α-Mangostin: Friend or Foe of the Immune System and the Gut Microbiota?

DISSERTATION

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By

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ABSTRACT

Chronic inflammation has been associated with the development of a myriad of diseases such as cancer and cardiovascular disease. Similarly, alterations in the normal composition of the gut microbiota are often associated with diseased states. Xanthones are polyphenolic compounds found in the pericarp of mangosteen, a fruit native to Southeast Asia, where it has been used in traditional medicine to treat inflammation, infection, wounds, and diarrhea. The use of supplements containing mangosteen has increased greatly in recent years as a result of aggressive marketing of their proposed health benefits. Mangosteen juice, for instance, has been promoted as beneficial for gastrointestinal health and immunity. However, scientific evidence supporting these and other claims is lacking.

α-Mangostin (α-MG) is the most abundant xanthone in the pericarp of mangosteen fruit. Data on the anti-inflammatory activity and cellular metabolism of α-MG in human cells is limited. First, I determined the cellular uptake and metabolism, as well as the anti-inflammatory activities of α-MG, using in vitro cultures of transformed cell lines and primary cells of human origin with hepatic, small intestinal, immune, and colonic phenotypes (Chapter 2). α-MG attenuated the secretion of pro-inflammatory mediators
by transformed cells, but actually increased the secretion of pro-inflammatory TNF-α by normal monocyte-derived macrophages. Phase II metabolites, as well as biotransformation of α-MG to other xanthones, was also observed. Degree of cellular retention of the xanthone and extent of metabolism were dependent on cell type.

Because bioavailability of xanthones is limited, the gastrointestinal tract is exposed to significant amounts of these compounds after oral ingestion. Therefore, I next examined the potential anti-inflammatory role of dietary α-MG during colitis, a disease characterized by chronic inflammation of the colon. Because α-MG exerts inhibitory activities against isolated bacterial species, its effect on the gut microbiota was also assessed (Chapter 3). The pathology of chemically-induced colitis in female C57BL/6J mice was exacerbated in animals that were fed diet with 0.1% α-MG compared to mice consuming a standard diet. Dietary α-MG induced greater colonic inflammation and injury, greater infiltration of CD3⁺ and F4/80⁺ cells, and increased content of myeloperoxidase in the colon, than controls. The colonic and cecal microbiota of non-colitic, healthy C57BL/6J mice fed α-MG also shifted to a reduced abundance of generally assumed beneficial bacterial phyla (Firmicutes and Bacteroidetes) and an increased abundance of pathogenic bacteria (Proteobacteria). This intestinal dysbiosis resembles that found in individuals with ulcerative colitis.

Host genetic are known to affect the composition of the gut microbiota. Thus, I sought to determine whether the induction of dysbiosis by dietary α-MG is strain-dependent or reflects a normal response of mice to the xanthone (Chapter 4). C3H, Balb/c, and Nude FoxN1nu, and C57BL/6J mice were fed standard diet or diet with 0.1% α-MG for 4
weeks. Pyrosequencing analysis showed that dietary α-MG significantly altered the cecal and colonic microbiota in mice in a strain-independent manner. α-MG was associated with reduced abundance of Firmicutes and increased abundance of Proteobacteria. Dietary α-MG also promoted increased proliferation of colonic epithelial cells and infiltration of immune cells, as well as increased fluid content in stool, in all four mouse strains.

Despite the numerous *in vitro* and *in vivo* studies on the bioactivities of mangosteen xanthones, further research is needed to evaluate their safety and health benefits before they can be recommended for preventive or therapeutic purposes.
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CHAPTER 1

REVIEW OF LITERATURE AND SPECIFIC AIMS

1.1 Introduction

Inflammation is a normal biological response to infection, tissue injury, or chemical irritation. Thus, when controlled properly, the inflammatory response is a defense mechanism for maintaining health. In contrast, prolonged and dysregulated inflammation is associated with development of chronic diseases such as cancer and cardiovascular disease (Calder and others 2009). Ulcerative colitis (UC) is a chronic disease of the colon characterized by mucosal and sub-mucosal inflammation. Persistent progressive or relapsing inflammation, bloody diarrhea, and abdominal distress are hallmarks of UC (Khor and others 2011). Patients with UC have greater relative risk of developing colorectal cancer compared to the general population (Clapper, Cooper, Chang 2007). Because medical therapy for the treatment of UC has only modest success and many adverse side effects (Lanzoni and others 2008), UC patients often seek complementary and alternative medicine (CAM) to manage their disease. However, clinical evidence supporting the use of CAM products is lacking at this time.
The etiology of UC remains only partially understood, but it is thought to result from a
dysregulated immune response to the gut microbiota in a genetically susceptible host
(Ordás and others 2012). The gut microbiota is the collection of bacteria, viruses, and
single-cell eukaryotes in the gastrointestinal (GI) tract and plays a critical role in the
metabolism of dietary nutrients and xenobiotics such as phytochemicals and drugs
(Sekirov and others 2010). Dysbiosis, an imbalance between putative species of
“protective” versus “harmful” intestinal bacteria, has been implicated in UC (Tamboli
and others 2004).

In vitro and in vivo studies have shown that dietary components can protect against
inflammation (Wu and Schauss 2012). Polyphenols (plant-derived phytochemicals) may
modify inflammatory processes and have been reported as beneficial for the prevention of
inflammation and the attenuation of the severity of pathology associated with excessive
inflammation (Romier-Crouzet and others 2009). Xanthones are polyphenolic
compounds with purported health benefits found mainly in the pericarp of mangosteen, a
fruit native to Southeast Asia where it has been used in traditional medicine (Pedraza-
Chaverri and others 2008). α-Mangostin (α-MG) is the most abundant xanthone in
mangosteen (Walker 2007) and the majority of studies have been focused on its anti-
oxidant, anti-inflammatory, antimicrobial, and anti-tumorigenic activities (Gutierrez-
Orozco and Failla 2013; Pedraza-Chaverri and others 2008). Aggressive marketing of
mangosteen’s health promoting benefits has led to increased sales of beverages and other
nutraceuticals containing mangosteen fruit or its components (Sloan 2010). However,
scientifically sound data in support of these claims is limited.

2
In order for α-MG and other dietary xanthones to exert their purported health-promoting activities, these compounds and/or their metabolites need to be delivered to target tissues. Other than a single report using differentiated cultures of Caco-2 human intestinal cells (Bumrungpert and others 2009a), no information exist on the uptake and metabolism of α-MG by cells of animal or human origin. Moreover, the chemical stability of α-MG in cell culture media and the metabolism of this compound during inflammatory conditions have not been addressed. The reported anti-inflammatory activity of xanthones in cells of human origin has been limited to primary cultures of adipocytes (Bumrungpert and others 2009b) and the transformed U937 macrophage-like cell line (Bumrungpert and others 2010). The effect of α-MG on the inflammatory response by normal (non-transformed) cells has not been assessed.

The bioavailability of mangosteen xanthones is limited (Chitchumroonchokchaisri and others 2012). Therefore, the GI tract is exposed to relatively high amounts of these compounds after oral ingestion of mangosteen pericarp and related products. However, the potential anti-inflammatory role of α-MG in the context of colonic inflammation, such as that found in UC, has not been previously investigated. Because α-MG exerts inhibitory activities against isolated bacterial species (Pedraza-Chaverri and others 2008), the impact of this xanthone on the gut microbiota also merits investigation. My dissertation has addressed the following: a) cellular uptake and metabolism of α-MG under normal and inflammatory conditions; b) in vitro anti-inflammatory activity of α-MG in cultures of normal and transformed human cells; c) effect of dietary α-MG on
inflammation during experimental colitis; and, d) the impact of orally consumed α-MG on the gut microbiota.

