammatory activities of mango, we investigated the further *in vitro* treatment with antagomiR of miR-126. After the transfection of miR-126 antagomiR, cells were treated with the mango extract (10mg GAE/L) and 1μg/ml LPS to induce inflammation. In results, the antigomiR treatment reduced the expression of miR-126, and mango extract partially reversed the effect of antagomiR-126 on transfected cells (Figure 14A). In addition, the reversed expression of miR-126 by mango treatment was accompanied by a decreased expression of PI3K (p85β) mRNA (Figure 14B). It indicated that miR-126 has an important role in the modulation of PI3K signaling by mango treatment.

Discussion

Ulcerative colitis, the most common form of IBD, is a multi-factorial disorder, and the development of this disease is affected by several factors, such as genetic susceptibility, uncontrolled intestinal microbiota, immune dysregulation and environmental factors (5). Imbalance of the immune system causes the overproduction of reactive oxidative stress (ROS) and pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF-α through NF-κB activation, and leads to chronic inflammation and mucosa damage in the large intestine (157). Although colitis is rarely lethal on its own, it is at high risk for developing colon cancer (40). Drugs have been mainly used for the

treatment of colitis, but natural compounds, in particular, polyphenols become as

promising candidates due to their anti-inflammatory effects (50, 139).
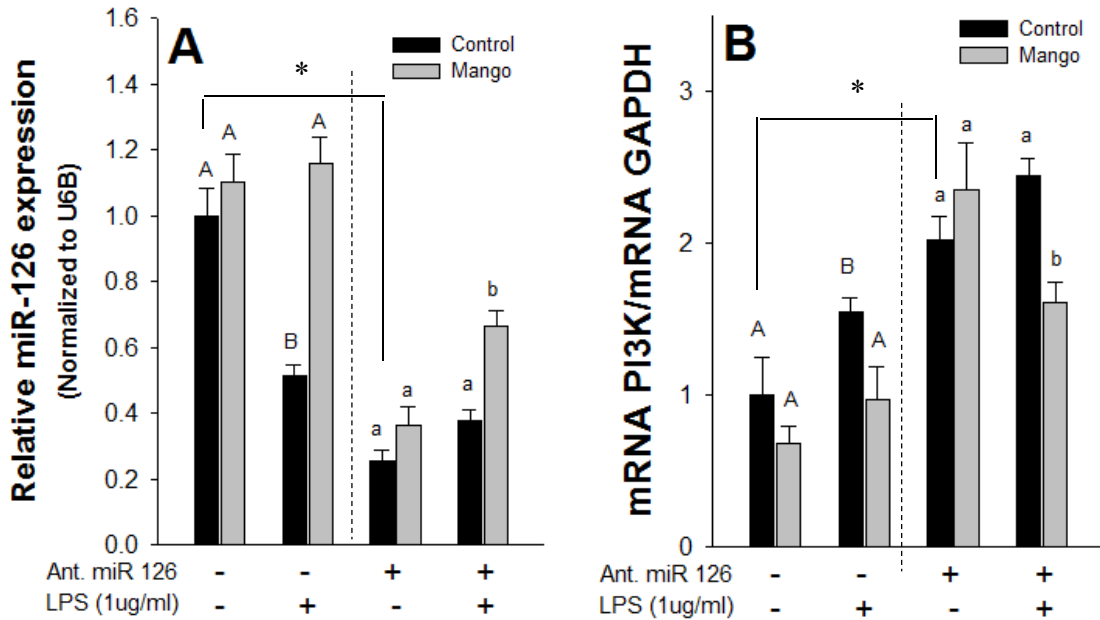
Figure 14. Effects of mango extract on miR-126 and PI3K mRNA levels in transfected CCD-18Co cells with a miR-126 antagomiR.
CCD-18Co cells were transfected with 20nmol/ml of miR-126 antagomiR. After that, cells were treated with the mango extract (10mg GAE/L) and LPS (1ug/ml) for 24h. (A) Mango extract reversed the effect of miR-126 antagomiR on transfected cells. (B) The reversed expression of miR-126 was accompanied by a decreased expression of PI3K (p85β) mRNA. miR-126 was analyzed as a ratio to the miR-NU6B endogenous control, and PI3K was assayed as ratio to GAPDH. All experiments were performed at least three times, and result were expressed as mean ± SEM (n=3). Different letters indicate significance at $p<0.05$. *$p < 0.05$.

Mango is a highly valued source of polyphenolics, such as gallic acid and gallotannins, which have been shown to have antioxidant, anti-inflammatory, and anticancer properties (140, 141, 143, 158). It has already shown that mango extracts play an anti-inflammatory role through reduction of inflammation related proteins such as iNOS, COX-2, and TNF-α in murine colitis (84). However, the mechanisms underlying the anti-inflammatory activities of mango against colitis remains to be unclear. In this study, we demonstrated that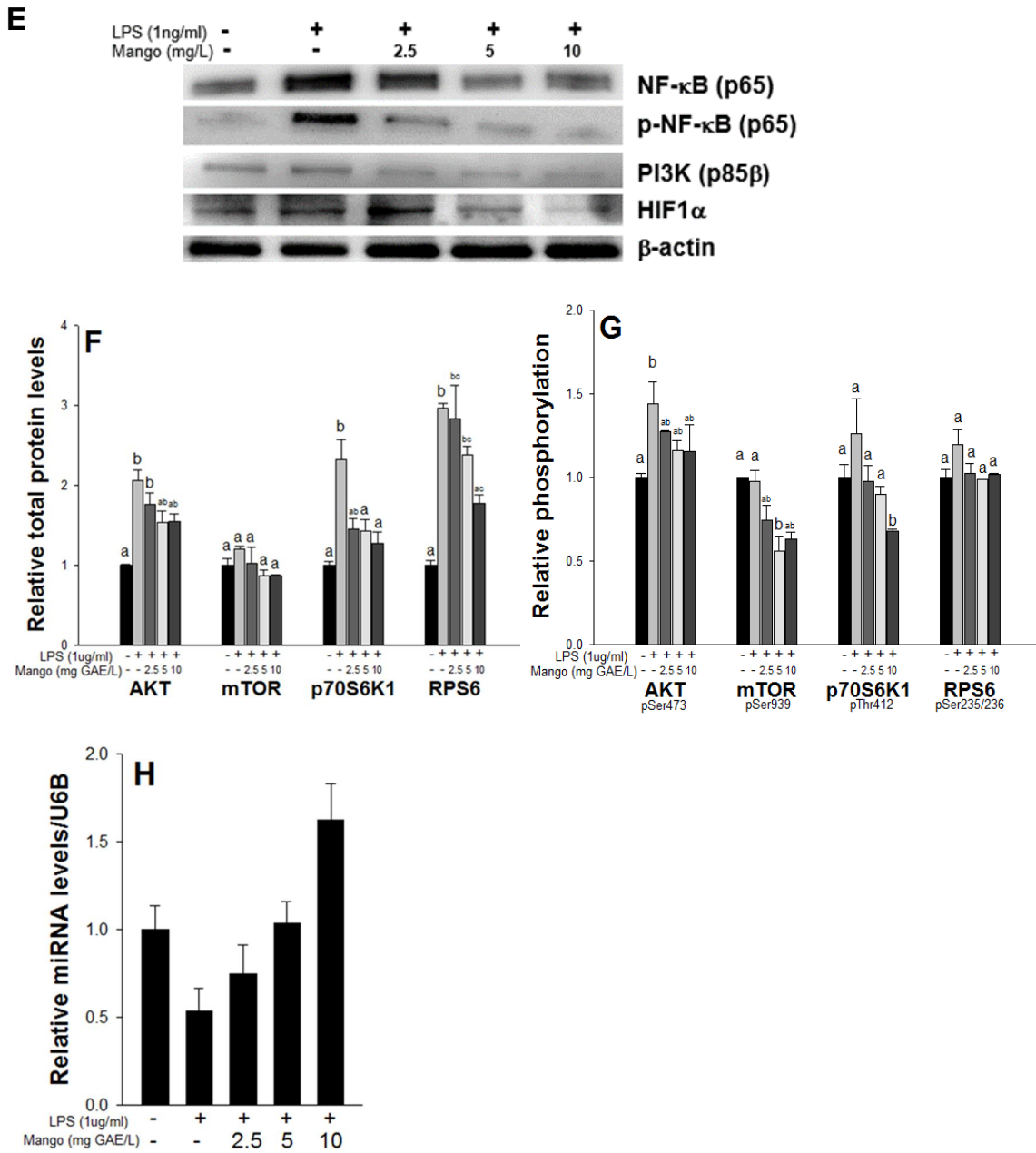 mango intake reduced inflammatory response in colitis *in vivo* and *in vitro* through modulation of mTOR pathway and its post-translational modification.

In this study, mango juice showed anti-inflammatory effects, as found in other studies (84). Mango intake slightly decreased the inflammatory scores (Figure 10A), and significantly suppressed cell proliferative index (Figure 10B) compared to control juice in DSS-treated rats. In the early process of colitis, LPS produced by bacteria and interferons (IFNs) by macrophage trigger signal transduction cascades, such as NF-κB and mTOR pathways (56), that induce cell proliferation in colitis (159). The NF-κB activation transcriptionally increases the expression of genes that encode pro-inflammatory cytokines, IL-1β, IL-6, TNF-α, COX-2 and iNOS in macrophages and epithelial cells (160). Induction of COX-2 and iNOS expression produce inflammatory mediators, such as free radical NO and PGE_2 (161). Mango juice reduced the expressions of pro-inflammatory cytokines mRNA (TNF-α and IL-1β, Figure 11A), and inflammation-related markers NF-κB protein, COX-2 and iNOS mRNA and proteins (Figure 11B, C) in the rat colitis model. In addition, mango extract also reduced the

expression of TNF-α and IL-6 mRNA (Figure 13B), and NF-κB mRNA and protein

(Figure 13C and E) in LPS-treated *in vitro* model.

The mammalian target of rapamycin (mTOR) pathway plays a central role in the

regulation of cell proliferation and inflammation that lead to induce colitis (162). During

colitis, LPS and IFN-γ stimulate PI3K/AKT signaling and mTOR pathway (56).

Activation of mTOR leads to the activation of 4E-BP1 and p70S6K (57), that induce

HIF-1α protein synthesis (163). The transcription factor HIF-1α regulates genes related

to angiogenesis, cell growth, and proliferation (164). In DSS-induced colitis model, it

was reported that induction of HIF-1α triggers inflammatory response by inducing pro-

inflammatory mediators (165). In a few studies, mTOR inhibitors have shown be

effective to suppress DSS-induced colitis by inhibiting T cell function (60, 62). Targeted

suppression of mTOR pathways during colitis by natural compounds may provide an

important strategy for the prevention of colitis (166). In this study, mango suppressed the

PI3K (p85β) mRNA and protein (Figure 12A, B), and the total and phosphorylated

expression of AKT and mTOR proteins in rat colitis model (Figure 12C, D). This result

was consistent with *in vitro* studies where mango extract suppressed the PI3K (p85β)

mRNA and protein (Figure 13D, E), and the mRNA and phosphorylation of mTOR

(Figure 13D, G). Downstream markers of mTOR pathway such as p70S6K, RPS6 and

HIF-1α protein were also down regulated by mango intake compared to control juice

(Figure 12B, C, and D).

miRNAs modulate the inflammation due to the regulation of gene expressions

by inducing mRNA degradation or blocking translation (167). The mTOR pathway is a

promising target by miRNAs for anti-colitis and anti-cancer therapeutics (68). miR-126

has target site in the promoter region of phosphatidylinositol 3-kinase regulatory subunit

beta (PI3Kp85β), an upstream component of the mTOR pathway (64). Accordingly,

miR-126 may be a potential anti-inflammatory target via the inhibition of angiogenesis,

vascular integrity, inflammation, and proliferation (69). Our previous studies with red

wine showed a significant suppression in inflammatory markers in LPS-treated CCD-

18Co cells, where also miR-126 was involved in the underlying mechanisms (168). In

addition, we demonstrated that pomegranate polyphenolics decreased inflammation and

reduce the PI3K/AKT pathways via modulation of miR-126 expression in AOM-treated

rats and in colon cancer cell lines (152).

In this study, mango decreased expression of PI3K (p85β) mRNA and protein

(Figure 12A, B and 13D, E) which was accompanied by up-regulation of miR-126 *in*

vivo (Figure 12E) and *in vitro* (Figure 13H). PI3K (p85β) is regulated by miR-126

though a target-binding site in the 3'-UTR region of the PI3K (p85β) mRNA (64). To

confirm the relationship between miR-126 and PI3K (p85β), cells were transfected to

decrease the expression of miR-126 with the antagomiR for miR-126, whose

concentration (20nM) were low enough to be overcome by the effects of mango. The

effects of mango were significantly reversed the effect of inhibitor (Figure 14A),

accompanied with the reduction of PI3K (p85β) mRNA (Figure 14B).

Certain limitations will require further experimentation in the future. The first

limitation is that mango juice slightly but not significantly suppress the intestinal

inflammation; however, several inflammatory markers were also evaluated in control

and mango juice group. In addition, the next approach can be to perform negative controls (control juice without DSS treatment) to compare the level of intestinal inflammation between the negative control and the mango juice group. A further limitation is that we did not use semi-purified diet but used rat chow pellets in this study. The chow diet typically contains soy protein, so it is not fully known all possible synergistic effects of mango polyphenolics and compounds in the soy protein, such as genistein, on the inflammation. In addition, colitis was induced in rats by DSS diluted in the experimental juice in this study. However, there can be a potential binding of DSS compounds on polyphenolics in the juice chemically, so DSS might not show the same toxicity in the control and mango juice group. Thus, investigating the detailed interaction among mango polyphenolics, soy proteins in the chow diet, and DSS compounds is essential for further understanding the effects of mango polyphenolics on colitis.

Despite these limitations, our data from *in vivo* rat studies provide evidence of a causal role for mango juice in attenuating the level of inflammatory markers by suppressing the PI3K/AKT/mTOR signaling pathway in part though up-regulation of miRNA-126 expression. Interaction of mango with miR-126/PI3K/AKT/mTOR pathway was identified as the underlying mechanisms that is, at least in part, involved in the anti-inflammatory of mango juice, and mango may be a potential therapeutic agent for colitis.

CHAPTER III

POMEGRANATE POLYPHENOLICS REDUCE INFLAMMATION IN INTESTINAL

COLITIS - POTENTIAL INVOLVEMENT OF THE MIR-145/P70S6K/HIF1-

α PATHWAY IN VITRO AND IN VIVO

Summary

Pomegranate polyphenolics including ellagic acid, ellagitannins, flavonoids and anthocyanins, have previously been demonstrated effective in the prevention of inflammation. However, anti-inflammatory mechanisms relevant to the prevention of colitis have not been well investigated.

This study investigates the potential role of the HIF1-α signaling pathway in the anti-inflammatory effects of pomegranate juice on dextran sodium sulfate (DSS)-induced colitis in Sprague Dawley rats and in human CCD-18Co colon-myofibroblastic cells. Animals were administered control juice (15.7g sugar and 0.05g citric acid/100ml) or pomegranate juice (total phenolic content of 2698.99mg/L GAE), and exposed to three cycles of 3% DSS. The a potential relationship between miR-145 and p70S6K1 was elucidated by the treatment of 10mg GAE/L pomegranate extract and 1ug/ml LPS in transfected cell lines with a miR-145 antagomiR. The mRNA and protein levels were assessed in colonic mucosa by RT-PCR, western blotting and multiplex bead assay.

Compared to the control, pomegranate suppressed cell proliferation (37.5%), and protected against DSS-induced colon inflammation and injury (50% and 66.7%). Pomegranate significantly down-regulated phosphorylation of p70S6K1, and decreased

HIF1-α expression. p70S6K1 is the direct target of miR-145. Induction of miR-145 by pomegranate juice (2.0 fold) compared to control juice corresponds to the down-regulation of p70S6K1. Pomegranate significantly attenuated the pro-inflammatory cytokines (TNF-α and Il-1β), COX-2 and iNOS. The involvement of miR-145/p70S6K/HIF1α was additionally investigated in CCD18Co, non-cancer colon fibroblasts, where the involvement of this pathway was confirmed using the specific antagomiR of miR-145, where the effects of pomegranate extract were in part reversed by the antagomiR.

These results suggest that pomegranate intake attenuated DSS-induced colitis by modulating the miR-145/p70S6K/HIF1 α pathway and inhibiting inflammatory responses, and it may be a potential therapeutic for colitis.

Introduction

Ulcerative colitis is a chronic and relapsing inflammation of the mucosal layer of the large intestine, such as the colon and rectum (43). Development of this disease is affected by multi-factorial conditions, such as genetic susceptibility, intestinal microbiota, immune dysregulation and environmental factors (e.g., smoking and diets high in fat and sugar) (3-5). A serious of long-term chronic inflammation causes loss of the lining of the colon, and produces high levels of pro-inflammatory cytokines and inflammatory molecules such as COX-2 and iNOS, which increases the risk of cancer. Accordingly, interventions that reduce intestinal inflammation may potentially reduce ulcerative colitis and related colon cancer risk.