1.2 Mangosteen (*Garcinia mangostana*)

Juice blends and other products containing exotic fruits also known as *superfruits* have been aggressively marketed for their proposed health benefits. This has resulted in a steady rise in sales of superfruit juices and products to consumers interested in their personal health. Mangosteen is one such superfruit that is produced by *Garcinia mangostana* L. The genus *Garcinia* is native to Asia and Africa and includes more than 300 distinct species from which several families of bioactive compounds such as xanthones, flavonoids, triterpenoids, and benzophenones have been isolated and characterized (Chin and Kinghorn 2008). Although many *Garcinia* species including *G. mangostana*, *G. schomburgkiana*, *G. dulcis*, *G. cowa*, *G. atroviridis*, *G. hanburyi*, *G. bancana*, *G. xanthochymus*, *G. thorelii*, *G. hombroniana*, and *G. speciosa* bear edible fruits, mangosteen has captured the most attention in the market place (Yapwattanaphun and others 2002). The mangosteen tree is mainly cultivated in Indonesia, Malaysia, the Philippines, and Thailand. Mature mangosteen trees range from 6 to 25 m. Production of the fruit generally requires 10 or more years with a yield of around 400 fruits per tree that increases in older trees. Mangosteen fruit is round, dark purple or reddish, and has a white juicy pulp possessing a slightly acidic and sweet flavor. This has resulted in mangosteen also being referred to as the “queen of fruits”. The pericarp of mangosteen
fruit has been used in traditional medicine in Southeast Asia for centuries to treat infection, wounds, inflammation and diarrhea (Pedraza-Chaverri and others 2008).

Products containing mangosteen juice or extract are a fast growing segment of the functional beverages market. Aggressive marketing of the proposed health benefits of mangosteen has resulted in sales of mangosteen products in the US exceeding $200 million in 2008 (Sloan 2010). Oftentimes, products marketed as mangosteen juice are a blend of numerous fruit juices with mangosteen being one of the less abundant components. For example, Xango®, one of the bestselling mangosteen products in the US, contains mangosteen puree, and a blend of juices from grape, pear, apple, blueberry, strawberry, raspberry, cranberry, and cherry.

1.3 Mangosteen xanthones

Xanthones are secondary metabolites that have been isolated from the pericarp of mangosteen and are attributed to the medicinal properties of the fruit. Xanthones have a unique chemical structure composed of a tricyclic aromatic system (C₆–C₃–C₆). Isoprene, methoxyl and hydroxyl groups located at various locations on the A and B rings result in numerous xanthone compounds. Xanthones are found in a select few higher plant families. At least 68 distinct xanthones have been identified in different parts of the G. mangostana plant with 50 present in the fruit’s pericarp at higher concentrations than in the aril, i.e., the edible portion of the fruit (Obolskiy and others 2009). The most abundant xanthones in the pericarp of mangosteen fruit are α- and γ-mangostin (Figure 1.1) (Walker 2007). Other xanthones in mangosteen pericarp include β-mangostin, gartanin,
8-deoxygartanin, garcinones A, B, C, D and E, mangostinone, 9-hydroxycalabaxanthone, and isomangostin. Details regarding the extraction and identification of these and other xanthones have been reviewed elsewhere (Obolskiy and others 2009).

Interest in the mangosteen fruit and xanthones has greatly increased in recent years as readily demonstrated by the number of scientific reports. A search of available literature using mangosteen and xanthones as terms in Pubmed, Science Direct, Google Scholar, and Scirus, retrieved 158 reports in the period of 1980–2008. In contrast, there have been 454 published articles from 2008 through March 2013 (Figure 1.2). By far, the most studied xanthone is α-mangostin (α-MG) for which anti-oxidant, anti-proliferative, pro-apoptotic, anti-inflammatory, anti-carcinogenic, and anti-microbial activities have been reported. Pertinent literature has been previously reviewed (Table 1.1). This chapter focuses primarily on recent reports considering the bioavailability and cellular metabolism of xanthones, their anti-cancer, anti-inflammatory, and antimicrobial activities, and their reported effects on cellular signaling pathways.
Figure 1.1. Chemical structures of two most abundant xanthones in mangosteen.
Figure 1.2. Number of publications related to mangosteen and their xanthones from 1980 to 2013. Search words: mangosteen, xanthones. Search performed on April 24 2013, including ahead of print publications. Databases: Pubmed, Science Direct, Google Scholar, Scirus.
1.3.1 Metabolism and bioavailability

The first report on xanthone bioaccessibility and metabolism was performed using the coupled in vitro digestion/Caco-2 human intestinal cell model. Optimal bioaccessibility of α- and γ-MG xanthones was dependent on incorporation into bile salt mixed micelles. In addition, α-MG was transported across the apical surface of enterocyte-like Caco-2 cells and partially converted to phase II metabolites. Both unconjugated α-MG and its phase II metabolites were effluxed across the basolateral membrane supporting the possibility that xanthones were absorbed. Transepithelial transport was enhanced by addition of products of lipid digestion in the apical compartment, suggesting that absorption was dependent on the assembly and secretion of chylomicrons. Xanthone metabolites also were retro-transported across the apical membrane into the simulated gut luminal compartment (Bumrungpert and others 2009a) (Figure 1.3).

Bioavailability is defined as the fraction of an orally ingested or administered compound in a food, beverage or supplement that reaches systemic circulation. The bioavailability and metabolism of α-MG have been reported in several recent studies using laboratory rodents. It was previously reported that intravenously injected α-MG (2 mg/kg) in rats was slowly eliminated from blood and rapidly distributed to tissues. However, the bioavailability of orally administered α-MG (20 mg/kg dose) dissolved in an aqueous solution containing 2% ethanol and 2% Tween 80 was estimated as only 0.4% (Li and others 2011). In a similar study, α-MG (40 mg/kg) dissolved in corn oil was orally administered to rats. The maximum plasma concentration (4.8 µg/mL) was reached within 63 min (Syamsudin and others 2009).
Table 1.1. Available reviews on chemical properties and bioactivities of xanthones in mangosteen.

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<td>enzyme modulation, anti-tumor activity, anti-microbial, central nervous system (CNS) depressants, CNS stimulants, neurological disorders, anti-convulsant, analgesic, anti-arrhythmic, anti-hypertensive, anti-inflammatory, anti-allergic and immunomodulatory activities</td>
<td>(Pinto, Sousa, Nascimento 2005)</td>
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<td>xanthones isolated from pericarp, whole fruit, trunk, leaves and branches</td>
<td>anti-oxidant, anti-tumor, anti-inflammatory, anti-allergic, anti-bacterial, anti-fungal, anti-viral and anti-malarial activities</td>
<td>(Pedraza-Chaverri and others 2008)</td>
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<td>structural characterization of mangosteen xanthones in whole fruit, stem, aril, seeds, heartwood, leaves</td>
<td>anti-oxidant, anti-bacterial, anti-fungal, anti-malarial, anti-HIV, cytotoxic, aromatase inhibitory, anti-cancer and anti-inflammatory activities</td>
<td>(Chin and Kinghorn 2008)</td>
</tr>
<tr>
<td>chemical constituents and methods of isolation from pericarp, whole fruit, stem, aril, seeds, heartwood, leaves</td>
<td>anti-oxidant, anti-fungal, anti-bacterial, cytotoxic, anti-histamine, anti-HIV, CNS-depressant, cardiovascular, anti-inflammatory and anti-ulcerative activities</td>
<td>(Oboliskiy and others 2009)</td>
</tr>
<tr>
<td>xanthones from mangosteen extracts</td>
<td>anti-cancer, anti-inflammatory, pro-apoptotic, cell cycle arresting, anti-invasive and anti-metastatic activities</td>
<td>(Shan and others 2011)</td>
</tr>
</tbody>
</table>
Figure 1.3. Transport and metabolism of mangosteen xanthones across intestinal epithelium. MG: xanthones; MG-X: xanthone phase II metabolites; MG\textsubscript{YZ}: bioconversion products of xanthones.
Xanthones were detected in plasma, liver and HT-29 subcutaneous tumors of athymic nude mice fed a diet containing 900 mg/kg α-MG (94% purity). Serum xanthones were extensively conjugated in these mice, whereas hepatic xanthones were primarily free. Interestingly, a xanthone tentatively identified as β-mangostin was the most abundant xanthone detected in liver despite its very low abundance in the diet (85% vs. 0.3% of total xanthones, respectively). α-, β- and γ-Mangostin, 9-hydroxycalabaxanthone, 8-deoxygartanin, gartanin, and garcinone E, were also detected in the HT-29 colon xenograft (Chitchumroonchokchhai and others 2013). The presence of the xanthones in the tumor of mice fed diet with α-MG was associated with a 40% reduction in tumor mass of mice. The presence of high concentrations of α-MG (and metabolites) in feces of mice fed diet with α-MG suggests that the epithelium in the cecum and the colonic tissue is exposed to these xanthones. When α-MG was administered to C57BL/6 mice by oral gavage in an oil suspension (100 mg/kg), a maximum plasma concentration of 1.38 µmol/L was reached within 30 min. In addition, mono- and di-glucuronide metabolites of α-MG were detected in plasma. α-MG was detected in plasma 24 h after oral administration suggesting a slow elimination pattern (Ramaiya, Petiwala, Johnson 2012).

There have also been several reports addressing the bioavailability of xanthones in human subjects. Healthy subjects consumed 59 mL of a xanthone-rich mangosteen juice product containing 94.2 mg xanthones. The maximum plasma concentration of α-MG (3.12 ± 1.47 ng/mL) was reached within 1 h. This study was limited by the fact that plasma samples were only collected for 6 h after ingestion of the mangosteen product and xanthone metabolites were not considered in the analysis. Plasma antioxidant capacity as
measured by the oxygen radical absorbance capacity, ORAC, in these subjects was increased by as much as 18% after ingestion of the mangosteen product compared to subjects ingesting a placebo product. However, the contribution of α-MG to this increase in ORAC value is unknown because the beverage also contained green tea, aloe vera, and supplements including minerals, and vitamins A, B, C, D and E (Kondo and others 2009).

In a more recent human study, xanthones from 100% mangosteen juice (containing both liquid and pericarp particles) were found to be absorbed and partially conjugated by healthy adults ingesting a single dose (60 mL) of the mangosteen juice (containing 130 mg of xanthones) with a high fat Western-style breakfast. Both free and glucuronidated/sulfated xanthones (α- and γ-MG, garcinones D and E, 8-deoxygatanin and gartanin) were detected in serum and urine. Variation in maximum concentration of α-MG in serum (113 ± 107 nmol/L), as well as in time to maximum concentration (3.7 ± 2.4 h), was noted for the 10 subjects. Urinary excretion of xanthones accounted for 2% of the ingested dose (Chitchumroonchokchai and others 2012). Xanthones were still present in plasma 24 h after juice ingestion, suggesting slow turnover as reported for mice after oral administration (Ramaiya, Petiwala, Johnson 2012).

### 1.3.2 Anti-cancer activities

*In vivo* studies examining the anti-tumorigenic activities and *in vitro* anti-proliferative and pro-apoptotic activities of mangosteen xanthones with cancer cell lines are summarized in Tables 1.2 and 1.3. The effects of mangosteen xanthones on mammary cancer have been examined in two studies using mammary BJMC3879 cancer cells xenografted into
Balb/c mice. Subcutaneous α-MG (Shibata and others 2011) and dietary Panaxanthone (75%–85% α-MG and 5%–15% γ-MG) (Doi and others 2009) significantly suppressed tumor volumes and metastatic expansion in this cancer model. *In vitro*, α-MG induced apoptosis, cell cycle arrest, activation of caspases-3 and -9, cytochrome c release and the loss of mitochondrial potential in BJMC3879 cells (Shibata and others 2011; Doi and others 2009).

Two recent reports addressed the anti-tumorigenic effects of α-MG in glioblastoma and prostate xenograft mouse models. Intraperitoneal administration of α-MG inhibited tumor growth by 50% in a GBM8401 glioblastoma xenograft model and this effect was associated with increased phosphorylation of AMPK (AMP-activated protein kinase) and induction of autophagy (Chao and others 2011). Oral administration of α-MG to athymic mice bearing 22Rv1 prostate tumors five times a week following implantation of cancer cells significantly decreased tumor volume. *In vitro*, α-MG induced cell cycle arrest and apoptosis in 22Rv1 prostate cancer cells through activation of caspase-3. By using a cell free assay, α-MG also was shown to inhibit cyclin/cyclin-dependent kinase 4, which is involved in cell cycle progression (Johnson and others 2012).

The majority of *in vivo* studies examining the anti-cancer activity of mangosteen xanthones have focused on colon cancer. Dietary administration of α-MG significantly inhibited the induction and development of aberrant crypt foci (ACF) in a chemically-induced rat model of colon carcinogenesis (Nabandith and others 2004). The growth of COLO 205 xenografts was completely suppressed when mice were injected intratumorally with 3 mg of a mangosteen extract containing α- and γ-MG. Caspase-
mediated apoptosis was detected in the tumor cells. Lower doses of the extract also reduced tumor volume. Induction of COLO 205 cell apoptosis was also confirmed *in vitro* (Watanapokasin and others 2010). Dietary administration of an extract from mangosteen pericarp containing α- and γ-MG inhibited the growth of colorectal HCT116 xenografts in mice. *In vitro*, α-MG reduced HCT116 cell viability and induced activation of caspases and loss of mitochondrial potential. In addition, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), Myc/Max and p53 signaling was enhanced by 71%, 48% and 30%, respectively, after treatment of cells with α-MG. Jun N-terminal kinase (JNK) pathway also increased, although the change failed to achieve statistical significance. Nuclear factor kappa-B (NF-κB) activity also was reduced by 30% (Aisha and others 2012). Balb/c mice bearing colon cancer NL-17 xenografts showed 50%–70% reduction in tumor size when intraperitoneally treated with an extract from mangosteen pericarp containing 25% α-MG. Anti-proliferative activity of the extract on NL-17 cells was also confirmed *in vitro* (Kosem and others 2013). Oral administration of α-MG also reduced growth of colon cancer Her2/CT26 xenografts in mice. The anti-tumor effect of α-MG was ascribed to autophagic activation rather than induction of endoplasmic reticulum stress as the xanthone was found to activate autophagy in the small intestine (Kim and others 2012). Finally, dietary α-MG reduced tumor mass and levels of Bcl-2 and β-catenin in colon cancer HT-29 xenografts. *In vitro* analysis confirmed that α-MG inhibited HT-29 proliferation and decreased expression of Bcl-2 and β-catenin (Chitchumroonchokchai and others 2013).
Table 1.2. *In vivo* anti-tumorigenic activities of mangosteen xanthones.

<table>
<thead>
<tr>
<th>Cancer cell type</th>
<th>Animal model</th>
<th>Tested compound</th>
<th>Delivery route</th>
<th>Dose</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJMC3879 (murine mammary adenocarcinoma)</td>
<td>Balb/c</td>
<td>panaxanthone (75%–85% α-MG, 5%–15% γ-MG)</td>
<td>diet</td>
<td>5000 ppm</td>
<td>suppression of tumor volume and lung metastasis; decreased microvessel density</td>
<td>(Doi and others 2009)</td>
</tr>
<tr>
<td>BJMC3879 (murine mammary adenocarcinoma)</td>
<td>Balb/c</td>
<td>α-MG</td>
<td>subcutaneous</td>
<td>20 mg/kg/day</td>
<td>decreased tumor growth and metastatic expansion; increased apoptosis; activation of caspase-3; decreased microvessel density; cytochrome c release from mitochondria; cell cycle arrest</td>
<td>(Shibata and others 2011)</td>
</tr>
<tr>
<td>GBM8401 (human malignant glioblastoma)</td>
<td>nude Balb/c A-v (v/v)</td>
<td>α-MG</td>
<td>intraperitoneal</td>
<td>2 mg/kg/day</td>
<td>inhibition of tumor growth by 50%; increased phosphorylation of AMPK; induction of autophagy</td>
<td>(Chao and others 2011)</td>
</tr>
<tr>
<td>22Rv1 (human prostate carcinoma)</td>
<td>Athymic nu/nu mice</td>
<td>α-MG</td>
<td>oral gavage</td>
<td>100 mg/kg, 5 times/week</td>
<td>decreased tumor growth</td>
<td>(Johnson and others 2012)</td>
</tr>
</tbody>
</table>

Continued
Table 1.2. Continued.