Epidemiological and clinical studies suggested that polyphenolics derived from dietary sources are highly considered to be sources of natural antioxidant and the anti-inflammatory properties by suppressing NF-κB, COX-2 and iNOS activation (74, 75). The conventional therapeutics strategies reported a number of side effects, and thus, polyphenolics could contribute as complementary approaches to the management of colitis. Pomegranate is rich in polyphenols, including ellagic acid, ellagitannins, flavonoids and anthocyanins. It was shown that pomegranate and its polyphenolics exert antioxidant (169) and anti-inflammatory properties (170). Previously it was demonstrated that pomegranate extract reduced colitis severity via modulation of microbiota in guts (*Bifidobacterium*, *Lactobacillus*, and *Clostridium*) and decreasing COX-2, iNOS, PTGES, and PGE_2 in DSS-induced colitis models (97). However, the underlying mechanisms relevant to reducing intestinal inflammation have not been well investigated.

HIF1-α is a heterodimeric nuclear transcription factor, and it plays an important regulator of epithelial barrier protection during colitis (171). On the other hand, during colitis, HIF1-α expression was induced, and it was correlated with more severe colitis and increase in inflammatory infiltrates in colon. Furthermore, activation of HIF1-α increased expression of pro-inflammatory mediators (165). Targeted suppression of HIF1-α during colitis by natural compounds may provide an important strategy for colitis and colon cancer prevention (166). The mechanism by which the expression of HIF1-α can be regulated is unclear. The mTOR/p70S6K1 is one of potential regulator by

inducing the transduction of HIF1-α, and this pathway might be an important target for colitis treatment (57).

miRNAs are a class of small non-coding RNA molecules that regulate mRNA degradation or repress their translation, and they are potential post-transcriptional regulators for the modulation of inflammatory and immune responses (63). miR-145 has target site in the promoter regions of p70S6K1, an downstream molecules of the mTOR pathway, and regulate the expression of HIF1-α (70). miR-145 may be a potential anti-inflammatory target for prevention and treatment of colitis and colon cancer. Furthermore, it was reported that dietary polyphenolics can be a potential post-transcriptional regulator by modulating the expression of miRNAs (71).

In this study, we investigated the molecular targets involved in anti-inflammatory activities of pomegranate juice on dextran sodium sulfate (DSS)-induced colitis in Sprague Dawley rats and in lipopolysaccharide (LPS)-treated human CCD-18Co colon-myofibroblastic cells. We hypothesized that pomegranate polyphenolics has anti-inflammatory activity in part due to the modulation of the miR-145/p70S6K1/HIF1-α pathway, which plays an important role in the inflammatory response in ulcerative colitis.

Materials and methods

Chemicals, antibodies, and reagents

DSS was purchased from MP biomedicals (Solon, OH). Antibody against Ki-67 was purchased from BD Pharmingen (San Jose, CA). Antibodies against p70S6K1,

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COX-2 were purchased from Cell signaling technology (Beverly, MA). The antibody for iNOS was purchased from Cayman chemical (Ann Arbor, MI). The antibody for HIF1-α was purchased from Abcam (Cambridge, MA). Lipofectamine 2000 was purchased from Invitrogen (Grand Island, NY). mirVana™ miRNA Isolation Kit, reverse transcription and real-time PCR amplification kits were purchased from Applied Biosciences (Foster City, CA). All primers were purchased from Integrated DNA Technologies (San Diego, CA). AntagomiR of miR-145 was purchased from from Dharmacon (Lafayette, CO).

Pomegranate chemistry

Pomegranate juice for the *in vivo* study was purchased from the POM wonderful (Los Angeles, CA). The pomegranate extract for the *in vitro* study was prepared as described in our previous literature (81). The pomegranate juice mainly contained sugar and polyphenolics, while pomegranate extract had only total polyphenolics from pomegranate. Total phenolic contents in the pomegranate juice and extract were measured spectrophotometrically by the Folin-Ciocalteu assay against an external standard of gallic acid. Individual pholyphenolics in the juice were analyzed on a Waters Alliance 2690 HPLC-MS system (Milford, MA). In addition, identification by mass spectrometric analyses were performed on a Thermo Finnigan LCQ Deca XP Max mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA) (81).

Animal treatment and tissue sampling

The DSS-induced colitis model is the most effective in producing the clinical

and histological features of UC characteristics in experimental animals (148). In

particular, epithelial homeostasis, regeneration and wound healing in DSS-treated rats is

similar to human IBD (127). Sprague-Dawley rats (10 week old) supplied from Harlan

Teklad (Houston, TX) were acclimated for a week, and were randomly distributed into

control juice and pomegranate juice groups. For calorie and pH adjustment, 15.7g sugar

and 0.05g citric acid were added in 100ml of control juice. After 3 weeks of rat chow

pellets and experimental juices *ad libitum*, all rats were administered 3% (w/v) DSS (3

cycles, 14 d separation, n=10/diet) in the experimental juices for 48 h. Rats were

sacrificed 2 weeks after three times of DSS administration, and colon tissues collected

(Figure 15). One centimeter sections were cut from the distal end of each rat colon, and

fixed in 4% paraformaldehyde (PFA) for embedding in paraffin. The remained colon

was gently scraped for collecting protein and RNA samples (149). The animal use

protocol was approved by the Institutional Animal Care and Use Committee of Texas

A&M University.
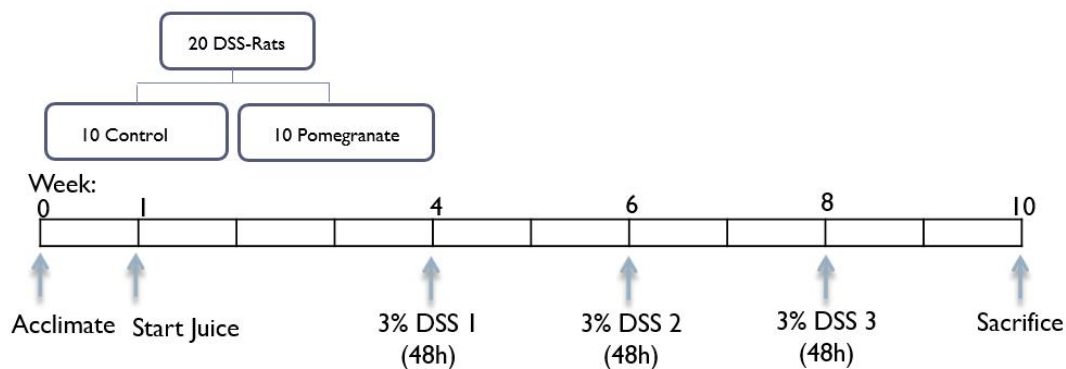
Figure 15. Experimental design.

Quantifying inflammation and cell proliferation assay

For histopathology and immunohistochemistry analysis, colon tissues were dehydrated, embedded in paraffin and serially sectioned into 4 μm thick. Hematoxylin and eosin (H&E) stained slides were then examined by a board-certified pathologist (C.P.) in blinded manner for accessing the histological colitis level. The inflammation score was assessed on a scale of 0-3, where 0 is none observed and 3 is severe inflammation (150). The ulceration score was also assessed on a scale of 0-3; no damage was scored as 0 and several sites of ulceration or one large ulcer >1cm was scored as 3 (172). For Ki-67 immunohistochemical staining, the primary antibody against Ki-67 (Dilution 1:50) were treated on the sections, and sections were incubated with biotinylated anti-mouse IgG from the Vectastain ABC Elite kit (Vector Lab, Burlingame, CA). Twenty-five crypt columns per rat were selected for quantitative analysis. The number and position of labeled cells were recorded. Proliferative index was calculated as 100 times the number of labeled cells divided by the total number of cells per crypt column.

Cell culture

Human colon CCD-18Co myofibroblastic cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA), and cultured as recommended by the ATCC. Cell were seeded for cell proliferation assay onto a 24-well plate (15,000 cells/well), for RNA extractions onto a 12-well plate (150, 000 cells/well), and for the protein analysis onto a 6-well plates (300,000 cells/well). Cells were pre-

incubated for 24h before exposing the treatment of the pomegranate extract (0-10mg GAE/L). The level of cell viability was quantified with a cell counter after 48h (Z2™ Beckman coulter, Fullerton, CA). RNA was extracted for gene expression analysis after 3h, and Protein was extracted after 24h.

Quantitative RT-PCR

For mRNA analysis, total and micro RNA from the colon mucosal scraping was isolated using the mirVana™ miRNA Isolation Kit according to the manufacturer's protocol. Equal amount (1ug) of mRNAs was converted to cDNA using a reverse transcription kit (Invitrogen, Grand Island, NY). Real-time PCR reations were performed using 2uL of cDNA using a Reverse Transcription Kit (Invitrogen, Grand Island, NY). SYBR Green PCR master Mix (Applied biosystems, Foster City, CA) was used for the qPCR analyses on Applied Biosystems 7900HT Fast Real-Time PCR system (Applied biosystems, Foster City, CA). The sequence of primers were designed using Primer3, online primer design tool and were obtained from Integrated DNA Technologies (Coralville, IA). GAPDH was used as the endogenous loading control. Quantification if miR-145 was measured using the Taqman MicroRNA reverse Transcription kit (Applied Biosystems, Foster City, CA) and qRT-PCR reaction using Taqman 2X Universal PCR master mix (No AmpErase UNG) (Applied Biosystems, Foster City, CA). The miR-4.5S and miR-NU6B were used as endogenous control for micro RNA expression.

Multiplex bead assay

The protein extract (20ug) from each tissue was used to determine the relative abundance of the total protein and phosphorylation status in the AKT/mTOR signaling (AKT, mTOR, p70S6 kinase, and RPS6) using the multiplex kits (Millipore, Billerica, MA) according to the manufacturer's protocol. Multiplex analysis was performed by the Luminex L200 instrument (Luminex, Austin, TX), and data was analyzed by Luminex xPONENT software.

Western blotting

The mucosal scrapings were homogenized in 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. After 24h pomegranate extract treatment, cells were lysed in RIPA buffer added 1% protease and proteinase inhibitor cocktail (Pierce/Thermo Scientific, Rockford, IL), and centrifuged (151). 60ug of protein was loaded onto acrylamide gel, followed by transfer onto PVDF membranes. The membranes were incubated with primary antibodies against iNOS, COX-2, p70S6K1, HIF1-α and β-actin.

Transfection with antagomiR of miR-145

Cells seeded into 12-well plates were transfected with 20nM antagomiR of miR-145 with using lipofectamin 2000 according the manufacturer's protocol. For the

control, a nonspecific oligonucleotide was used. After transfection for 4h, the transfection mix was replaced with medium with 10mg GAE/L of pomegranate extract, and after 1h, cells were treated with medium with pomegranate extract and 1nM of LPS for 24h as previously described (151).

Statistical analysis

Quantitative data represent mean values with standard error. Data were analyzed by student t-test and one way ANOVA using Tukey's post hoc test ($p < 0.05$) using SAS version 9 (SAS Institute Inc., Cary, NC).

Results

Polyphenolic composition of pomegranate juice and the juice consumption of DSS-treated rats

The concentration of total soluble phenolics of pomegranate juice used for this study was 2698.99mg gallic acid equivalents (GAE)/L. HPLC-MS analysis of pomegranate juice showed the abundance of ellagitannins (Figure 16A), flavonoids (Figure 16B), and anthocyanins (Figure 16C). There was the most predominant ellagitannins, such as punicalins (peak 1), punicalagin (peak 2), ellagic acid glucoside (peak 3), and ellagic acid (peak 4) with peak absorption at 280nm (Figure 16A). However, the overall the pomegranate juice consumption and final body weight in the pomegranate group were significantly lower compared to those in the control group (Table 4). The reason might be that it took time for rats to become used to the taste of

tannins in the early stage of this study (Figure 16D). The total caloric intakes were not significantly different, because the food intake in the pomegranate group was increased (Table 4). In addition, during the DSS administration, the intake of pomegranate juice containing 3% DSS was significantly higher than the control juice, potentially due to the improvement of the bitter taste of DSS (Table 4). When a rat (440.41g body weight) drank 81.468ml of pomegranate juice (2698.99mg GAE/L), the dose was equivalent to 499.27 mg GAE/kg/day in rats. The human equivalent amount of pomegranate juice consumed by rats was calculated with the body surface area normalization method (human equivalent dose (mg/Kg)=rat dose (mg/Kg)×rat K_m/human K_m(6/37)) (153, 154). The human equivalent dose of pomegranate juice was 5.6g GAE/day of polyphenolics or 2L/day of pomegranate juice in 70kg human. There were no symptoms (for example, severe diarrhea or bloody stool) of severe inflammation after DSS treatment.

Table 4. Effects of pomegranate juice on final body weight, food intake, juice intake, and caloric intake.

Group	Final Body weight (g)	Food intake (g/day)	Juice intake (ml/day)	DSS containing Juice intake (ml/day)	Caloric intake (kcal/day)
Control	475.61 ± 7.18	8.725 ± 0.281	91.242 ± 0.993	38.667 ± 1.545	82.193
Pomegranate	440.41 ± 5.57*	11.345 ± 0.64*	81.468 ± 2.042*	43.024 ± 1.378*	83.893

Each value is a mean ± SEM (n=10 per group). Values are statistically significant at *p<0.05. Food intake measured as the mean (± SEM) weight (g) of food intake per 48 hour period at 8 weeks. Juice intake was calculated as the mean (± SEM) volume (ml) of juice consumed for whole study. Caloric intake was calculated on the basis of 3 kcal/g of pellet and 0.612 kcal/ml of juice.
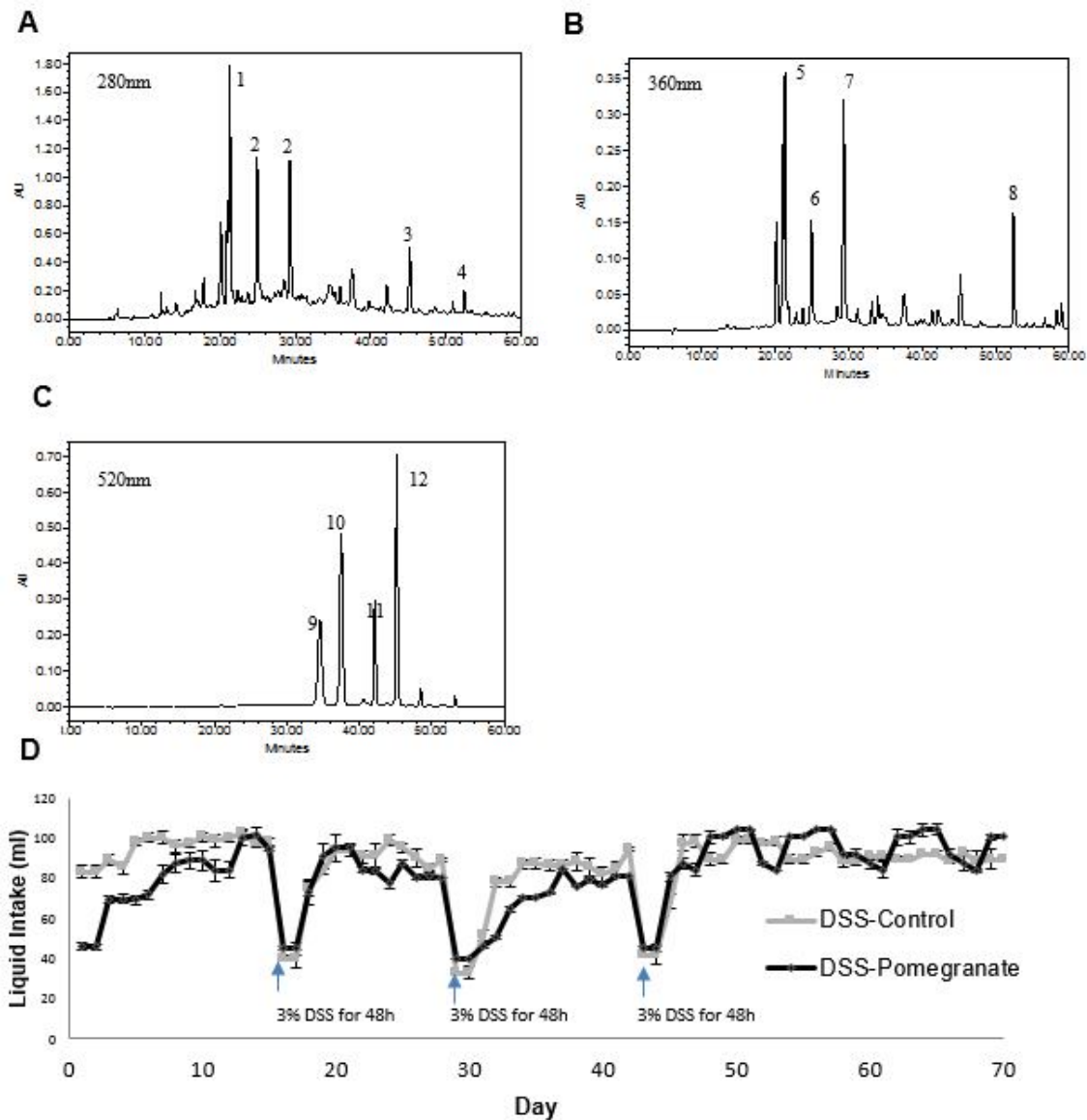
Figure 16. Representative chromatographic profiles at 280nm, 360nm and 520nm of phenolic compounds in pomegranate juice (POM wonderful) and the daily liquid consumption by rats.
(A, B, and C) Total phenolic content of the pomegranate juice was 2698.99mg GAE/L. Tentative peak assignments: 1 Punicalins; 2 Punicalagins; 3 Ellagic acid glucoside; 4 Ellagic acid; 5 Chlorogenic acid; 6 Quercetin-3-rutinoside; 7 Quercetin; 8 Flavonols; 9 Cyanidin-3,5-diglucoside; 10 Delphinidin-3,5-diglucoside; 11 Delphinidin-3-glucoside; 12 Cyanidin-3-glucoside. (D) The daily liquid consumption by rats was measured every day. Values are mean ± SEM (n=10 per group).