<table>
<thead>
<tr>
<th>Cancer cell type</th>
<th>Animal model</th>
<th>Tested compound</th>
<th>Delivery route</th>
<th>Dose</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLO205 (human colorectal adenocarcinoma)</td>
<td>Athymic NCr nu/nu mice</td>
<td>mangosteen pericarp extract containing 48 mg α-MG and 6.4 mg γ-MG per gram of extract</td>
<td>intratumorally</td>
<td>0.024–3.0 mg per tumor</td>
<td>complete suppression of tumor growth at 3 mg extract/tumor; apoptotic cells, nuclear fragmentation and chromatin condensation; activation of caspases-3 and -8</td>
<td>(Watanapokasin and others 2010)</td>
</tr>
<tr>
<td>HCT116 (human colorectal carcinoma)</td>
<td>Athymic NCR nu/nu nude mice</td>
<td>extract of mangosteen pericarp (81% α-MG and 16% γ-MG)</td>
<td>diet</td>
<td>0.25% and 0.5% extract: food ratio (wt/wt)</td>
<td>inhibition of tumor growth; fewer blood vessels in tumor</td>
<td>(Aisha and others 2012)</td>
</tr>
<tr>
<td>NL-17 (murine colon adenocarcinoma)</td>
<td>Balb/c</td>
<td>pericarp methanolic extract (25% α-MG)</td>
<td>intraperitoneal</td>
<td>100–200 mg/kg</td>
<td>reduced tumor mass by 50%–70%</td>
<td>(Kosem and others 2013)</td>
</tr>
<tr>
<td>Her2/CT26 cells (murine colon carcinoma)</td>
<td>Balb/c</td>
<td>α-MG</td>
<td>oral</td>
<td>20 mg/kg</td>
<td>reduced subcutaneous growth</td>
<td>(Kim and others 2012)</td>
</tr>
</tbody>
</table>
Table 1.2. Continued.

<table>
<thead>
<tr>
<th>Cancer cell type</th>
<th>Animal model</th>
<th>Tested compound</th>
<th>Delivery route</th>
<th>Dose</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29 (human colon adenocarcinoma)</td>
<td>Athymic Balb/c</td>
<td>α-MG</td>
<td>diet</td>
<td>900 mg/kg</td>
<td>40% reduction in tumor mass; decreased Bcl-2 and β-catenin</td>
<td>(Chitchumroonchokchai and others 2013)</td>
</tr>
<tr>
<td>Chemically-induced (1,2dimethylhydrazine) colon cancer</td>
<td>F344 rats</td>
<td>α-MG</td>
<td>diet</td>
<td>0.02% and 0.05% in CE-2 basal diet</td>
<td>inhibition of induction and development of ACF; decreased dysplastic foci and β-catenin accumulated crypts; lower proliferating cell nuclear antigen in colon</td>
<td>(Nabandith and others 2004)</td>
</tr>
</tbody>
</table>

**Chemically induced cancer model**
Table 1.3. *In vitro* pro-apoptotic and anti-proliferative activities of mangosteen xanthones.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Tested compound</th>
<th>Dose</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJMC3879 (murine mammary adenocarcinoma)</td>
<td>α-MG</td>
<td>8 µM</td>
<td>induction of apoptosis; cell cycle arrest; activation of caspase-3 and -9; loss of mitochondrial potential</td>
<td>(Doi and others 2009)</td>
</tr>
<tr>
<td>PC3, and 22Rv1 (human prostate carcinoma)</td>
<td>α-MG</td>
<td>2.5–15 µM</td>
<td>suppressed cell viability and colony formation; cell cycle arrest; activation of caspase-3</td>
<td>(Johnson and others 2012)</td>
</tr>
<tr>
<td>COLO205 (human colorectal adenocarcinoma)</td>
<td>mangosteen extract: 48 mg α-MG and 6.40 mg γ-MG/g extract</td>
<td>30 µg/mL</td>
<td>induction of apoptosis; activation of caspase-3 and -8; release of mitochondrial cytochrome c</td>
<td>(Watanapokasin and others 2010)</td>
</tr>
<tr>
<td>HCT116 (human colorectal carcinoma)</td>
<td>extract of mangosteen pericarp (81% α-MG and 16% γ-MG)</td>
<td>10–20 µg/mL</td>
<td>reduced cell viability; increased activities of caspase-3/7 and-9; loss of mitochondrial potential; enhanced activity of MAPK/ERK, Myc/Max and p53 signaling; increased JNK; decreased NF-κB</td>
<td>(Aisha and others 2012)</td>
</tr>
<tr>
<td>NL-17 (murine colon adenocarcinoma)</td>
<td>pericarp methanol extract (25% α-MG)</td>
<td>&gt;25 µg/mL</td>
<td>anti-proliferative activity</td>
<td>(Kosem and others 2013)</td>
</tr>
<tr>
<td>HT-29 (human colon adenocarcinoma)</td>
<td>α-MG</td>
<td>6–12 µM</td>
<td>anti-proliferative activity; decreased Bel2 and β-catenin</td>
<td>(Chitchumroonchokchai and others 2013)</td>
</tr>
</tbody>
</table>
1.3.3 Anti-inflammatory activities

The reported *in vitro* anti-inflammatory activities of mangosteen xanthones are summarized in Table 1.4. α-MG attenuated lipopolysaccharide (LPS)-induced expression of inflammatory mediators such as tumor necrosis factor α (TNF-α) and interleukin (IL-) 6 in human U937 macrophage-like cells. α-MG also decreased activation of several signaling pathways including IL-1, mitogen-activated protein kinase kinase (MEK), JNK, ERK, signal transducer and activator of transcription 1 (STAT-1), and activator protein 1 (AP-1) in these cells (Bumrungpert and others 2010; Liu and others 2012). Concentrations of α-MG used in these studies ranged from 6–12 nM (Liu and others 2012) to 10–30 µM (Bumrungpert and others 2010) and the LPS insult also differed in these reports. Inhibition of activation of MAPK, NF-κB, and AP-1 and attenuation of expression of pro-inflammatory cytokine genes also was observed in LPS-stimulated primary human adipocytes in response to α-MG treatment (Bumrungpert and others 2009b).

α- and γ-MG inhibited nitric oxide (NO) and prostaglandin E₂ (PGE₂) production in murine RAW 264.7 macrophages. These effects were associated with reduced amounts of iNOS inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA (Chen, Yang, Wang 2008; Tewtrakul, Wattanapiromsakul, Mahabusarakam 2009). Suppression of histamine release by α-, β- and γ-MG was observed in IgE-sensitized rat basophilic leukemia RBL-2H3 cells (Itoh and others 2008). γ-MG also dose dependently inhibited basal and A23187-induced release of PGE₂ in C6 rat glioma cells (Nakatani and others 2002). These effects were associated with reduced COX-2 mRNA and protein
expression, and NF-κB activation. Garcinone B also had similar effects in C6 cells by interfering with activation of NF-κB (Yamakuni and others 2006). Contrary to these reports, an aqueous extract containing polyphenolic compounds from mangosteen pericarp stimulated the inflammatory response in cultures of Caco-2 cells treated with IL-1β (Romier-Crouzet and others 2009b). This difference may be due to the absence of the hydrophobic xanthones in the extract.