Pomegranate reduced the inflammation and ulceration scores and suppressed cell

proliferation in DSS-treated rats

The DSS-induced colitis model is the most effective in producing the clinical and histological features of ulcerative colitis characteristics in experimental animals. This model is a valuable tool for investigating the involvement of dietary factors into in pathogenesis of IBD and evaluating different therapeutic options (128). The 3 cycles of 3% DSS administration for 48h was sufficient to induce inflammation but not severe pathological ulceration (149, 154, 156). Histologic evaluation was done to access colonic inflammation and ulceration. Pomegranate juice slightly reduced the inflammation score, and significantly decreased ulceration scores by 50% and 66.7% compared to the control juice ($p=0.059$ and <0.05, Figure 17A, B). The significant reduction in Ki-67 labeling in the pomegranate group was detected compared to the control group (37.5% reduction; $P <0.05$) (Figure 17C, D).
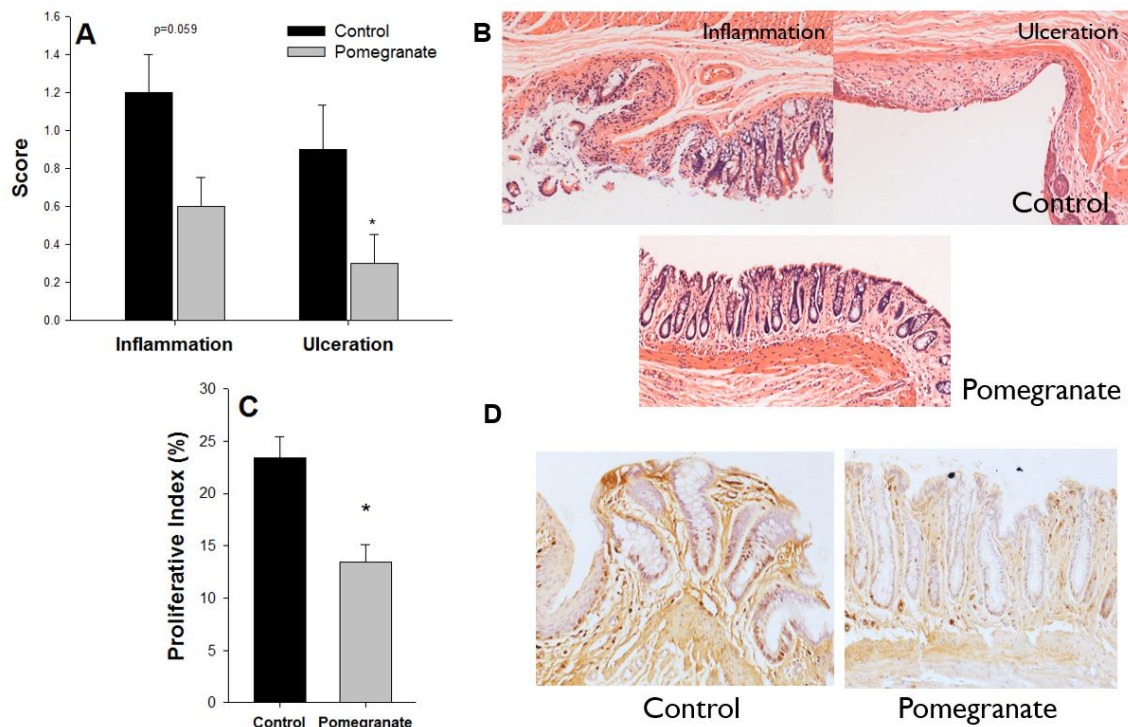
Figure 17. Effects of pomegranate juice on DSS-induced colonic inflammation and ulceration (A) and colonocyte proliferation (B) in DSS-treated rats.
Rats were fed a control juice or a pomegranate juice, and were exposed to three cycles of DSS. (A) The inflammation score was slightly decreased, and the ulceration score was significantly decreased by the pomegranate juice compared to the control juice intake in DSS-treated rats (p=0.059). Colonic inflammation and ulceration was assessed by a board-certified pathologist (C.P.) in a blinded manner. Scores were assessed on a scale of 0-3. Values are mean ± SEM (n=10 per group). (B) Pomegranate juice significantly suppressed colonocyte proliferation compared to control juice in DSS-treated rats. The measurement of Ki-67 was used to determine cell proliferation. Values are mean ± SEM (n=10 per group). *p < 0.05.

Pomegranate suppressed markers of inflammation

There is increasing evidence that ellagic acid, a dominant juice in pomegranate, targets key players in inflammation (93, 94). Based on this information, we supposed that pomegranate intake may suppress inflammation. To further quantify the effect of pomegranate juice on inflammatory markers *in vivo*, we examined the inflammatory

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cytokines, iNOS and COX-2 expression. Pomegranate juice inhibits mucosal levels of the inflammatory cytokines TNF-α and IL-1β mRNA (Figure 18A). The expression of iNOS and COX-2 mRNA and protein levels were reduced in DSS-treated rats consuming pomegranate compared to the control group in colon epithelial cells (Figure 18A and B).
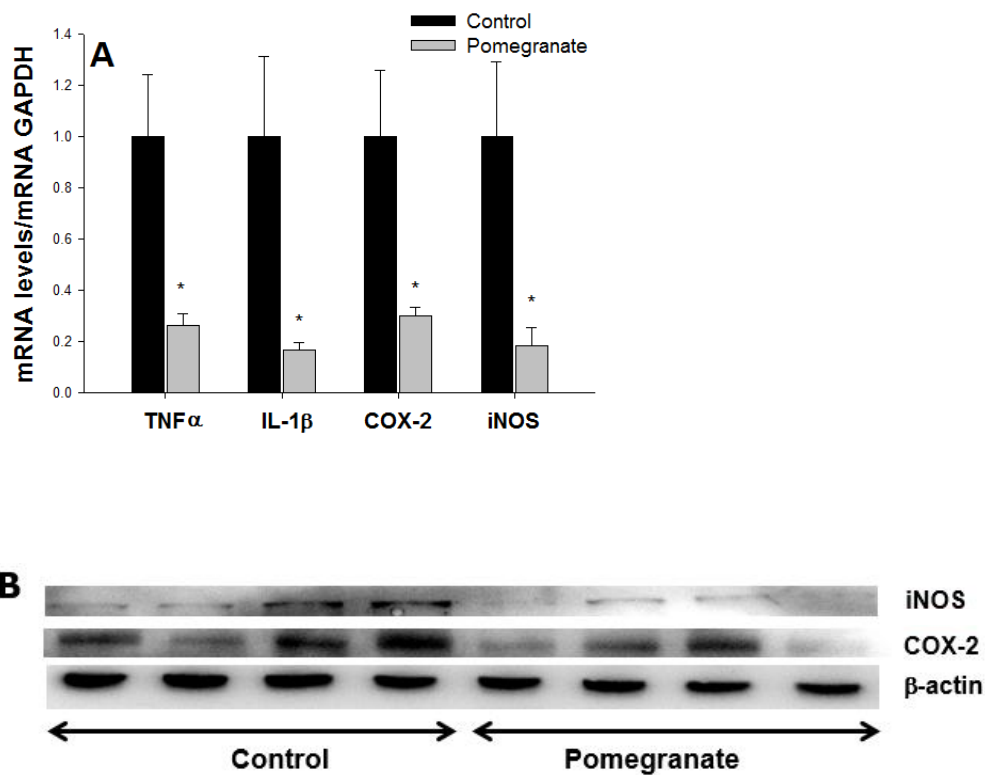
Figure 18. Effects of pomegranate juice on pro-inflammatory cytokines, COX-2 and iNOS in DSS-treated rats.
Rats were fed a control juice or a pomegranate juice, and were exposed to three cycles of DSS. Pomegranate juice decreased the expressions of (A) TNFα, IL-1β, COX-2 and iNOS mRNA in DSS-treated rats. Values are mean ± SEM (n=10 per group). *p<0.05. (B) Pomegranate juice suppressed the expression of iNOS and COX-2 protein in DSS-treated rats.

Pomegranate modulated the p70S6K1 expression and increased the expression of miR-145 in DSS-treated rats

The HIF-1α plays a central role in the regulation of cell growth, cell proliferation and inflammation in colitis. Activation of the mTOR/p70S6K1 promotes cell growth and cell proliferation via downstream transduction of proliferative signals, such as HIF-1α (57). The suppression of the HIF1-α may represent a key role in the anti-inflammatory against colitis (165). Pomegranate juice decreased the expression of mTOR, p70S6K, and HIF1-α mRNA in DSS-treated rats (Figure 19A). In addition, pomegranate juice suppressed the expression of p70S6K total protein and phosphorylated protein, RPS6 phosphorylated protein (Figure 19B and C), and HIF1-α protein levels in DSS-treated rats (Figure 19D). miR-145 is known to target p70S6K1, which in turn regulates the HIF1-α (70). We have already shown that pomegranate juice suppressed inflammation in AOM-treated rats via the induction of miR-126 expression (152). However, in this study, pomegranate juice increased the expression of miR-145 compared to control juice (Figure 19E), which may be correspondingly accompanied by suppression of p70S6K1 mRNA and protein expression in DSS-treated rats (Figure 19A, B and C).
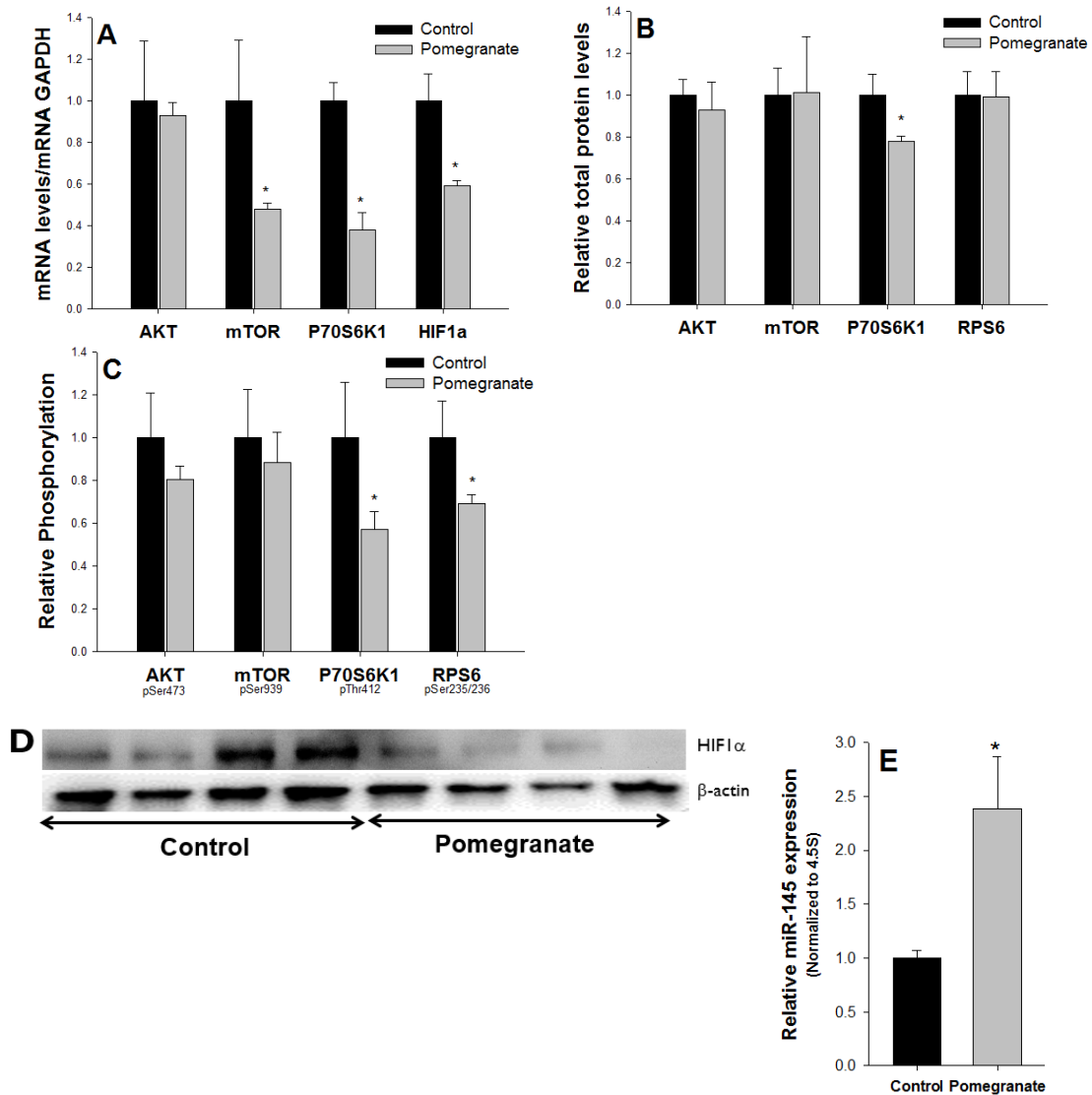
Figure 19. Effects of pomegranate juice on the mTOR signaling pathway in DSS-treated rats.

Rats were fed a control juice or a pomegranate juice, and were exposed to three cycles of DSS. (A) Pomegranate juice decreased the expression of mTOR, p70S6K1 and HIF1-α mRNA in DSS-treated rats. (B) Pomegranate juice suppressed the total protein of p70S6K1, (C) the phosphorylated protein of p70S6K1 and RPS6, (D) the expression of HIF1-α protein in DSS-treated rats. (E) Pomegranate juice increased the expression of miR-145 compared to control juice in DSS-treated rats. Values are mean ± SEM (n=10 per group). *p<0.05.

Pomegranate decreased the markers in the inflammatory and the mTOR signaling

pathway in vitro

Previously, we have shown the anticancer effects on pomegranate juice in the breast and colon cancer models (89, 152). However, the specific mechanisms of the anti-inflammatory effects of pomegranate in colitis were not elucidated. Pomegranate extract did not affect cell viability in CCD-18 cells (Figure 20A). Similar results of *in vivo* study were obtained in LPS treated CCD-18Co cell lines. Pomegranate extract suppressed the expression of inflammatory marker, iNOS mRNA at 10mg GAE/L in LPS-treated CCD-18 cells (Figure 20B). In addition, pomegranate extract also modulated the mTOR/p70S6K1/HIF1-α in LPS-treated CCD-18 cells. Pomegranate extract suppressed the expression of mTOR, p70S6K1 and HIF1-α mRNA at 10mg GAE/L (Figure 20C), and p70S6K1 and HIF1-α protein expression (Figure 20D) in LPS-treated CCD-18Co cells. Pomegranate extract (0~10mg GAE/L) also increased the expression of miR-145 in a dose-dependent manner in LPS-treated CCD-18 cells (Figure 20E).
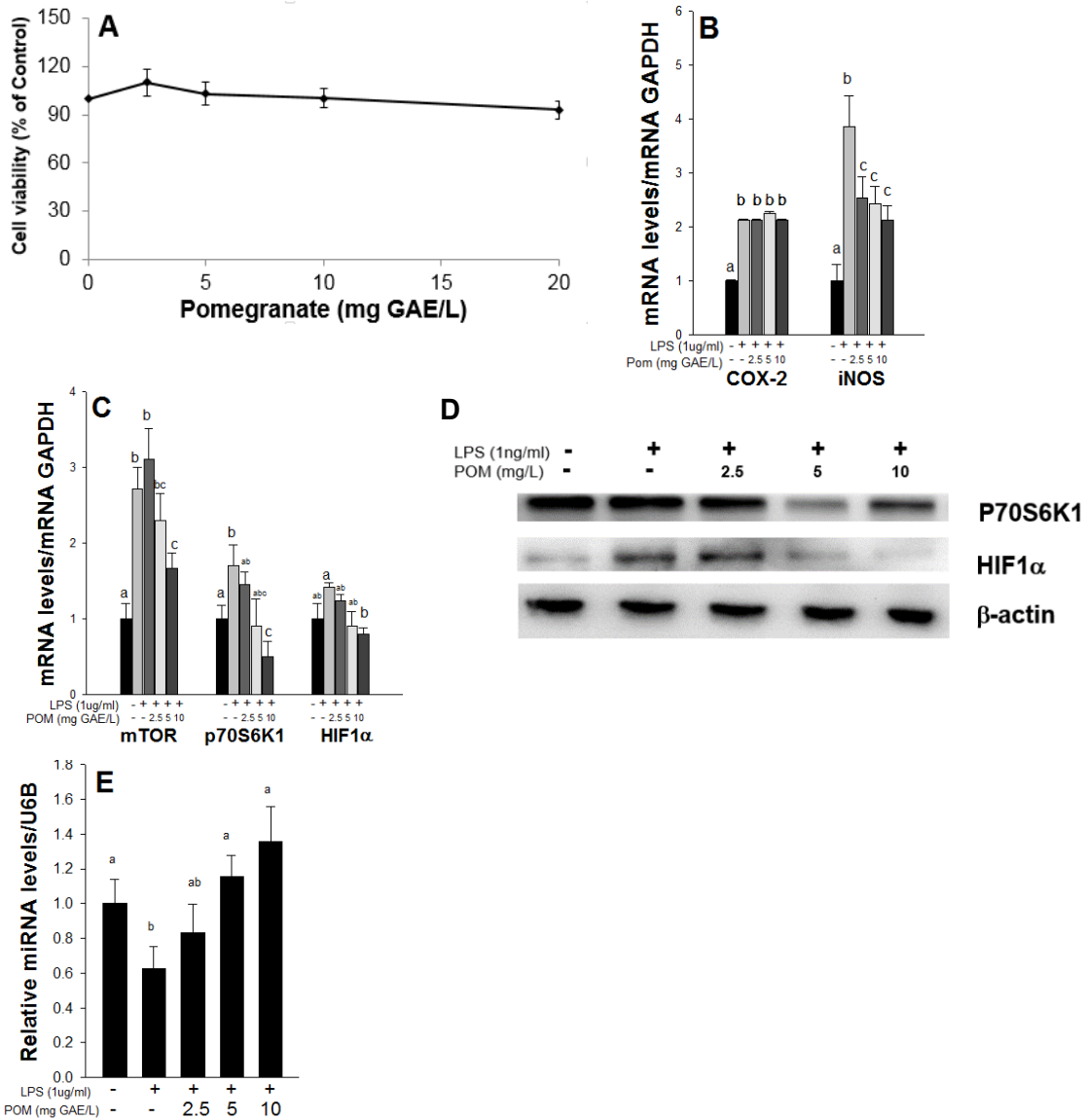
Figure 20. Effects of pomegranate extract on the inflammatory and the HIF1-α signaling pathway in LPS-treated CCD-18 cells.
(A) Pomegranate extract did not affect cell viability in CCD-18 cells. (B) Pomegranate extract suppressed the expression of inflammatory markers, iNOS mRNA in LPS-treated CCD-18 cells. (C) Pomegranate extract suppressed the expression of mTOR, p70S6K1, and HIF1-α mRNA, and the expression of p70S6K1 and HIF1-α proteins in LPS treated CCD-18 cells. (E) Pomegranate extract increased the expression of miR-145 in LPS-treated CCD-18 cells. Cells were treated different concentration of pomegranate extract (0-10 mg GAE/L) and LPS (1ug/ml) for 3h (RNA extraction) or 24h (Protein extraction). All experiments were performed at least three times, and result were expressed as mean ± SEM (n=3). Different letters indicate significance at $p<0.05$.