The *in vivo* anti-inflammatory activities of mangosteen xanthones are summarized in Table 1.5. Early studies showed that both intraperitoneal and oral administration of α-MG, 1-isomangostin, or mangostin triacetate had anti-inflammatory activities in several rat models of inflammation (Shankaranarayan, Gopalakrishnan, Kameswaran 1979). The *in vivo* anti-inflammatory activity of γ-MG has been confirmed in the carrageenan-induced hind paw edema model in rats when the xanthone was administered intraperitoneally 30 minutes prior to inflammatory insult (Nakatani and others 2004). α-MG exhibited similar anti-inflammatory effects with this model (Nguemfo and others 2009). Oral administration of α-MG also inhibited paw edema formation in mice (Chen, Yang, Wang 2008). Orally administered α- and γ-MG also exhibited anti-inflammatory activity in a mouse model of ovalbumin (OVA)-induced allergic asthma. Both xanthones had similar efficacy (Jang and others 2012).

Information about the anti-inflammatory activity of mangosteen xanthones in humans is limited to three reports. Topical application of a gel containing extract of mangosteen pericarp decreased periodontal inflammation suggesting that the formulation may be useful as an adjuvant. However, the xanthone content and composition in the gel was not
reported for this study (Rassameemasmaung and others 2008). Ingestion of a blended mangosteen juice decreased serum C-reactive protein (CRP) levels. However, other markers of inflammation were increased in subjects consuming the mangosteen product compared to placebo (Tang and others 2009). It was also reported that CRP levels in obese subjects consuming 18 oz of a mangosteen juice blend per day for 8 weeks were lower than those in the placebo group. However, levels of the pro-inflammatory interferon-inducible protein 10 (IP-10) and macrophage inflammatory protein-1 β (MIP-1 β) were increased in subjects consuming the high volumes of mangosteen juice blend (Udani and others 2009).

1.3.4 Antimicrobial activities

Preparations of mangosteen pericarp are used in traditional medicine for the treatment of skin infections and wounds (Pedraza-Chaverri and others 2008; Yapwattanaphun and others 2002). In addition, pericarp extracts are added to herbal preparations and cosmetics to prevent or treat acne (Pothitirat, Chomnawang, Gritsanapan 2010). The inhibitory activities of mangosteen xanthones against bacteria, fungi and virus have been previously reviewed (Obolskiy and others 2009; Pedraza-Chaverri and others 2008). For example, α-MG inhibited the growth of pathogenic bacteria, such as *B. subtilis* (IC<sub>50</sub> 3.9 µM), *S. aureus* (IC<sub>50</sub> 7.8 µM), but didn’t affect other pathogens such as *E. coli* and *C. albicans* (IC<sub>50</sub> >200 µM) (Al-Massarani and others 2013). α-MG also has been shown to inhibit the growth of bacterial species such as methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococci*, *Mycobacterium tuberculosis*, and *Helicobacter pylori* at
concentrations in the range of 1.6-12.5 µg/mL (Chin and Kinghorn 2008). A dichloromethane extract of mangosteen pericarp inhibited the growth of *Propionibacterium acnes* and *Staphylococcus epidermidis* with minimum inhibitory concentrations (MIC) of 3.9 and 15.6 µg/mL, respectively (Pothitirat, Chomnawang, Gritsanapan 2010). α-MG had strong inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA) with MIC of less than 1.6 µg/mL (Koh and others 2013).

A crude extract of mangosteen pericarp inhibited the growth of *E. coli*, *Salmonella* spp., *Shigella* spp., and *Vibrio* spp. at MIC ranging from 6.2-100 mg/mL (Sindermsuk and Deekijsermphon 1989). These investigators also reported inhibition of intestinal commensals using similar concentrations. A mangosteen pericarp extract has also been used in a mouthwash preparation to treat periodontal disease (Rassameemasmaung and others 2008). α-MG, along with γ–MG and garcinone B, inhibited *Mycobacterium tuberculosis* (Suksamrarn and others 2003).

Mangosteen xanthones also have antifungal activity against phytopathogenic fungi such as *Fusarium oxysporum vasinfectum*, *Alternaria tenuis*, and *Dreschlera oryzae* (Gopalakrishnan, Banumathi, Suresh 1997). α-MG inhibited *Epidermdophyton floccosum*, *Alternaria solani*, *Mucor* sp., *Rhizopus* sp., and *Cunninghamella echinulata* (Sundaram and others 1983). α-MG and γ–MG also inhibited HIV-1 protease potentially affecting viral replication (Chen, Wan, Loh 1996).
Table 1.4. *In vitro* anti-inflammatory activities of mangosteen xanthones.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Pro-inflammatory insult</th>
<th>Tested compound</th>
<th>Dose</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human U397 macrophage-like cells and primary adipocytes</td>
<td>LPS (100 µg/L) for 3 h</td>
<td>α- and γ-MG</td>
<td>α and γ-MG (2 h-pretreatment) with 10 or 30 µmol/L</td>
<td>α- and γ-MG decreased expression of IL-6, TNF-α, IFN-γ-inducible protein (IP)-10 in macrophage-like cells; decreased phosphorylation of MEK, JNK, ERK and p38; only γ-MG pretreatment attenuated LPS-mediated IκBα degradation; α- and γ-MG pretreatment decreased phosphorylation of c-Jun, Elk-1 and ATF-2; α- and γ-MG attenuated LPS-induced PPAR-γ suppression; γ-MG reduced inflammation and insulin resistance in adipocytes</td>
<td>(Bumrungpert and others 2010)</td>
</tr>
</tbody>
</table>
Table 1.4. Continued

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Pro-inflammatory insult</th>
<th>Tested compound</th>
<th>Dose</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human primary adipocytes</td>
<td>LPS, 10 µg/L for 3 h</td>
<td>α- and γ-MG</td>
<td>α- or γ-MG (24 h pretreatment with 3 µmol/L)</td>
<td>α- and γ-MG attenuated LPS-induced inflammatory gene expression of TNF-α, IL-1β, IL-6, IL-8, MCP-1, and Toll-like receptor-2; α- and γ-MG decreased MAPK activation by suppressing phosphorylation of JNK, p38, and ERK; γ-MG attenuated IκBα degradation and NF-κB activation induced by LPS; xanthones inhibited phosphorylation of c-Jun and transcriptional activity of AP-1; γ-MG blocked LPS-induced suppression of PPARγ (peroxisome proliferator-activated receptor γ) and its target genes</td>
<td>(Bumrungpert and others 2009b)</td>
</tr>
<tr>
<td>Cell type</td>
<td>Pro-inflammatory insult</td>
<td>Tested compound</td>
<td>Dose</td>
<td>Outcomes</td>
<td>Reference</td>
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</tr>
<tr>
<td>Human U397 macrophage-like cells</td>
<td>LPS (0.1 ng/mL) for 4 h</td>
<td>α-MG</td>
<td>6–12 nM for 30 min</td>
<td>α-MG attenuated LPS-stimulated TNF-α secretion by U937 macrophage-like cells and suppressed expression of genes related to immune responses and inflammatory processes such as cytokine production, Th1 and Th2 differentiation, and IL-1 signaling; α-MG decreased activation of p38, ERK1/2, JNK, STAT1, c-Fos and c-Jun</td>
<td>(Liu and others 2009)</td>
</tr>
<tr>
<td>Murine RAW 264.7 macrophage-like</td>
<td>LPS (100 µg/mL)</td>
<td>pericarp ethanol extract, α- and γ-MG</td>
<td>pericarp ethanol extract, (3–100 µg/mL), α- and γ-MG (3–100 µM)</td>
<td>α-MG and γ-MG inhibited NO and PGE₂ production with moderate inhibitory effects on secretion of TNF-α and IL-4; expression of iNOS and COX-2 mRNA suppressed by α-MG; γ-MG inhibited transcription of iNOS</td>
<td>(Tewtrakul, Wattanapiromsakul, Mahabusarakam 2009)</td>
</tr>
<tr>
<td>Murine RAW 264.7 macrophage-like</td>
<td>LPS (0.5–1 µg/mL)</td>
<td>α- and γ-MG</td>
<td>3–25 µM</td>
<td>inhibition of NO and PGE₂ production by α- and γ-MG; iNOS expression reduced by both compounds; COX-2 expression and iNOS enzymatic activity were not affected</td>
<td>(Chen, Yang, Wang 2008)</td>
</tr>
<tr>
<td>Cell type</td>
<td>Pro-inflammatory insult</td>
<td>Tested compound</td>
<td>Dose</td>
<td>Outcomes</td>
<td>Reference</td>
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<tr>
<td>Rat RBL-2H3 basophilic leukemia</td>
<td>bovine serum albumin</td>
<td>α-, β-, and γ-MG</td>
<td>20 µM</td>
<td>α-MG significantly inhibited histamine release and blocked cytoplasmic Ca²⁺ elevation; γ-MG significantly reduced reactive oxygen species; suppressed phosphorylation of Syk, phospholipase C γ1 and γ2 by all mangostins; complete suppression of phosphorylation of Erk ½; JNK ½ and p38 MAPK signaling not altered; slight suppression of p-Akt; decreased phosphorylation of ERK and cytosolic phospholipase A₂</td>
<td>(Itoh and others 2008)</td>
</tr>
<tr>
<td>Rat C6 glioma cells</td>
<td>A23187 calcium ionophore (10 µM)</td>
<td>γ-MG</td>
<td>1–30 µM</td>
<td>inhibition of COX-1 and -2 activities and PGE₂ release by γ-MG; no effects on MAPK/ERK phosphorylation</td>
<td>(Nakatani and others 2002)</td>
</tr>
<tr>
<td>Rat C6 glioma cells</td>
<td>LPS (10 µg/mL)</td>
<td>γ-MG</td>
<td>1–30 µM</td>
<td>inhibition of LPS-induced PGE₂ release, COX-2 mRNA and protein expression; no effect on COX-1; inhibition of IκB kinase activity; inhibition of IκB degradation; decreased NF-κB activation</td>
<td>(Nakatani and others 2004)</td>
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Table 1.4. Continued

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Pro-inflammatory insult</th>
<th>Tested compound</th>
<th>Dose</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat C6 glioma cells</td>
<td>A23187 calcium ionophore (10 µM) and LPS (1 µg/mL)</td>
<td>Garcinone B</td>
<td>10–20 µM</td>
<td>inhibition of COX-1 and COX-2 activities and PGE₂ release; inhibition of IKK activity and NF-κB-dependent transcription</td>
<td>(Yamakuni and others 2006)</td>
</tr>
<tr>
<td>Human Caco-2 enterocyte-like cells</td>
<td>IL-1β (25 µg/mL) and LPS (1 µg/mL) containing polyphenolic compounds</td>
<td>aqueous extract of mangosteen pericarp containing polyphenolic compounds</td>
<td>50 µmol gallic acid equivalents/L</td>
<td>stimulation of basal PGE₂ secretion; no effect on IL-8 secretion or activation of ERK, JNK, and NF-κB</td>
<td>(Romier-Crouzet and others 2009b)</td>
</tr>
</tbody>
</table>
Table 1.5. *In vivo* anti-inflammatory activities of mangosteen xanthones.

<table>
<thead>
<tr>
<th>Model</th>
<th>Tested compound/product</th>
<th>Delivery route</th>
<th>Dose</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rats: carrageenan-induced hind paw edema, cotton pellet implantation, granuloma pouch technique</td>
<td>α-MG, 1-isomangostin, mangostin triacetate</td>
<td>intraperitoneal, oral</td>
<td>50 mg/kg</td>
<td>reduction in paw edema volume, granuloma weight, and granuloma pouch exudate</td>
<td>Shankaranarayan, Gopalakrishnan, Kameswaran 1979</td>
</tr>
<tr>
<td>rat carrageenan-induced hind paw edema</td>
<td>γ-MG</td>
<td>intraperitoneal</td>
<td>10 and 30 mg/kg</td>
<td>concentration dependent inhibition of edema formation</td>
<td>(Nakatani and others 2004)</td>
</tr>
<tr>
<td>rat carrageenan-induced paw edema</td>
<td>α-MG isolated from <em>Allanblackia monticola</em></td>
<td>not specified</td>
<td>9.4 mg/kg</td>
<td>inhibition of edema</td>
<td>(Nguemfo and others 2009)</td>
</tr>
<tr>
<td>mouse carrageenan-induced paw edema</td>
<td>α- and γ-MG</td>
<td>oral</td>
<td>20 mg/kg</td>
<td>inhibition of paw edema formation by α-MG, but not by γ-MG</td>
<td>(Chen, Yang, Wang 2008)</td>
</tr>
<tr>
<td>mouse OVA-induced allergic asthma</td>
<td>α- and γ-MG</td>
<td>oral</td>
<td>10 and 30 mg/kg</td>
<td>both xanthones attenuated inflammatory cell recruitment into the airway; reduced airway hyper-responsiveness; lower levels of Th2 cytokines; attenuated PI3K activity, Akt phosphorylation, and NF-κB activation</td>
<td>(Jang and others 2012)</td>
</tr>
</tbody>
</table>

Continued
**Table 1.5. Continued**

<table>
<thead>
<tr>
<th>Model</th>
<th>Tested compound/product</th>
<th>Delivery route</th>
<th>Dose</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>human subjects with periodontal pockets</td>
<td>pericarp extract</td>
<td>topical</td>
<td>not specified</td>
<td>clinical improvement in periodontal inflammation; subgingival microbial composition altered from diseased to healthy state</td>
<td>(Rassameemasmaung and others 2008)</td>
</tr>
<tr>
<td>healthy adults</td>
<td>mangosteen supplement containing mangosteen juice, vitamins, minerals, aloe vera, and green tea</td>
<td>oral</td>
<td>59 mL/day for 30 days</td>
<td>decreased levels of serum CRP levels; increased ratio of T helper to cytotoxic T cells; elevated serum levels of IL-1α and IL-1β, and complement components C3 and C4</td>
<td>(Tang and others 2009)</td>
</tr>
<tr>
<td>obese subjects</td>
<td>mangosteen juice blend (mangosteen, apple, pear, grape, blueberry, raspberry, strawberry, cranberry and cherry)</td>
<td>oral</td>
<td>6, 12, and 18 oz/day for 8 weeks</td>
<td>Decreased CRP levels in subjects consuming 18 oz of blended juice; increased levels of IP-10 in subjects consuming 6 and 18 oz of blended juice; no differences in F2 isoprostane and IL-12p70 levels; increased MIP-1 beta in subjects ingesting 18 oz blended juice</td>
<td>(Udani and others 2009)</td>
</tr>
</tbody>
</table>
1.3.5 **Modulation of pro-apoptotic, anti-proliferative and anti-metastatic signaling pathways**

A series of reports focused on the mechanisms of anti-proliferative and pro-apoptotic activities of xanthones in cultured cells have appeared recently and are summarized in Table 1.6. Mangosteen xanthones have been shown to mediate their pro-apoptotic effects by activating caspase cascade signaling in various cell types. Furthermore, mangosteen xanthones have been shown to disrupt mitochondrial membrane potential and release of cytochrome c from mitochondria into the cytoplasm. Less evidence and somewhat controversial findings have been reported on the effects of xanthones on other signaling pathways such as ERK1/2 and JNK1/2 with stimulation or inhibition of their activation, depending on cell type. α-MG was also shown to downregulate the levels of p-Akt, a protein kinase associated with cell survival. The anti-proliferative activity of α-MG in colorectal cancer cells were explained by inhibition of TCF/β-catenin transcriptional activity by the xanthone. Less is known about the effects of mangosteen xanthones on the cell cycle, although arrest at the G1 phase and downregulation of cyclins have been demonstrated in several studies.