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The involvement of miR-145 in the Pomegranate-induced modulation of p70S6K1

In order to determine the involvement of miR-145 and its target gene, p70S6K1 in the anti-inflammatory activities of pomegranate, we investigated the further *in vitro* treatment with antagomiR of miR-145. After the transfection of miR-145 antagomiR, cells were treated with the pomegranate extract and LPS to induce inflammation. In results, pomegranate extract partially reversed the effect of miR-145 antagomiR on transfected cells (Figure 21A). The reversed expression of miR-145 was accompanied by a decreased expression of p70S6K1 mRNA (Figure 21B) and protein (Figure 21C). It indicated that miR-145 has an important role in the modulation of p70S6K1 signaling by pomegranate treatment.

Discussion

Pomegranate polyphenolics is a highly valued, functional food that has antioxidant and anti-inflammatory activities *in vivo* and *in vitro* (169, 170). It was already reported that pomegranate and its polyphenolics play an anti-inflammatory role in colitis. Ellagic acid inhibited NF-κB activity through induction of IkB phosphorylation and suppression of reduced IL-8 secretion in Caco-2 (93), and reduced colitis severity via reduction of MPO and TBARS activity in DSS-treated rats (94). Dietary pomegranate showed the anti-inflammatory properties through inhibition of MPO activity and TNF α levels in chronic TNBS-induced colitis. In addition, pomegranate decreased COX-2 and iNOS expression, and reduced MAPK pathway (p38MAPK, JNK, and ERK1/2) and NF-κB activities in the TNBS-treated rats (95, 96).

In DSS-induced colitis models, pomegranate extract reduced colitis severity via modulation of microbiota in guts (*Bifidobacterium*, *Lactobacillus*, and *Clostridium*) and decreasing COX-2, iNOS, PTGES, and PGE_2 (97).
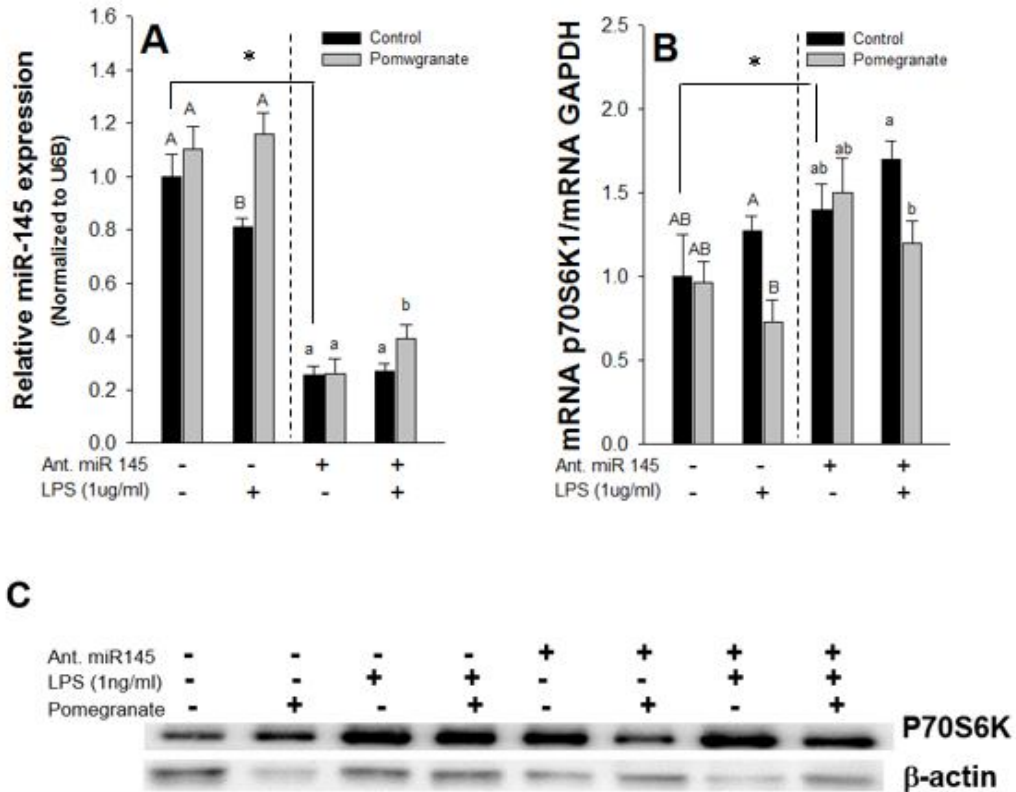
Figure 21. Effects of pomegranate extract on miR-145 and p70S6K1 mRNA levels in in LPS-treated CCD-18 cells.
CCD-18Co myofibroblast colon cells were transfected with 20nmol/ml of miR-145 antagomiR. After that, cells were treated with the pomegranate extract (10mg GAE/L) and LPS (1ug/ml) for 24h. (A) Pomegranate extract reversed the effect of miR-145 antagomiR on transfected cells. The reversed expression of miR-145 was accompanied by a decreased expression of (B) p70S6K1 mRNA and (C) protein. miR-145 was analyzed as a ratio to the miR-NU6B endogenous control, and p70S6K1 was assayed as ratio to GAPDH. All experiments were performed at least three times, and result were expressed as mean $\pm$ SEM (n=3). Different letters indicate significance at p<0.05. *p < 0.05.

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These anti-inflammatory properties of pomegranate may help protect against human cancer. In the human lung carcinoma cells and lung tumors in mice, pomegranate polyphenols down-regulated inflammation by suppressing PI3K/AKT pathway and decreased the activation of NF-κB. In addition, pomegranate extracts have shown to induce cytotoxicity and anti-inflammatory properties in colon cancer *in vitro* and *in vivo* by inhibiting PI3K/AKT pathway and the activation of NF-κB (102). However, the mechanisms underlying the anti-inflammatory activities of pomegranate against colitis is unclear. The objective of the present study is to understand the anti-inflammatory effects of pomegranate polyphenolics on colitis *in vivo* and *in vitro* through modulation of HIF1-α pathway and its post-translational modification.

Ulcerative colitis is the most common form of IBD, characterized by a chronic and relapsing inflammation of the mucosal layer of the colon. Although colitis is rarely lethal on its own, it is at high risk for developing colon cancer (40). DSS-induced colitis is a well-established model whose symptoms and colonic histopathology are phenotypically similar to human colitis (155). This model is characterized by high levels of nitric oxide produced by the induction of iNOS expression during the inflammation status. Our results showed that pomegranate juice suppressed iNOS induction caused by DSS treatment. In this study, pomegranate juice intake slightly decreased the inflammatory score, and significantly decreased the ulceration score (Figure 17A) and cell proliferative index (Figure 17C) compared to control juice in DSS-treated rats. Moreover, pomegranate juice suppressed the expressions of pro-inflammatory cytokines

(TNF-α, IL-1β), and inflammation-related proteins COX-2 and iNOS (Figure 18A, B, and 20B) in the rat colitis model and in LPS treated *in vitro* model.

HIF1-α plays a central role in the regulation of angiogenesis, cell proliferation and inflammation that lead to induce colitis (165). During colitis, HIF1-α expression was induced, and it was correlated with more severe colitis and increase in inflammatory infiltrates in colon. Furthermore, activation of HIF1-α increased expression of pro-inflammatory mediators (165), and it was known that the target genes of the transcription factor HIF-1 are VEGF, HO-1, iNOS (173) and COX2 (174). HIF-1α inhibitors have shown be effective to suppress DSS-induced colitis by inhibiting pro-inflammatory cytokines (165). The mTOR/p70S6K1 is an important signaling pathway in the regulation of inflammation via downstream transduction of HIF1-α (57). Targeted suppression of the mTOR/p70S6K1/HIF1-α during colitis by natural compounds may provide an important strategy for colitis (166).

In previous studies, pomegranate polyphenols have been shown to anti-inflammatory properties in lung and colon cancer by suppressing the PI3K/AKT pathway and decreasing the activation of NF-κB (98, 100-102). Based on these studies, we hypothesized that the inhibition of the PI3K/AKT pathway by pomegranate juice may modulate the expression of mTOR and determined whether the inhibition of inflammatory pathway by pomegranate was due in part by suppressing the mTOR pathway. In the results, pomegranate suppressed the pro-inflammatory cytokines, iNOS and COX expression. This finding agrees with previous findings where it was demonstrated that pomegranate juice decreased inflammation and suppress the COX-2

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and iNOS in DSS-induced colitis models (97). In addition, pomegranate juice reduced the expression of the p70S6K1 mRNA and protein (Figure 19A, B, C), and HIF-1α proteins in rat colitis model (Figure 19D), and this was consistent with *in vitro* studies where pomegranate extract suppressed the p70S6K1 mRNA and protein, and the HIF-1α (Figure 20C, D).

microRNAs are emerging as a potential link between inflammation and cancer due to the modulation of gene expressions by inducing mRNA degradation or blocking translation (63). The HIF-1α is a promising target by miRNAs (175). miR-145 is an colon cancer suppressor genes, and plays an important role in regulating colon cell fate (176). It was reported that miR-145 is an important regulator in the innate immune system, and loss of miR-145 increased colitis (177, 178). Our previous studies with pomegranate showed the anti-cancer properties in breast and colon cancers through miRNAs modulations. In this study, pomegranate increased expression of p70S6K1 which was accompanied by up-regulation of miR-145 *in vivo* (Figure 19E) and *in vitro* (Figure 20E). p70S6K1 is regulated by miR-145 though a target-binding site in the 3'-UTR region of the p70S6K1 mRNA . To confirm the relationship between miR-145 and p70S6K1, cells were transfected with the antagomiR for miR-145 to slightly reduce the expression of miR-145, where the effects of pomegranate were slightly reversed the effect of inhibitor (Figure 21A), accompanied with the suppression of p70S6K1 mRNA and protein (Figure 21B, C). The concentration of antagomiR (20nM) were low enough to be overcome by the effects of pomegranate. This indicates that miR-145 is involved in

the pomegranate-induced down-regulation of HIF-1α and may modulate inflammation and proliferation.

In conclusion, pomegranate juice attenuated DSS-induced colitis by suppressing the p70S6K1/HIF-1α signaling pathway in part though up-regulation of miRNA-145 expression. Interaction of pomegranate with miR-145/ p70S6K1/HIF-1α pathway was identified as the underlying mechanisms that is, at least in part, involved in the anti-inflammatory of pomegranate juice, and pomegranate may be a potential therapeutic agent for colitis.

CHAPTER IV

COMPARISON OF ANTI-INFLAMMATORY MECHANISMS OF MANGO

(MANGIFERA INDICA L.) AND POMEGRANATE (PUNICA GRANATUM L.) IN

DSS-INDUCED COLITIS

Summary

Polyphenolics from mango (gallic acid, galloyl derivatives, flavonol glycosides and benzophenone derivatives), and pomegranate (ellagic acid, ellagitannins, flavonoids, and anthocyanins) have been shown to have potent antioxidant and anti-inflammatory properties. Ulcerative colitis, a chronic inflammation of the large intestine, may increase risk of human colorectal cancer. Polyphenolics have been shown to suppress inflammation through different mechanisms, including inhibition of the mTOR signaling pathway.

To determine the anti-inflammatory effects and possible mechanisms of mango and pomegranate juice in DSS-induced colitis in rats, Sprague Dawley (SD) rats were administered control juice (15.7g sugar and 0.05g citric acid/100ml), mango juice (Total phenolic content of 475.80mg/L GAE), or pomegranate juice (Total phenolic content of 2698.99mg/L GAE), and were exposed to three cycles of 3% dextran sodium sulfate (DSS) in the drinking juice followed by 2-week recovery period. Colon inflammation and ulceration scores were assessed. The levels of protein involved in the AKT/mTOR pathway were analyzed by multiplex bead assay, while the transcriptional variation with

the mTOR pathway were analyzed by low density PCR arrays. In addition, the levels of several miRNAs involved in the mTOR pathway were measured.

Both mango and pomegranate juice reduced DSS-induced colon inflammation during chronic colitis in rats compared to control juice. Mango juice suppressed the IGF-1R-AKT/mTOR signaling axis via up-regulation of miR-126 and down-regulation of IR, while pomegranate decreased p70S6K by up-regulating miR-145 and down-regulation of the MEK-ERK1/2 pathway.

These results suggest that polyphenolics of different predominant structure may differentially regulate inflammation-involved pathways while attenuating DSS-induced colitis. For the further study, there may be possible synergistic interactions between both polyphenolics classes in the reduction of inflammation.

Introduction

Ulcerative colitis (UC) is a chronic relapsing inflammatory condition in the colon. Active immune cells destroy the intestinal barrier through releasing reactive oxygen species (ROS), and pro-inflammatory cytokines (2). 1.4 million people in the United States and 2.2 million people in Europe suffer from these diseases (3). The most serious complications of UC is high risk of developing human colorectal cancer (40). The risk of colorectal cancer increased with duration of the disease with a cancer risk of 2% by 10 years, 8% by 20 years and 18% by 30 years, and it has high mortality (> 50%) (41, 42). Therefore, it is important to identify the mechanisms underlying suppression of the intestinal inflammation.

The mTOR pathway may play a key role during ulcerative colitis (60-62). Once PI3K is activated by insulin, IGF-1, or TLR4, it starts a signal transduction cascade via the AKT/mTOR pathway. mTOR exists in two complexes within the cell: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Activation of mTORC1 regulates protein synthesis (HIF1α), cell growth, and cell proliferation via downstream transduction of proliferative signals, such as 4E-BP1 and p70S6K (57). The transcription factor HIF-1 has target genes, such as VEGF, HO-1, iNOS (173) and COX2 (174). This pathway is a promising target of miRNAs for anti-colitis and anti-cancer therapeutics (68). Targeted suppression of these activated pathways during colitis using natural compounds provides an important strategy for colitis and colon cancer prevention.

Mango is rich in polyphenols, such as gallic acid and gallotannins. It was reported that mango extract has anti-inflammatory properties via reduction of iNOS, COX-2, TNFα, and TNFR-2 in a DSS-induced model of colitis (84). Pomegranate is rich in ellagic acid, ellagitannins, flavonoids, and anthocyanins. Pomegranate showed the anti-inflammatory properties through suppression of COX-2 and iNOS expression, MAPK pathway (p38MAPK, JNK, and ERK1/2), and NF-κB activities in chemically-induced colitis (95, 96). However, the mechanisms underlying anti-inflammatory activities of polyphenolics is unclear yet.

It was reported that different classes of polyphenolics may have different anti-inflammatory activities, possibly due to the structural variations or the different cellular targets (179). In the above described studies with DSS-induced colitis, we were shown that mango and pomegranate juice differently modulated the mTOR pathway. The

results suggest that juice of different structure may differentially regulate inflammation-involved pathways while attenuating DSS-induced colitis.

Proteomic and gene expression analysis allows us to figure out the potential therapeutic target in prevention of inflammatory disease and to elucidate the underlying mechanisms how the polyphenolics ameliorate the inflammatory response (180). In addition, we can compare the gene expression changes altered by different polyphenolics intake in vivo models for investigating the mechanisms underlying the anti-inflammatory activities. Therefore, in this study, we determined how mango and pomegranate juice influence colonic inflammation, proteomics and mucosal gene expression involved in the mTOR pathway. In addition, we have compared the underlying mechanisms altered by mango and pomegranate juice.