The anti-metastatic potential of mangosteen xanthones was shown to be mediated by the inhibition of matrix metalloproteinase (MMP) activities which is expected to result in less adhesion, invasion and migration of cancer cells treated with α-MG. This suppressive effect was associated with an inhibition of IκBα degradation, as well as activation of the αvβ3 integrin/FAK/ERK pathway which is one of the main upstream regulators of NF-κB that inhibits its nuclear translocation.
Table 1.6. Modulation of signaling pathways related to apoptosis, cell cycle and metastasis by mangosteen xanthones

<table>
<thead>
<tr>
<th>Biological activity</th>
<th>Target/messenger/process</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apoptosis</strong></td>
<td>*↓p-ERK1/2; ↓p-JNK1/2</td>
<td>chondrosarcoma SW1353</td>
<td>(Krajarng and others 2011)</td>
</tr>
<tr>
<td></td>
<td>↓Ψ&lt;sub&gt;m&lt;/sub&gt;</td>
<td>leukemia HL60; prostate cancer PC12; colorectal cancer DLD-1; melanoma SK-MEL-28; colorectal HCT116; malignant glioblastoma GBM 8401</td>
<td>(Aisha and others 2012; Chang and others 2010; Matsumoto and others 2004; Nakagawa and others 2007; Sato and others 2004; Wang, Sanderson, Zhang 2011)</td>
</tr>
<tr>
<td></td>
<td>↑Caspase-3</td>
<td>chondrosarcoma SW1353; colon cancer COLO205; leukemia HL60; prostate cancer PC12; melanoma SK-MEL-28; colorectal HCT116; breast cancer MDA-MB231</td>
<td>(Aisha and others 2012; Krajarng and others 2011; Kurose and others 2012; Matsumoto and others 2004; Sato and others 2004; Wang, Sanderson, Zhang 2011; Watapokasin and others 2010)</td>
</tr>
<tr>
<td></td>
<td>↑Caspase-8</td>
<td>chondrosarcoma SW1353; colon cancer COLO205; breast cancer MDA-MB231</td>
<td>(Krajarng and others 2011; Kurose and others 2012; Watapokasin and others 2010)</td>
</tr>
<tr>
<td></td>
<td>↓Bcl-2; ↑Bax</td>
<td>chondrosarcoma SW1353</td>
<td>(Krajarng and others 2011)</td>
</tr>
<tr>
<td></td>
<td>↑cytochrome c release</td>
<td>chondrosarcoma SW1353; colon cancer COLO205; leukemia HL60; prostate cancer PC12; breast cancer MDA-MB231</td>
<td>(Krajarng and others 2011; Kurose and others 2012; Matsumoto and others 2004; Sato and others 2004; Watapokasin and others 2010)</td>
</tr>
</tbody>
</table>

Continued
<table>
<thead>
<tr>
<th>Biological activity</th>
<th>Target/ messenger/process</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>↓Akt</td>
<td>chondrosarcoma SW1353; colorectal cancer DLD-1</td>
<td>(Krajarng and others 2011; Nakagawa and others 2007)</td>
</tr>
<tr>
<td></td>
<td>†p-JNK1/2</td>
<td>prostate cancer PC12; colorectal cancer DLD-1</td>
<td>(Nakagawa and others 2007; Sato and others 2004)</td>
</tr>
<tr>
<td></td>
<td>†Endonuclease G</td>
<td>colorectal cancer DLD-1</td>
<td>(Nakagawa and others 2007)</td>
</tr>
<tr>
<td></td>
<td>†p-ERK1/2</td>
<td>colorectal cancer DLD-1 and HCT116</td>
<td>(Nakagawa and others 2007; Watapokasin and others 2010)</td>
</tr>
<tr>
<td></td>
<td>†microRNA-143</td>
<td>colorectal cancer DLD-1</td>
<td>(Nakagawa and others 2007)</td>
</tr>
<tr>
<td></td>
<td>↓NF-κB</td>
<td>colorectal HCT116</td>
<td>(Watapokasin and others 2010)</td>
</tr>
<tr>
<td></td>
<td>†Myc, Max, p53</td>
<td>colorectal HCT116</td>
<td>(Watapokasin and others 2010)</td>
</tr>
<tr>
<td></td>
<td>↓β-catenin</td>
<td>colorectal HCT116 and SW480</td>
<td>(Yoo and others 2011)</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>G1 arrest</td>
<td>colorectal cancer DLD-1; melanoma SK-MEL-28; breast cancer MDA-MB231</td>
<td>(Kurose and others 2012; Matsumoto and others 2005; Wang, Sanderson, Zhang 2011)</td>
</tr>
<tr>
<td></td>
<td>↓cyclins, cdc2</td>
<td>colorectal cancer DLD-1; breast cancer MDA-MB231</td>
<td>(Kurose and others 2012; Matsumoto and others 2005)</td>
</tr>
<tr>
<td>Metastasis</td>
<td>↓MMP-2, MMP-9</td>
<td>prostate carcinoma PC-3; breast adenocarcinoma MCF-7; lung adenocarcinoma A549</td>
<td>(Hung and others 2009; Lee and others 2010; Shi and others 2010)</td>
</tr>
<tr>
<td></td>
<td>↓u-PA⁺</td>
<td>prostate carcinoma PC-3</td>
<td>(Hung and others 2009)</td>
</tr>
<tr>
<td></td>
<td>↓p-JNK1/2</td>
<td>prostate carcinoma PC-3</td>
<td>(Hung and others 2009)</td>
</tr>
</tbody>
</table>
### Table 1.6. Continued

<table>
<thead>
<tr>
<th>Biological activity</th>
<th>Target/messenger/process</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastasis</td>
<td>↓NF-κB</td>
<td>prostate carcinoma PC-3; breast adenocarcinoma MCF-7; lung adenocarcinoma A549</td>
<td>(Hung and others 2009; Lee and others 2010; Shi and others 2010)</td>
</tr>
<tr>
<td></td>
<td>↓AP-1</td>
<td>prostate carcinoma PC-3; breast adenocarcinoma MCF-7</td>
<td>(Hung and others 2009; Lee and others 2010; Shi and others 2010)</td>
</tr>
<tr>
<td></td>
<td>↓p-ERK1/2</td>
<td>breast adenocarcinoma MCF-7; lung adenocarcinoma A549</td>
<td>(Hung and others 2009; Lee and others 2010; Shi and others 2010)</td>
</tr>
<tr>
<td></td>
<td>↓ανβ3 integrin/FAK</td>
<td>lung adenocarcinoma A549</td>
<td>(Hung and others 2009; Lee and others 2010; Shi and others 2010)</td>
</tr>
</tbody>
</table>

*↓, decrease; ↑, increase; ↓Ψ<sub>m</sub>: loss of mitochondrial membrane potential; *u-PA: urokinase-plasminogen activator*
1.4 Inflammation

Inflammation is a normal biological process in response to infection, tissue injury or chemical irritation. Redness, swelling, heat and pain are the cardinal signs of acute inflammation. When controlled properly, inflammation is a defense mechanism to maintain health and homeostasis. It is self-limiting and resolves rapidly due to negative feedback mechanisms that include the secretion of anti-inflammatory cytokines, the termination of pro-inflammatory signaling events, and the activation of regulatory cells (Calder and others 2009). Innate immune responses are the first immediate defense against infections in mammals. Macrophages have an important role in innate immunity as they secrete cytokines that stimulate inflammation to protect the host against foreign organisms, harmful substances and tissue injury (Gordon 2007). Mast cells, dendritic cells, and natural killer cells also participate in the innate immune response (Xu and Schauss 2012). Through the secretion of chemical mediators such as pro-inflammatory cytokines, chemoattractants (chemokines), and reactive oxygen species, the adaptive immune response is initiated. T and B lymphocytes are recruited and work in conjunction with innate immune cells and chemical mediators to eliminate the pathogen and/or repair the tissue, which terminates the inflammatory response.