Materials and methods

Fruit preparation

Mango (Keitt, Mexico) was homogenized and centrifuged for obtaining the experimental mango juice. After that, the supernatant was stored at -20°C to prevent oxidation. Pomegranate was purchased from the POM wonderful (Los Angeles, CA). The concentration of total polyphenolics were mango juice (475.90mg GAE/L) and pomegranate juice (2698.99mg GAE/L). The control juice was adjusted its calorie and pH to Brix 15.7 and pH 3.4 (Table 5).

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Table 5. The nutritional analysis for the experimental juices.

	Control juice	Mango juice	Pomegranate juice
Brix	15.7	15.6	15.8
pH	3.4	3.3	3.5
Total polyphenolics (mg GAE/L)		475.90	2698.99
Vitamin C (mg/L)		6.13	10.17

Animal treatment and tissue sampling

DSS-induced colitis is an excellent preclinical model of colitis that exhibits many phenotypic characteristics such as epithelial homeostasis, regeneration and wound healing, relevant to the human disease (127, 148). This model is a valuable tool for investigating involvement of dietary factors into the pathogenesis of IBD and evaluate different therapeutic options (128). Ten week-old Sprague-Dawley rats obtained from Harlan Teklad (Houston, TX) were acclimated for a week and randomly grouped (n=10) by weight into control, mango and pomegranate juice groups. After 3 weeks of rat chow pellets and experimental juices *ad libitum*, all rats were administered 3% (w/v) DSS (MP Biomedicals, Solon, OH) (3 cycles, 14 d separation, n=10/diet) in the experimental juices for 48 h. Rats were sacrificed 2 weeks after three times of DSS administration, and the entire colon was removed. One centimeter sections were cut from the distal end of each rat colon, and fixed in 4% paraformaldehyde (PFA) for embedding in paraffin. The remained colon was gently scraped for collecting protein and RNA samples (149). The animal use protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Histological scoring

For histopathology analysis, sections were stained with hematoxylin and eosin (H&E). Slides were examined by a board-certified pathologist (C.P.) in blinded manner for accessing the degree of inflammation (score, 0-3) and ulceration (score, 0-3) (150). The presence of rare inflammatory cells in the lamina propria was scored as 0; increased number of inflammatory cells was scored as 1; predominant inflammatory cells as 2; and the inflammatory infiltrate as 3 (181). For the evaluation of ulceration, no damage was scored as 0; One linear ulcer <1cm length was scored as 1; Two linear ulcer <1cm length was scored as 2; More sites of ulceration or one large ulcer >1cm was scored as 3 (172).

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 mTOR signaling (Akt, GSK3β, GSK3α, IGF1R, IR, IRS1, mTOR, p70 S6 kinase, PTEN, RPS6, and TSC2) using the multiplex kits (Millipore, Billerica, MA) according to the manufacturer's protocol. Multiplex analysis was performed by the Luminex L200 instrument (Luminex, Austin, TX), and data was analyzed by Luminex xPONENT software.

miRNAs analysis

miRNAs were isolated from scraped mucosa using the mirVana™ miRNA Isolation Kit (Applied Biosciences, Foster City, CA) by following the manufacturer's protocol. Quantifications of miRNAs relevant to the mTOR pathway, such as Let7a,

miR-21, miR-126, miR-143, miR-145, and miR-155, were measured using the Taqman MicroRNA reverse Transcription kit (Applied Biosystems, Foster City, CA). The miR-4.5S was used as endogenous control for microRNAs expression (89).

Low density PCR array (mTOR and MAPK pathways)

mRNA (400ng) was converted to cDNA by using a RT_2 First-strand cDNA synthesis kit (Qiagen, Valencia, CA). RT-PCR for mTOR and MAPK pathways were performed by using RT_2-Profiler PCR array (n=5/group; Qiagen, Valencia, CA) by using ABI 7900HT PCR system (Applied biosystems, Foster City, CA). The low density PCR array takes advantages of SYBR Green-based RT-PCR and microarray. Each plate contains gene specific primer sets for 84 relevant pathway genes. Analysis of the results was performed by the zRMicroArray. Only genes that passed the significance test ($p<0.05$) were considered.

Statistical analysis

Data were expressed as means with their standard errors. 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) or ANOVA R-package with is embedded in zRMicroArray. P values <0.05 were accepted as significant.

Results

Pomegranate but not mango juice protected against intestinal ulceration in DSS treated rats

To explore the effects of mango and pomegranate juice on DSS-induced colitis, rats were treated with three cycles of 3% DSS for 48h, and colonic inflammation and ulceration were assessed. Inflammation and ulceration scores represent the degree of immune cell infiltration and ulceration, respectively. Mango and pomegranate juice slightly decrease colonic inflammation scores (p=0.062 and 0.059). With respect to the ulceration score, the pomegranate juice significantly decreased ulceration score (p<0.05) compared with control (Figure 22). This data suggest that mango and pomegranate may affect the pathology of DSS-induced colitis by modulating immune cell infiltration. In addition, pomegranate juice showed more protective effects compared to mango juice.
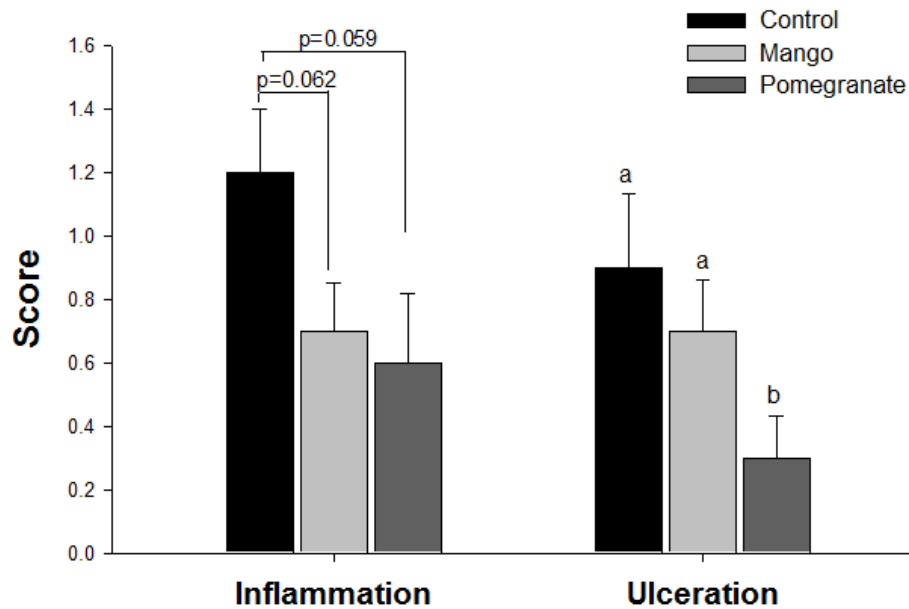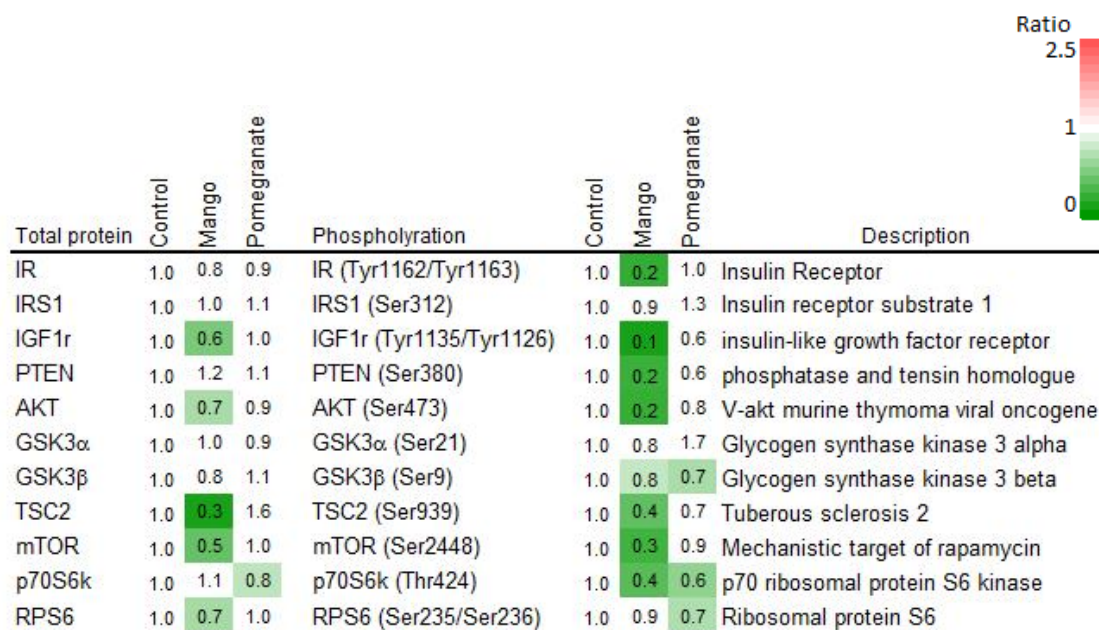
Figure 22. Effects of mango and pomegranate juice on DSS-induced colonic inflammation and ulceration in rats.
Rats were fed a control, mango, or pomegranate juice, and were exposed to three cycles of DSS. The inflammation scores were slightly decreased by the experimental juice intake compared to the control juice intake (p=0.062 and 0.059). The ulceration scores were significantly reduced by pomegranate juice. Colonic inflammation and ulceration was assessed by a board-certified pathologist (C.P.) in a blinded manner. Scores were assessed on a scale of 0-3. Values are mean ± SEM. Different letters indicate significant differences among groups (n=10; p<0.05).

The mTOR pathway may play an important role in colitis (60-62). The pathway is activated by hormones, mitogens and growth factors, and the activation may regulate protein synthesis, cell growth, and cell proliferation (57). Mango juice decreased the expression of IGF1R, AKT, TSC2, mTOR, and RPS6 total protein, and the expression of IR, IGF1R, PTEN, AKT, GSK3β, TSC2, mTOR, and p70S6K phosphorylated protein. Meanwhile, pomegranate juice decreased the expression of p70S6K total protein, and the expression of GSK3β, p70S6K, and RPS6 phosphorylated protein (Figure 23).

Total protein	Control	Mango	Pomegranate	Phospholyration	Control	Mango	Pomegranate	Description
IR	1.0	0.8	0.9	IR (Tyr1162/Tyr1163)	1.0	0.2	1.0	Insulin Receptor
IRS1	1.0	1.0	1.1	IRS1 (Ser312)	1.0	0.9	1.3	Insulin receptor substrate 1
IGF1r	1.0	0.6	1.0	IGF1r (Tyr1135/Tyr1126)	1.0	0.1	0.6	insulin-like growth factor receptor
PTEN	1.0	1.2	1.1	PTEN (Ser380)	1.0	0.2	0.6	phosphatase and tensin homologue
AKT	1.0	0.7	0.9	AKT (Ser473)	1.0	0.2	0.8	V-akt murine thymoma viral oncogene
GSK3α	1.0	1.0	0.9	GSK3α (Ser21)	1.0	0.8	1.7	Glycogen synthase kinase 3 alpha
GSK3β	1.0	0.8	1.1	GSK3β (Ser9)	1.0	0.8	0.7	Glycogen synthase kinase 3 beta
TSC2	1.0	0.3	1.6	TSC2 (Ser939)	1.0	0.4	0.7	Tuberous sclerosis 2
mTOR	1.0	0.5	1.0	mTOR (Ser2448)	1.0	0.3	0.9	Mechanistic target of rapamycin
p70S6k	1.0	1.1	0.8	p70S6k (Thr424)	1.0	0.4	0.6	p70 ribosomal protein S6 kinase
RPS6	1.0	0.7	1.0	RPS6 (Ser235/Ser236)	1.0	0.9	0.7	Ribosomal protein S6

Ratio: 2.5 — 1 — 0

Figure 23. Proteomic profiling altered by mango and pomegranate juice in DSS treated rats.
Rats were fed a control, mango, or pomegranate juice, and were exposed to three cycles of DSS. The intensity of the green color indicated the degree of down-regulation (green, low abundance; white, not significant; n=10 per group; $p<0.05$).

MicroRNAs targeting the mTOR pathway were altered by mango and pomegranate juice

miRNAs are potential post-transcriptional regulators for the regulation of inflammatory and immune responses (63). The mTOR pathway is a promising target of miRNAs for anti-colitis and anti-cancer therapeutics (68). Moreover, polyphenolics are known to modulate post-transcriptional regulation by regulating the expression of miRNAs (71). Mango juice significantly increased the expression of Let7a, miR-126, and miR-143, while pomegranate juice decreased the expression of miR-126 and miR-143 compared to the levels of the control group. In addition, pomegranate increased the miR-145 levels (Figure 24). miR-126 targets PI3Kp85β, an upstream component of the mTOR pathway (64), and miR-145 targets p70S6K1, a downstream target of the mTOR pathway (70). Increased miR-126 and miR-145 by juice could provide a rationale for the suppression of the mTOR pathway.
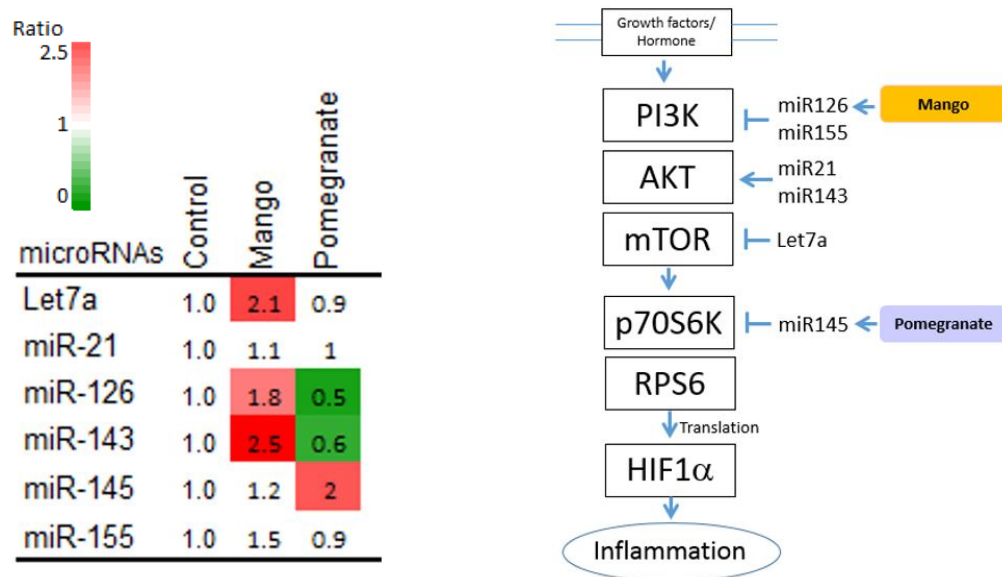
Figure 24. miRNAs expression altered by mango and pomegranate juice in DSS treated rats.

Rats were fed a control, mango, or pomegranate juice, and were exposed to three cycles of DSS. Mango juice increased the expression of Let7a, miR-126, and miR-143, and pomegranate juice decreased the expression of miR-126, miR143, and increased miR-145 compared to control juice in DSS treated rats. A green–red gradient represents abundance differences (green, low abundance; red, high abundance; white, not significant; n=10 per group; $p<0.05$).

Mango and pomegranate modulate mucosal gene expression involved in the mTOR

pathway

Gene expression analysis of mTOR pathway was determined by comparing the juice groups with control group. The mTOR pathway alteration by mango and pomegranate juice were compared in order to elucidate the different effects of different polyphenolics contributing the anti-inflammation. Among 84 genes involved in the mTOR pathway, each six genes had a significant differential expression by mango and pomegranate juice ($p < 0.05$). Of these, *Pld1, Ppp2r2b*, and *Tp53* genes were overlapped. The expression of *Ppp2r2b* and *Tp53* were up-regulated, while *Pld1* expression was down-regulated by mango and pomegranate juice. The gene expression of *Igf1, Insr*, and *Pik3cd* genes were down-regulated by mango juice, and *Rps6ka2*, *Rragd*, and *Vegfc* were differentially expressed by pomegranate juice (Table 6 and Figure 25).

Table 6. Differentially expressed genes in the mTOR pathway by mango and pomegranate juice.

Symbol	Gene name	GenebankID	Relative expression	p-value	Relative expression	p-value
			Mango		**Pomegranate**	
Ppp2r2b	Protein phosphatase 2 (formerly 2A), regulatory subunit B, beta isoform	NM_022209	2.993	0.026	2.792	0.037
Tp53	Tumor protein p53	NM_030989	2.585	0.045	2.367	0.049
Pld1	Phospholipase D1	NM_030992	0.342	0.049	0.350	0.044
Igf1	Insulin-like growth factor 1	NM_178866	0.573	0.038		
Pik3cv	Phosphoinositide-3-kinase, catalytic, beta polypeptide	NM_053481	0.351	0.045		
Insr	Insulin receptor	NM_017071	0.608	0.047		
Vegfc	Vascular endothelial growth factor C	NM_053653			0.428	0.007
Rps6ka2	Ribosomal protein S6 kinase polypeptide 2	NM_057128			0.455	0.035
Rragd	Ras-related GTP binding D	NM_001106641			2.241	0.040
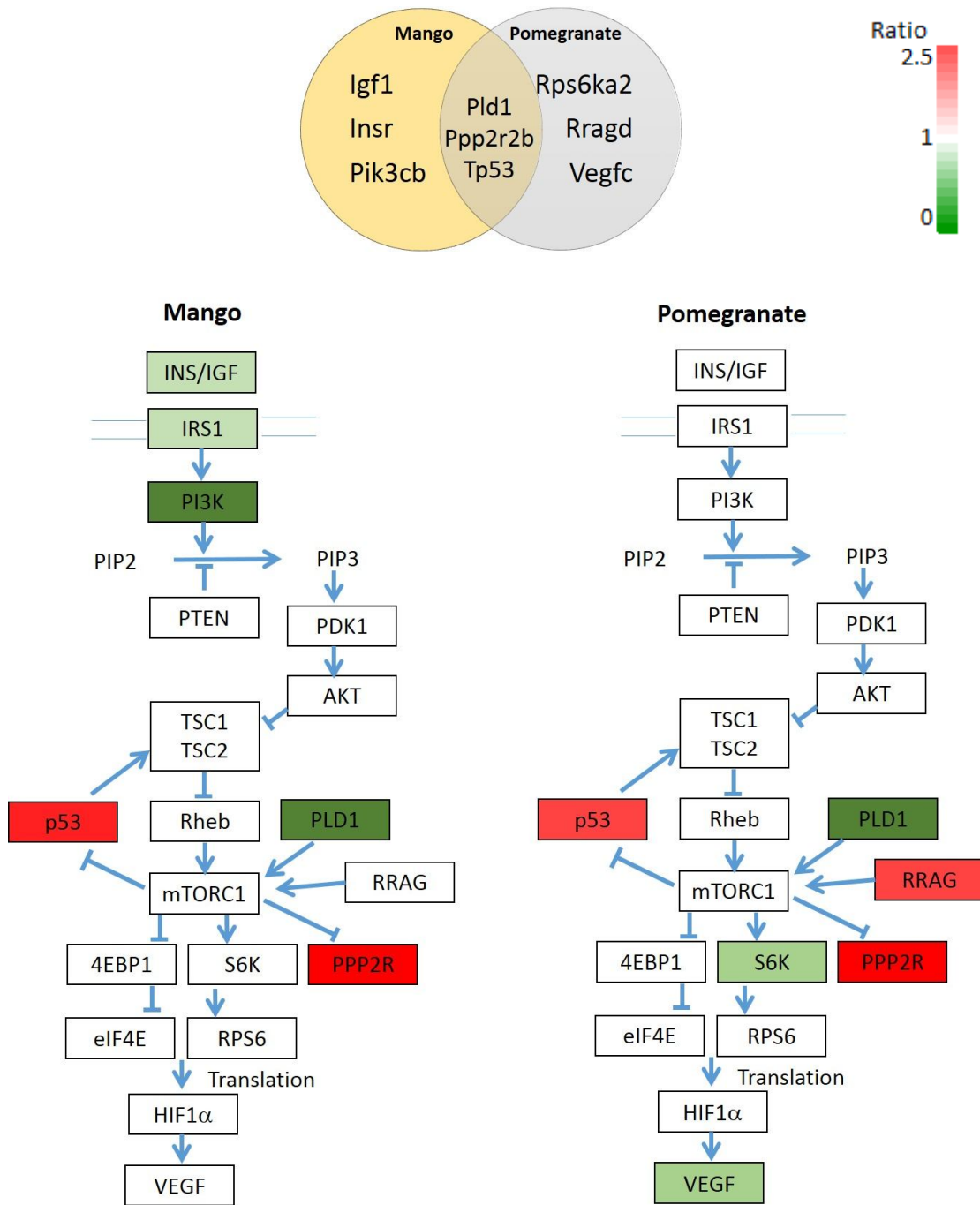
Figure 25. Differentially expressed genes involved in the mTOR signaling altered by mango or pomegranate juice.
Venn diagram categorized the differentially expressed gene sets. A green–red gradient represents abundance differences (green, low abundance; red, high abundance; white, not significant; n=5 per group; p<0.05).

Mango and pomegranate modulate mucosal gene expression involved in the MAPK

pathway

The MAPK pathway is an important regulator of the mTOR pathway. The MAPK pathway was reported to promote mTORC1 activity independently of the PI3K/AKT pathway (182). Gene expression analysis of the MAPK pathway was also determined by comparing the juice groups with control group. Among 84 genes involved in the MAPK pathway, seven and eight genes had a significant differential expression by mango and pomegranate juice, respectively (p<0.05). Of these, *Ccne1, Map3k2, Pak1, and Tp53* genes were overlapped. The expression of *Ccne1, Map3k2,* and *Pak1* were up-regulated, while *Tp53* expression was up-regulated by mango and pomegranate juice. The gene expression of *Mapk9, Mapk10,* and *Rac1* genes were down-regulated by mango juice, while *Cdc42, Map2k2, Mapk1,* and *sfn* were down-regulated by pomegranate juice (Table 7 and Figure 26).

Table 7. Differentially expressed genes in the MAPK pathway by mango and pomegranate juice.

| Symbol	Gene name	GenebankID	Relative expression	p-value	Relative expression	p-value
			Mango		**Pomegranate**	
Ccne1	Cyclin E1	M_00110082	0.460	0.022	0.532	0.023
Pak1	P21 protein (Cdc42/Rac)-activated kinase 1	NM_017198	0.456	0.042	0.480	0.044
Tp53	Tumor protein p53	NM_030989	3.991	0.045	3.093	0.026
Map3k2	Mitogen activated protein kinase kinase kinase 2	NM_138503	0.570	0.047	0.639	0.032
Rac1	Ras-related C3 botulinum toxin substrate 1	NM_134366	0.489	0.017		
Mapk10	Mitogen activated protein kinase 10	NM_012806	0.445	0.036		
Mapk9	Mitogen-activated protein kinase 9	NM_017322	0.564	0.049		
Map2k2	Mitogen activated protein kinase kinase 2	NM_133283			0.495	0.027
Cdc42	Cell division cycle 42 (GTP binding protein)	NM_171994			0.499	0.030
Mapk1	Mitogen activated protein kinase 1	NM_053842			0.432	0.036
Sfn	Stratifin	XM_232745			0.443	0.037
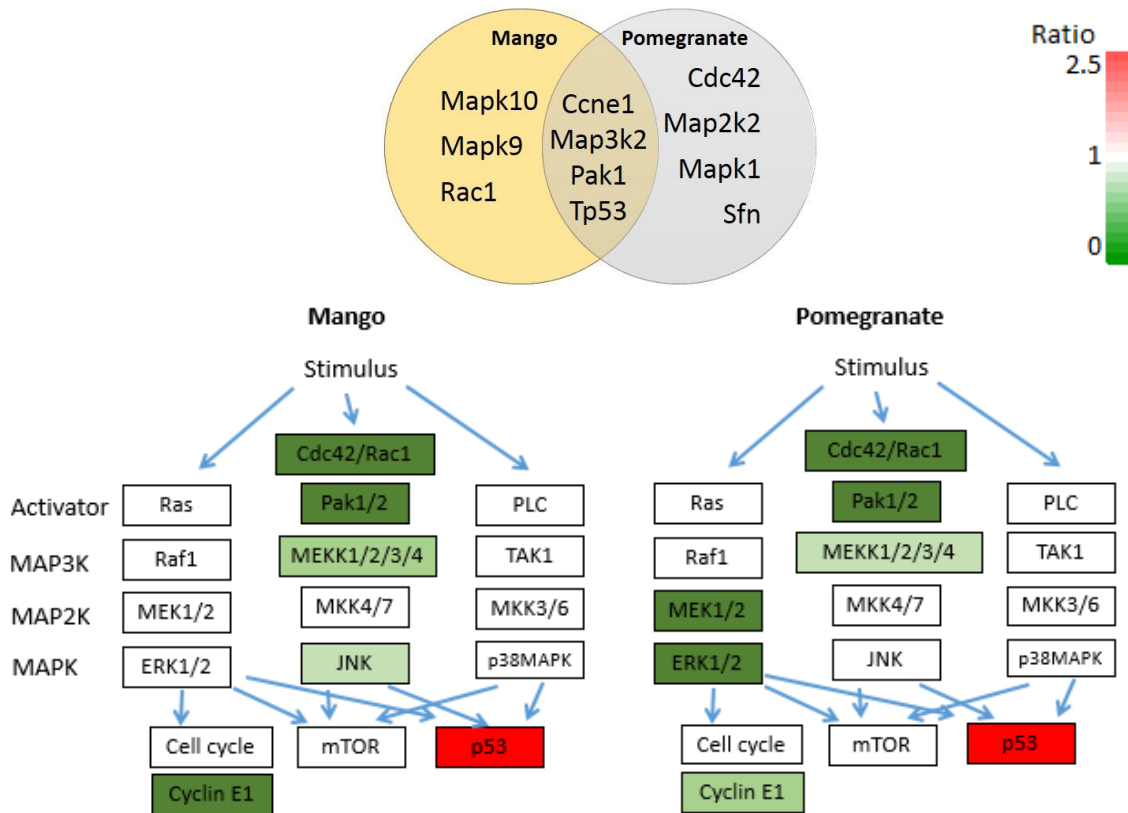
Figure 26. Differentially expressed genes involved in the MAPK pathway altered by mango or pomegranate juice.
Venn diagram categorized the differentially expressed gene sets. A green–red gradient represents abundance differences (green, low abundance; red, high abundance; white, not significant; n=5 per group p<0.05).

Confirmatory analysis of proteomic and gene expression analysis

To define an appropriate measure of similarity for gene expression patterns and total and phosphorylated protein levels, it was verified the results from the low density array with multiplex total and phosphorylated protein assay. In the correlation between total protein and mRNA and phosphorylated protein and mRNA, the R^2 values were $R^2=0.5956$ and $R^2=0.0906$ respectively (Figure 27).
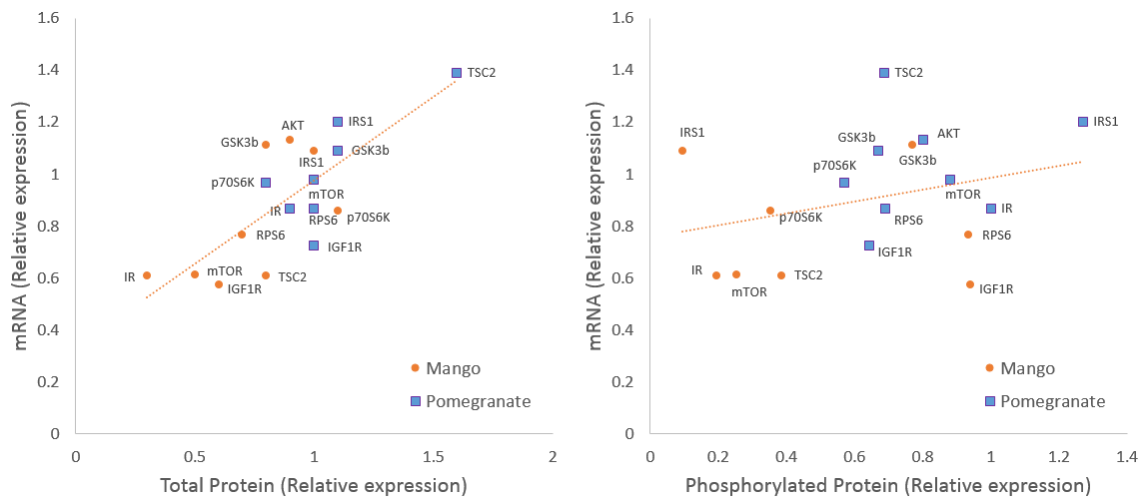
Figure 27. Correlation analysis of total or phosphorylated protein abundance and mRNA expression.
Data are expressed as the ratio of the experimental juice groups relative to the control group ($R^2=0.5956$ and 0.0906).

Discussion

Dietary mango and pomegranate polyphenolics have received substantial attention for their antioxidant and anti-inflammatory effects (84, 95, 96). In this study, we compared the anti-inflammatory properties of two bioactive compounds on experimental colitis in rats. Ulcerative colitis, the most common form of IBD, is

characterized by a chronic and relapsing inflammation of the mucosal layer of the large intestine, which includes the colon and rectum (5). It was shown that different classes of flavonoids may have different effects on inflammation at mRNA and protein level, possibly due to substantial structural differences (179) . In the above described studies with DSS-induced colitis, mango and pomegranate polyphenolics differently modulated the anti-inflammatory mechanisms. The results suggest that polyphenolics of different structure may differentially regulate inflammation-involved pathways while attenuating DSS-induced colitis. In this comparative study, we compared the mechanisms altered by mango and pomegranate juice involved in modulation of the mTOR pathway using proteomic and gene expression analysis.

Results demonstrate that both mango and pomegranate juice slightly decreased colonic inflammation scores, where only pomegranate juice significantly decreased the ulceration score compared to the control juice. This may be based on two reasons, a) difference in juice concentrations (475.90 and 2698.99mg GAE/L) and b) difference in predominant juice structure (gallotannins and ellagitanins).

The mTOR pathway plays an important role in colitis (60, 62, 147). Suppression of the mTOR pathways during colitis using natural compounds may provide an important strategy in the prevention of colitis. Our data show that mango juice decreased the up and downstream components of the mTOR pathway in the colonic mucosa, while pomegranate juice only decreased the downstream targets of the mTOR pathway (Figure 23, 25 and Table 6) even though the concentration of pomegranate juice was higher. In addition, the insulin/IGF-1 signaling pathway might be the main target of mango juice.

The insulin/IGF-1 receptor activates a number of signaling transduction pathway, including the PI3K/AKT pathway during colitis (183). Mango juice may suppress the insulin receptor, and modulate dependent intracellular signaling pathways and gene expression.

Polyphenolics are known to modulate post-transcriptional regulation by regulating the expression of miRNAs (71). The mTOR pathway is an important target of miRNAs in anti-colitis and anti-cancer therapeutics (68). Mango juice may suppress the AKT/mTOR signaling via up-regulation of miR-126 and Let7a, while pomegranate decreased p70S6K by up-regulating miR-145 (Figure 24).

Initially, we hypothesized that pomegranate juice may suppress inflammation at least in part through down-regulation of the mTOR pathway as mango juice did. However, the hypothesis was only partially acceptable (Figure 23, 25). In this study, pomegranate juice did not affect the activators of the mTOR pathway, however decreased the down-stream modulators of mTOR-regulated kinases involved in cell proliferation and inflammation. For this reason, alternative pathways involved in the regulation of mTOR-dependent kinases such as the MAPK pathway were investigated using gene expression. Previously, dietary pomegranate showed anti-inflammatory activities through inhibition of MAPK pathway (p38MAPK, JNK, and ERK1/2) in chronic TNBS-induced colitis (95, 96). Like the PI3K/AKT axis, the MAPK pathway is also a key modulator of mTOR pathway. Results indicate that low density arrays relevant to the MAPK pathway revealed that pomegranate juice may down-regulate the gene expressions involved in the MEK-ERK1/2 signaling compared to the control group

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(Figure 26). ERK1/2 is a regulator of p70S6K (184), and the suppression of ERK1/2 by pomegranate juice may contribute to the down-regulation of p70S6K. Results also show that both, mango and pomegranate juice down-regulated Cyclin E1 and up-regulation of P53 and that this may contribute to their anti-inflammatory functions (Figure 26).

When performing correlation-analysis between proteomic and gene expression analysis, the correlation between total protein and mRNA and phosphorylated protein and mRNA were $R^2=0.5956$ and $R^2=0.0906$ respectively. This may indicate that juice reduce inflammation mainly at the level of post-transcriptional modification. Gene expression analysis is a fast and cheap large-scale sequencing compared to proteomic assay. However, although gene expression analysis provides a critical information addressing the anti-inflammatory mechanisms of botanical compounds, proteomic assay may be more relevant to biological functions due to post-transcriptional modification (185).

In conclusion, data show that mango and pomegranate juice differentially modulate the mTOR pathway in DSS-induced colitis in rats. Mango juice appear to inhibit the entire IGF-1-PI3K/AKT-mTOR axis, while pomegranate juice regulate the downstream of the mTOR pathway through the reduction of ERK1/2. Results indicate further that there may be possible synergistic interactions between both juice classes in the reduction of inflammation.

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CHAPTER V

MANGO AND POMEGRANATE POLYPHENOLICS IN THE MODIFICATION OF

MICROBIOTA AND SHORT CHAIN FATTY ACIDS IN DSS-INDUCED COLITIS

Summary

Dysregulation of the interactions between gut microbiota and the immune system seems to be involved in triggering inflammation. Gut microbiota can be modulated by dietary compounds such as fiber and polyphenolics. To evaluate the effects of mango and pomegranate polyphenolics on fecal microbiota and short chain fatty acid (SCFAs) production, Sprague Dawley (SD) rats were administered control, mango, or pomegranate juice, and were exposed to three cycles of 3% dextran sodium sulfate (DSS) in the drinking juice followed by 2-week recovery period. Pro-inflammatory cytokines were analyzed in serum and mucosal samples. Fecal samples were collected one week before the end of the study to characterize the fecal microbiota composition using selected representative genus by qPCR and to measure SCFAs concentration. The mRNA levels involved in host energy metabolisms were assessed in colonic mucosa by RT-PCR.

Mango and pomegranate juice decreased the level of pro-inflammatory cytokines, such as IL-1b and GM-CSF in serum, and induced the level of IL-10. Pomegranate juice induced compositional changes in the fecal microbiota. In particular, the anti-inflammatory *Ruminococcaceae* significantly was increased by pomegranate juice in the feces but the concentrations of SCFAs were not significantly affected. In

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response to the mango juice, fecal SCFAs isovalerate and valerate were increased, while there was no significant change in the composition of the intestinal microbiota at the genus level. Polyphenolics decreased the expression of the TLR4 receptor that is triggered by lipopolysaccharide expressed in the membrane of gram negative bacteria, and mango juice also increased the GPR43 receptor which is a receptor for SCFAs. In addition, among the genes related energy metabolism and autophagy, autophagy marker Light chain 3 (*lc3*) and Lactate dehydrogenase A (*ldha*) that convert pyruvate to lactate was up-regulated while Monocarboxylate transporter 4 (*slc16a3*), which is lactate and butyrate transporter, was down-regulated by mango and pomegranate juice. In summary, mango juice induced changes in the SCFAs production while pomegranate juice induced changes in the composition of microbiota associated with the SCFAs production, however pomegranate did not alter the concentrations of SCFAs themselves.

Introduction

Ulcerative colitis (UC) is a kind of inflammatory bowel disease (IBD), which is characterized by chronic inflammation of the colonic mucosa. The development of this disease is affected by four factors: genetic susceptibility, intestinal microbiota, immune dysregulation, and environmental factors (e.g., smoking and diets high in fat and sugar) (3-5). The microbiota are integral in triggering and enhancing inflammation. For example, germfree IL-10 gene deficient mice had no evidence of intestinal inflammation or colitis (12).

There are more than 10^{14} microbiota, from more than 1000 species, in the gastrointestinal tract, mostly in the colon. Microbiota activate the immune system to produce reactive oxidative stress (ROS) or cytokines, and they are involved in the development of inflammatory disease and colon cancer. Gram-negative bacteria (*Bacteroides, Fusobacterium*, and *Escherichia coli*) containing lipopolysaccharide (LPS) partly though the induction of the toll-like receptors (TLR)s/MyD88/NF-κB signaling pathway (18) and overproduction of pro-inflammatory cytokines, such as TNF-α and IL-6 (19, 20). *Bifidobacterium* and *Lactobacillus* are considered to be probiotics that induce health benefits, and exert anti-inflammatory properties through inhibition of NF-κB activation and IL-8 secretion (21). Other gram-positive bacteria (*Ruminococcaceae, Blautia, Faecalibacterium,* and *Streptococcus*) were also shown to have anti-inflammatory effects, as the production of SCFAs blocked NF-κB activation and the induction of IL-10 (22).

SCFAs are a sub-group of fatty acids with 2 to 6 carbons, primarily acetate, propionate, butyrate, and valeric acid (23). SCFAs are metabolites produced from undigested carbohydrates, fibers, proteins and polyphenols by protective microbiota, and they seem to be beneficial in the suppression of inflammatory responses and UC (24). SCFAs, especially butyrate bind the G-protein coupled receptor 43 (GPR43) or GRP41 (FFAR3), and suppress inflammatory responses, as it decreases the production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-12, IL-17, inducible protein (IP)-10), increases the production of anti-inflammatory cytokine IL-10, and suppresses the activation of various immune cells such as macrophages, eosinophil, T cells, B cells, and

dendritic cells (24, 26). SCFAs might play a key role in the prevention and treatment of colitis.

Most polyphenolics pass from the small intestine to the colon, which indicates the importance of the colon in polyphenolics. Hydrolyzable tannins such as Gallotannins and ellagitannins are hydrolyzed by gut microbiota and then can be absorbed in the colon (186). It has been reported that polyphenolics and their metabolites influence the composition and function of the gut microbiota (107). The metabolism of the gut microbiota on polyphenolics may modify the immune response and the potential health effects.

Previously, it was shown that mango and pomegranate extracts had anti-inflammatory properties in DSS-induced colitis model (84, 97). However, the specific mechanisms is unclear. Mango is rich in polyphenols, including gallic acid and gallotannin. Increased levels of *Clostridium* and *Bacteroides* are a cause of colitis, and the growth of athogenic *E. col*i and *Clostridium perfringens* was strongly inhibited by gallic acid (110). Gallotannins showed anti-microbiota activity against most gram-positive bacteria except *E. coli, Pseudomonas,* and *lactic acid* (111). Pomegranate is rich in ellagic acid and ellagitannins. It was reported that pomegranate extract reduced colitis severity via modulation of the microbiota in the gut (*Bifidobacterium*, *Lactobacillus*, and *Clostridium*) in DSS-induced colitis models (97).

Based on this information, the objective of this study was to understand the interactions between the fecal microbiota and mango and pomegranate juice on DSS-induced colitis in Sprague Dawley rats. We hypothesized that mango and pomegranate

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juice may mitigate inflammation in DSS-induced colitis model in part by altering

microbiota composition and SCFAs production. In addition, we have investigated the

underlying mechanisms relevant to the prevention of colitis by the SCFAs production.

Materials and methods

Fruit preparation

Mango (Keitt, Mexico) was received as a kind gift from the National Mango

Board (Orlando, FL). For obtaining the experimental mango juice, full ripe mango was

peeled to remove skin and seed, and only the pulp was homogenized in a blender, and

heated with cellulase and pectinase to break down the fiber. After heating at 55°C for 1h,

the puree was centrifuged, and the supernatant was stored at -20°C to prevent oxidation.

Pomegranate was purchased from the POM wonderful (Los Angeles, CA). The

concentration of total polyphenolics were mango juice (475.90mg GAE/L) and

pomegranate juice (2698.99mg GAE/L). The sugar concentrations of juices were 15.6

and 15.8%, and the pH was 3.3 and 3.5, respectively. The control juice was adjusted its

calorie and pH to Brix 15.7 and pH 3.4.

Animal treatment and tissue sampling

DSS-induced model of colitis is an excellent preclinical model of colitis that

exhibits many phenotypic characteristics such as epithelial homeostasis, regeneration

and wound healing, relevant to the human disease (127, 148). This model is a valuable

tool for investigating the relationship between intestinal inflammation and microbiota

composition (114). Ten week-old Sprague-Dawley rats were obtained from Harlan Teklad (Houston, TX) and were acclimated for a week. After that, they were randomly grouped (n=10) by weight into control, mango and pomegranate juice groups. After 3 weeks of rat chow pellets and experimental juices *ad libitum*, all rats were administered 3% (w/v) DSS (MP Biomedicals, Solon, OH) (3 cycles, 14 d separation, n=10/diet) in the experimental juices for 48 h. Fresh fecal and serum samples were collected one week before the end of the study. Blood was collected from sublingual vein under anesthesia. At 10 weeks, rats were sacrificed and the entire colon was removed. The colon was gently scraped for collecting RNA samples (149). The animal use protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Multiplex bead assay

The serum and mucosal tissue samples were used to determine the levels of inflammatory marker, C-reactive protein (CRP) and the cytokines (TNFα, IL-1β, GM-CSF, IFNγ, IL-6 and IL-10) using the multiplex kits (Millipore, Billerica, MA) according to the manufacturer's protocol. Multiplex analysis was performed by the Luminex L200 instrument (Luminex, Austin, TX), and data was analyzed by Luminex xPONENT software.

Quantitative real-time PCR assays

qPCR targeting 16S rRNA genes is a useful tool for quantifying very low concentrations of bacterial targets in fecal samples. Bacterial DNA was extracted from

fecal samples using a bead-beating phenol-chloroform method as previously described. Further steps of DNA extraction and purification were performed using a commercial DNA extraction kit (Qiagen) according to the manufacturer's instructions (187). qPCR assays for selected bacterial groups were performed: total bacteria, Bacteroidetes, Fusobacteria, Blautia, Ruminococcaceae, Faecalibacterium spp., Turicibacter spp., Bifidobacterium spp., E coli, Lactobacillus, and Streptococcus. The qPCR data was expressed as log amount of DNA (fg) for each particular bacterial group per 10 ng of isolated total DNA (188).

SCFA analysis

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 top layer was repeated after adding diethyl ether and vortexing for 15min. After adding MTBSTFA(N-tert-butyldimethylsilyl-N ethytrifluoracetamide), samples was transferred to vials for the GC/MS injection. Dry matter weights of fecal samples were used to normalize the concentration of SCFAs.

Quantitative RT-PCR

For mRNA analysis related energy metabolism and autophagy, total RNA from the colon mucosal scraping was isolated using the mirVana™ miRNA Isolation Kit (Applied Biosciences, Foster City, CA) according to the manufacturer's protocol. Equal amount (1ug) of mRNAs was converted to cDNA using a reverse transcription kit (Invitrogen, Grand Island, NY). Real-time PCR reactions were performed using 2uL of cDNA using a Reverse Transcription Kit (Invitrogen, Grand Island, NY). SYBR Green PCR master Mix (Applied biosystems, Foster City, CA) was used for the qPCR analyses on Applied Biosystems 7900HT Fast Real-Time PCR system (Applied biosystems, 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.

Statistical analysis

Quantitative data represent mean values with standard error. Data were analyzed by student t-test and one way ANOVA using Tukey's post hoc test ($p < 0.05$) using SAS version 9 (SAS Institute Inc., Cary, NC). Differences in the proportions of microbiota between groups were analyzed using a non-parametric Kruskal-Wallis test with Dunn's multiple comparison test afterwards to compare individual groups (Prisms5, GraphPad Software Inc.). $p < 0.05$ was considered for statistical significance.

Results

Mango and pomegranate juice decreased pro-inflammatory cytokines

C-reactive protein (CRP) binds to phosphocholine expressed on the surface of dead cells, and it is used as a marker of inflammation (189). In this study, we measured the concentration of CRP and pro-inflammatory cytokine levels in serum and mucosal samples. Serum levels of IL-1β and GM-CSF were decreased in the mango and pomegranate groups compared to the control group (Figure 28A). In addition, the level of CRP and pro-inflammatory cytokines such as TNFα, IL1β, GM-CSF, and IL-6 in mucosal sample were lower in the juice group than the control group (Figure 28B).

Modification of the intestinal microbiota

An aberrant ratio of microbiota in the large intestine can be integral in triggering and enhancing IBD. Polyphenolics and their metabolites can influence the composition and function of the gut microbiota (107). Feces samples were collected from rats at 9 weeks to investigate the changes in the gut microbiota by qPCR. At the genus level, pomegranate juice resulted in fecal abundance of *Blautia*, *Fusobacterium*, and *Ruminococcaceae* ratio compared to the control group ($p < 0.05$). Interestingly, mango juice resulted in an increase of *Bacteroidetes* and *Lactobacillus* as well as a reduction of *Fusobacterium*, and *Ruminococcaceae* ratio in feces microbiota compared to the pomegranate group (Figure 29).

(A)
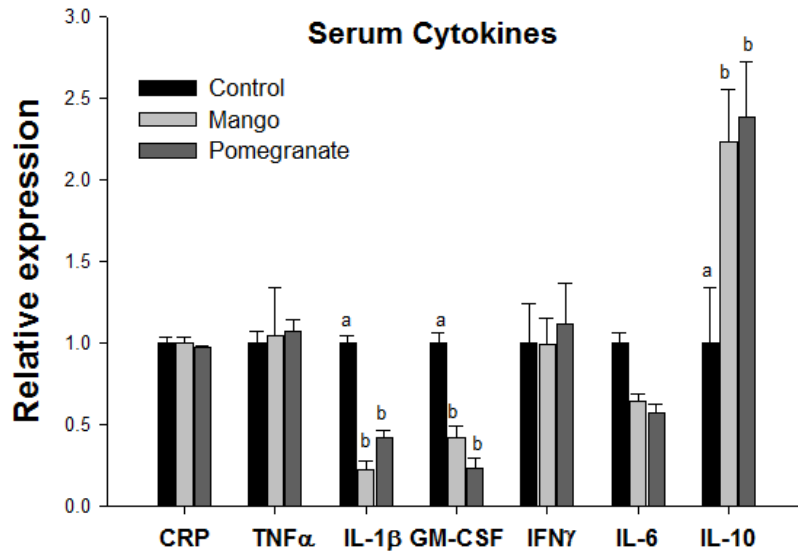
(B)
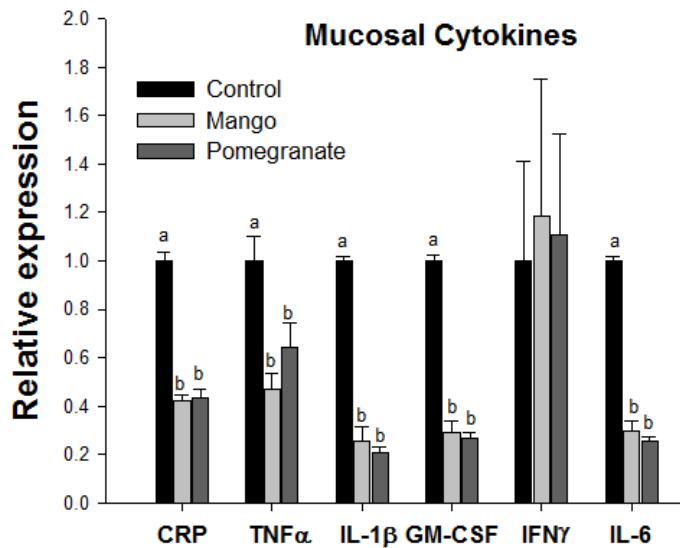
Figure 28. Effects of mango and pomegranate juice on CRP and pro-inflammatory cytokines in serum and DSS-induced colitis.
Rats were fed a control juice or a mango juice, and were exposed to three cycles of DSS. (A) Serum CRP and cytokine levels, (B) CRP and cytokines in colonic tissue. Values are mean ± SEM. Different letters indicate significant differences among groups (n = 10; $p<0.05$).
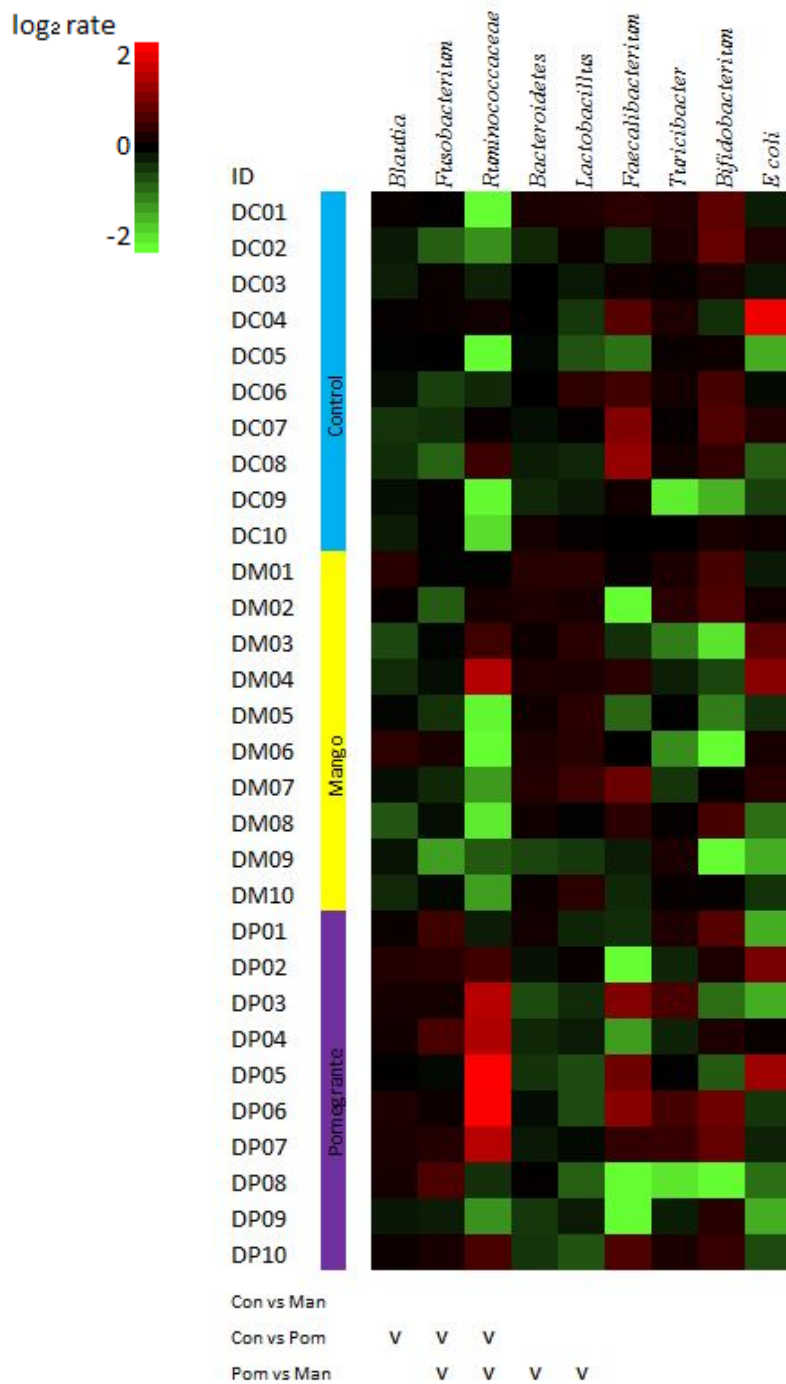
Figure 29. The influence of mango and pomegranate juice on microbiota.
Heatmap represents the relative log$_2$ ratio of each sample. A green–red gradient represents abundance differences (green, low abundance; red, high abundance; black, not significant).The "V" under the picture denote significant differences in relative proportions observed between each groups (based upon Kruskal-Wallis test; $p < 0.05$).

Modification of SCFAs production

SCFAs are produced by the anaerobic microbiota, therefore the change of microbiota composition may alter the SCFAs production. The major SCFAs is acetate (60%), propionate (25%), and butyrate (15%) (24). Mango juice resulted in an increase of isovalerate and valerate compared to the control group, as well as an increase of propionate compared to the pomegranate group ($p < 0.05$) (Figure 30).

Figure 30. SCFAs production by mango and pomegranate juice
Values are mean ± SEM. Different letters indicate significant differences among groups (n = 10; Kruskal-Wallis test; $p < 0.05$).

Mango and pomegranate juice modulate the expressions of TLR4and GPR43

The TLR4/MyD88 receptor responses LPS contained gram-negative bacteria (*Bacteroides, Fusobacterium*, and *Escherichia coli*), and causes inflammation through the induction of NF-κB signaling pathway (18) and overproduction of pro-inflammatory cytokines, such as TNF-α and IL-6 (19, 20). GPR41 and GPR43 are SCFA receptor, which are involved in the anti-inflammatory mechanisms, lipid and glucose metabolism (190). To determine the interaction between colonocytes, microbiota and SCFAs, we screened the gene expressions involved in the receptors, which ligand to the microbiota and SCFAs. Both mango and pomegranate juice decreased the level of TLR4 mRNA, while mango induced the expression of GRP43 mRNA (Figure 31).
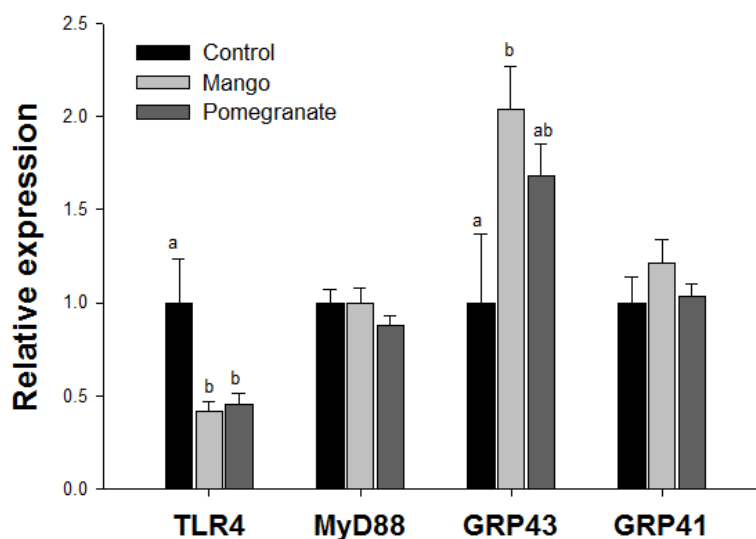
Figure 31. The level of TLR4, MyD88, GRP43, and GRP41 mRNA altered by mango and pomegranate juice.
Values are mean ± SEM. Different letters indicate significant differences among groups (n = 10; p<0.05).

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Mango and pomegranate juice altered gene expressions involved in the energy

metabolism and autophagy

SCFAs exert beneficial effects on host energy metabolism, because colonocytes prefer to derive their energy supply from SCFAs oxidation (191). The interaction of GRP receptors and SCFAs seem to affect glucose metabolism in colonocytes (191), and autophagy can be partially modulated the glucose metabolisms altered by SCFAs (192). We investigated the effect of juice on lactate metabolism and autophagy associated genes to explain the relationship between SCFAs modulation by juice and energy metabolism. Mango and pomegranate juice dramatically modulated an increase of LDHA as well as a decrease of MCT4 (Figure 32A), which are involved in the lactate metabolism where LDHA converts pyruvate to lactate and MCT4 is lactate and butyrate transporter. Among genes involved in the autophagy pathway, LC3, a representative marker of autophagosomes, were up-regulated by juice (Figure 32B).

(A)   (B)
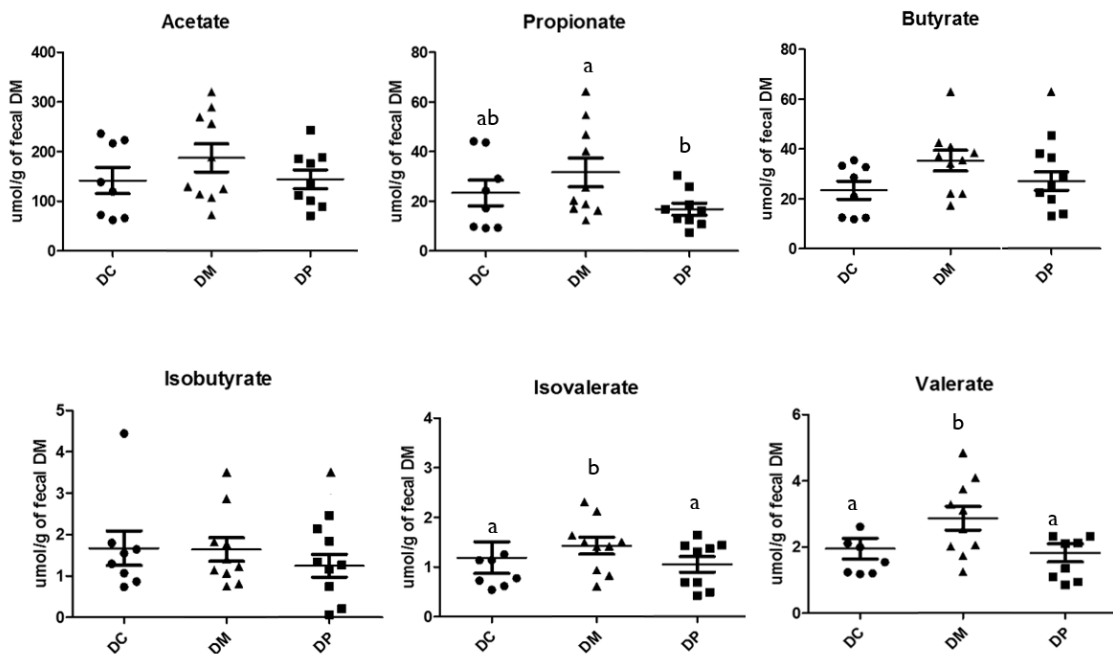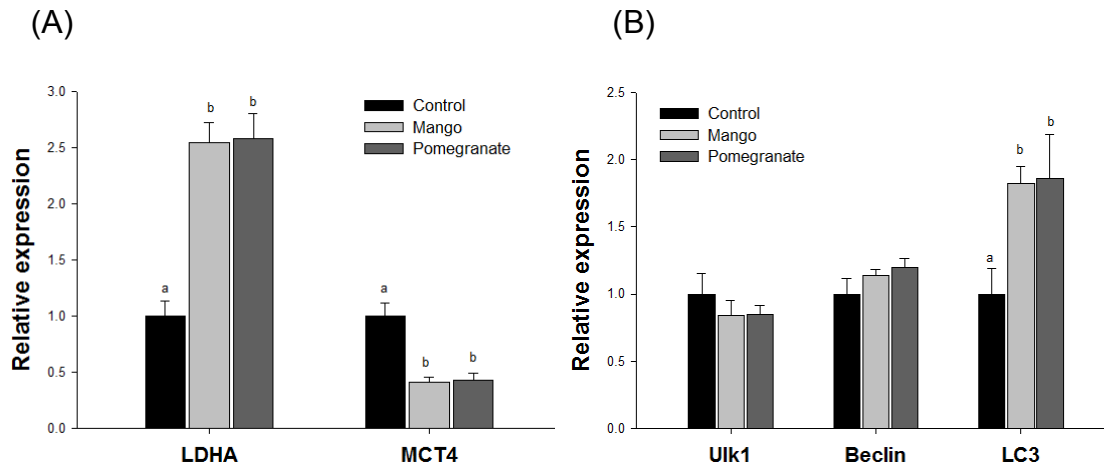

Figure 32. Gene expressions related to lactate metabolism and autophagy altered by mango and pomegranate juice.
Values are mean ± SEM. Different letters indicate significant differences among groups (n = 10; $p<0.05$).

Discussion

Recent studies have provided compelling evidences that the imbalanced regulation between the immune system and gut microbiota seems to be involved in triggering colitis. Furthermore, modulation of gut microbiota composition can be a key target of the prevention and treatment of colitis (5). SCFAs are produced by a bacterial fermentation of undigested fiber, and they are known to have a variety of health beneficial effects on intestine (21). Polyphenolics are metabolized by gut microbiota in colon, and produce a number of metabolites. Polyphenolics and their metabolites can influence the composition and function of the gut microbiota and SCFAs production (107). In this study, we investigated if the consumption of mango and pomegranate juice is capable of modifying the intestinal microbiota and SCFAs production.

This study showed that anti-inflammatory activities of mango and juice contribute to the prevention and treatment of colitis. Mango and pomegranate juice reduced colitis severity, and attenuated the pro-inflammatory cytokines such as TNFα, IL1β, GM-CSF, and IL-6 in mucosal sample, and serum IL-1β and GM-CSF (Figure 28).

In the results, pomegranate juice altered microbiota compositions. The genus level of *Blautia, Fusobacterium*, and *Ruminococcaceae* in feces were induced compared to the control group (p<0.05) (Figure 29). Human colonic butyrate producers are mainly gram-positive *firmicutes*, the genera *Clostridium*, *Eubacterium* and *Faecalibacterium* (193). *Blautia* and *Ruminococcaceae* are involved in the gram-positive bacteria, and they have anti-inflammatory effects (194) due to the production of butyrate, which repair the epithelium and regulate inflammation (195). Pomegranate juice may induce butyrate production through the induction of butyrate producer microbiota, *Blautia* and *Ruminococcaceae.*

SCFAs produced by the intestinal bacterial flora have various physiological actions involved in health, and it is important to determine the concentrations of fecal short-chain fatty acids and evaluate their relationship with large intestinal diseases. Butyrate is the most biologically potent of the SCFAs in colon epithelial cells, inhibiting human colon carcinoma cell proliferation and inducing apoptosis (196). In our results, mango juice significantly induced the amount of isovalerate and valerate compared to the control group. Valerate and isovalerate produce from the bacterial fermentation of inadequately digested branch chain amino acids. High amounts of these SCFAs suggest

the maldigestion of protein (197). In addition, mango juice slightly induced the amount of propionate and butyrate also (Figure 30).

There are several trans-membrane receptors that detect gut microbiota to initiate the intercellular response. Gram-negative bacteria containing LPS is a ligand for the TLR4/MyD88 receptor (18), and the binding induce the NF-κB signaling pathway. juice decreased the expression of TLR4. GPR41 and GPR43 are receptors for SCFAs, and they are key regulators of inflammation through the suppression of the NF-κB signaling pathway (24). Mango juice increased the expression of GRP43 (Figure 31). The modulation of trans-membrane receptors by juice may help to the changes of microbiota composition involved in the anti-inflammatory activities.

Inflamed cells display the same metabolism that involves a shift from mitochondrial oxidative phosphorylation to aerobic glycolysis like tumor cell, which known as the Warburg effect (198). SCFAs are used as colonocytes energy sources to maintain cell proliferation in normal, non-Warburg cells. However, in cancerous or inflamed Warburg cells, SCFAs cannot be used as colonocytes energy sources, are accumulated in the cells and trigger autophagy and apoptosis as an HDAC inhibitor (199). LDHA produces lactate from glucose and MCT4 is lactate and butyrate transporter. The up-regulation of LDHA and down-regulation of MCT4 by juice may cause the Warburg effects, and it creates aerobic glycolysis environment and induce apoptosis and autophagy by SCFAs (Figure 32).

The objective of this study was to elucidate whether juice have a significant influence on SCFAs and microbiota composition, and whether this would have an

influence on autophagy, and markers for energy metabolism. Real time PCR analysis of selected representative genus of the intestinal microbiota indicated that polyphenolics from pomegranate, but not mango significantly increased genus associated with butyrate production. In contrast, mango did not significantly alter the composition of intestinal microbiota, however mango juice significantly increased the concentration of valerate and isovalerate, while pomegranate juice did not significantly affect the composition of SCFAs. Mango and pomegranate juice may mitigate inflammation in DSS-induced colitis model in part by altering microbiota composition and SCFAs production.

CHAPTER VI

SUMMARY AND CONCLUSION

Ulcerative colitis is a chronic inflammation of the large intestine, and it may increase risk of human colorectal cancer. Therefore, a variety of extensive research has been conducted to determine inflammatory mechanisms and to develop the potential therapeutic strategies (200). Due to the many possible factors of these disease such as genetics, microbiota, and immune dysfunction, colitis remains an important topic of research.

The general scope of this study was to investigate the mechanisms underlying the anti-inflammatory properties of mango and pomegranate polyphenols in DSS-induced colitis in rats and in LPS-treated treated CCD-18Co, non-cancer colon fibroblasts cell lines. In addition, we investigated the differential effects of these polyphenolics on the post-transcriptional modulation and the changes of microbiota composition, which contribute to the prevention and treatment of colitis.

This study showed that anti-inflammatory activities of mango and pomegranate juice contribute to the prevention and treatment of colitis. Mango and pomegranate juice reduced colitis severity, and attenuated the pro-inflammatory cytokines such as TNFα, IL1β, GM-CSF, and IL-6 in mucosal sample, serum IL-1β and GM-CSF, pro-inflammatory cytokines (TNF-α and Il-1β) mRNA. In addition, mango and pomegranate juice suppressed COX-2 and iNOS mRNA and protein expressions.

Our lab has previously shown that polyphenolics, such as pomegranate, wine, and betulinic acid, reduced inflammation through the modulation of miRNA expression (151, 152, 168). In this study, we evaluated the ability of polyphenolics to the post-transcriptional modulation. Mango and pomegranate polyphenols were able to counteract the expression of HIF-1α, which is considered a key regulator of inflammation. The mTOR pathway influences the HIF-1α translation. Mango juice showed the capacity to suppress the PI3K/AKT/mTOR pathway through the up-regulation of miRNA-126 in colitis, whereas pomegranate juice suppressed the p70S6K1/HIF1α pathway through the up-regulation of miRNA-146.

Polyphenolics in mango and pomegranate differentially affected pathways involved in the anti-inflammatory effects. Proteomic and gene expression analysis was utilized to determine the potential therapeutic target in prevention of inflammatory disease and to elucidate the mechanisms underlying the anti-inflammatory response. Results showed that Mango juice decreased the PI3K/AKT-mTOR signaling axis via up-regulation of miR-126 and let7a, and down-regulation of IR, while pomegranate decreased p70S6K by up-regulating miR-145 and down-regulation of the ERK1/2 pathway. These results suggest that polyphenolics of different predominant structure may differentially regulate inflammation-involved pathways while both attenuating DSS-induced colitis. This may also indicate that a combination of both juice classes may have synergistic effects in mitigation of inflammation.

Real time PCR analysis of selected representative genus of the intestinal microbiota indicated that juice from pomegranate, but not mango, significantly increased

genus associated with butyrate production. In contrast, mango did not significantly alter the composition of intestinal microbiota, however mango juice significantly increased the concentration of valerate and isovalerate, while pomegranate juice did not significantly affect the composition of SCFAs. Both mango and pomegranate juice seemed to induce Warburg effects through the modulation of the aerobic glycolysis components (LDHA and MCT4), and may induce autophagy, possibly through the cumulative increase in SCFAs in colonocytes, which serves as an adaptive strategy of reducing inflamed cells in inflammation.

Certain limitations will require further experimentation in the future. The first limitation is that juice groups suppress the intestinal inflammation slightly but not significantly; however, several inflammatory markers were also evaluated in control, mango, and pomegranate juice groups. In addition, the next approach can be to perform negative controls (control juice without DSS treatment) to compare the level of intestinal inflammation between the negative control and the juice groups. A further limitation is that we did not use a semi-purified diet but used rat chow pellets in this study. The chow diet typically contains soy protein, so it is not fully known all possible synergistic effects of polyphenolics and compounds in the soy protein, such as genistein, on the inflammation. In addition, colitis was induced in rats by DSS diluted in the experimental juice in this study. However, there can be an association between DSS compounds and polyphenolics in the juice, so DSS might not show the same toxicity in the control and juice groups. Thus, investigating the detailed interaction among polyphenolics, soy

proteins in the chow diet, and DSS compounds, is essential for further understanding the effects of mango and pomegranate polyphenolics on colitis.

In summary, we have investigated the mechanisms underlying the anti-inflammatory effects of mango and pomegranate juice and potential role of miRNA and intestinal microbiota composition in colitis *in vivo* and *in vitro*. Mango and pomegranate juice exerted anti-inflammatory activities in colitis *in vivo* and *in vitro*.

Overall, this research will provide the scientific basis for comparing the effects of different polyphenolics on the mTOR pathway for the anti-inflammatory responses and prevention of colitis. In addition, fruit juice can directly influence the post-transcriptional modification or the intestinal microbiota and its relationship with the immune system. The mango and pomegranate polyphenolics are likely a promising countermeasure for protection against any potential inflammatory response. Additionally, both mango and pomegranate polyphenolics may have synergistic effects since both utilize differential molecular mechanism in the mitigation of inflammation.

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