In contrast, prolonged and dysregulated inflammation is associated with development of chronic diseases such as cancer, atherosclerosis, inflammatory bowel diseases, obesity, diabetes, and cardiovascular disease (Ferguson and Laing 2010; Hanahan and Weinberg 2011; Lee, Lee, Choue 2013; Libby 2012). Epidemiological evidence attributes 25% of all cancer to chronic inflammation (Balkwill and Mantovani 2001). Thus, attenuation of
the synthesis and secretion of pro-inflammatory mediators by preventive agents is expected to limit the initiation and propagation of immunologic and inflammatory responses. Epidemiological evidence suggests that diets that are rich in fruit and vegetables prevents or may delay the onset of chronic disease (Carter and others 2010; Dauchet and others 2006; Hung and others 2004; Riboli and Norat 2003). These effects are in part associated with attenuation of inflammatory processes by dietary components (Wu and Schauss 2012).

1.4.1 Inflammation and diet

In vitro and in vivo studies have shown that diet can protect against inflammation (Xu and Schauss 2012). Dietary components play a major role in modulating inflammation as evidenced by epidemiological and clinical studies. For instance, many studies support an inverse correlation between fruit and vegetable consumption and inflammatory status. Many of the health benefits associated with fruit and vegetable consumption have been attributed to plant-derived compounds possessing anti-inflammatory activities (Romier-Crouzet and others 2009). Among these compounds, polyphenols have been shown to inhibit enzymes involved in the inflammatory response, such as iNOS (inducible nitric oxide), COX-2 (cyclooxygenase-2), and LOX (lipoxygenase). Polyphenols have also been shown to inhibit the production of pro-inflammatory cytokines and cell adhesion molecules (Pan, Lai, Ho 2010). Further information on specific examples on the anti-inflammatory activity of polyphenols and their possible mechanisms are available in several comprehensive reviews (Romier-Crouzet and others 2009; Gonzalez and others...
2011). It is important to remember that dietary components also have the potential to promote inflammation (Basu, Devaraj, Jialal 2006).

1.4.2 Inflammatory bowel disease (IBD)

Crohn’s disease (CD) and ulcerative colitis (UC) are the two major forms of inflammatory bowel disease (IBD). While CD is associated with a non-continuous transmural inflammation of the gastrointestinal tract and the development of strictures, abscesses and fistulas, UC involves mucosal and submucosal inflammatory conditions limited to the colon with the presence of cryptitis and crypt abscesses (Khor, Gardet, Xavier 2011). Bloody diarrhea is a hallmark of UC. Incidence of UC is higher than that of CD and its prevalence in North America and northern Europe ranges from 156-291 cases per 100 000 people (Ordás and others 2012). Overall, these disorders are characterized by a persistent progressive or relapsing inflammatory state which is associated with an increased risk for the development of colon cancer. In fact, UC patients have up to 8 times-higher relative risk of developing colorectal cancer compared to the general population (Clapper, Cooper, Chang 2007). It has become evident that genetic and environmental factors play a critical role in the pathogenesis of IBD (Khor, Gardet, Xavier 2011). The innate immune system is among the key players in the initiation and progression of the disease, along with its interaction with the commensal microbiota and the adaptive immune system. Conventional medical therapies for the treatment of IBD have only modest success and are associated with many adverse side effects (Giacomo and others 2008). This likely contributes to the use of complementary
and alternative medicines such as herbal preparations by as many as 50% of patients with the disease (Rahimi, Mozaffari, Abdollahi 2009).

1.4.2.1. Models of IBD

Different experimental models of IBD have been developed over the last few years and are divided into broad categories (i.e., chemically induced, genetically engineered, cell transfer and spontaneous) depending on the tools used to induce the condition (Mizoguchi 2012). These categories define key cellular and molecular mechanisms of host microbiota crosstalk in the gastrointestinal tract.

Genetically engineered murine models such as IL-10\(^{-/-}\) and IL-12\(^{-/-}\) have been proven useful for elucidating disease pathogenesis. However, disease in the IL-10\(^{-/-}\) model affects not only the colon but also the small intestine, while systemic disease is developed in the IL-12\(^{-/-}\) mouse model (Kuhn and others 1993; Sadlack and others 1993). In addition, the length of time required for development of IBD, as well as reliance on very specific engineered pathways in these models, limits their applicability for testing of potential therapeutic agents. Although useful for identifying susceptible genes in IBD (Kosiewicz and others 2001; Sundberg and others 1994), the purchase and maintenance of the spontaneous models C3H/HeJBir and SAMP1/Yit is expensive (Mizoguchi 2012).

Chemically induced IBD remains the most commonly used protocol for studying possible therapies. In these models, administration of a chemical irritant such as dextran sulfate sodium (DSS) or trinitrobenzene sulfonic acid (TNBS) induces damage to the epithelium. Colitis is induced by administration of 2-5% DSS (30-50 kDa) in the drinking
water for 2-5 days (Boismenu and Chen 2000). Earlier studies suggested that a single cycle of DSS exposure would lead to acute colitis, while several cycles of DSS exposure were necessary for development of chronic colitis (Dieleman and others 1998). However, recent research shows that a single cycle of DSS administration is sufficient for the induction of chronic colitis in C57BL/6 mice (Melgar, Karlsson, Michaëlsson 2005).

**Table 1.7** presents key characteristics of the DSS model. Recent preclinical studies have shown the relevance of this model and supported its use as a system to study the role of chronic inflammation in the development of colorectal cancer given its similarities to the disease in humans (Clapper, Cooper, Chang 2007). Although the degree of inflammation in the DSS model tends to vary greatly from mouse to mouse and from region to region in an individual colon, such variability is also present in human IBD (Mizoguchi 2012).

DSS-induced colitis is one of the most commonly used models for evaluation of therapeutic agents due to its advantages over other systems such as time, cost, and similarity to many of the outcomes associated with the human disease (Clapper, Cooper, Chang 2007). Several factors affect the susceptibility to DSS-induced colitis, namely, murine strain, gender, microbiota, the molecular weight, concentration and batch of DSS, as well as the duration of administration of the chemical insult (Perse and Cerar 2012). For instance, DSS induces chronic colitis in the C57BL/6J mouse strain, but only acute inflammation and injury occurs when Balb/c mice are administered DSS (Melgar, Karlsson, Michaëlsson 2005). Furthermore, female mice are less sensitive to DSS insult than males (Ding and others 2012).
Table 1.7. Characteristics of the DSS model.

<table>
<thead>
<tr>
<th>Species</th>
<th>Response</th>
<th>Pathogenic factors</th>
<th>Localization</th>
<th>Key outcomes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>mice, rats,</td>
<td>acute: innate</td>
<td>genetic, microbial flora,</td>
<td>continuos,</td>
<td>epithelium damage, body weight loss, bloody diarrhea, rectal bleeding, ulceration, shortening of the colon, infiltration of pro-inflammatory cells, production of pro-inflammatory molecules</td>
<td>(Boismenu and Chen 2000; Clapper, Cooper, Chang 2007; Dieleman and others 1998; Okayasu and others 1990)</td>
</tr>
<tr>
<td>hamsters,</td>
<td>immunity</td>
<td></td>
<td>colonic mucosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>guinea-pigs</td>
<td>chronic: innate and adaptive immunity (Th1 and Th2 response)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.5 The gut microbiota and its role in health and disease

The gastrointestinal (GI) tract is inhabited by a collection of bacteria, viruses, and single-cell eukaryotes commonly referred to as the gut microbiota. These microbes play an essential role in the development of immunity both at the intestinal mucosa and systemic levels, and in the functional and structural maturation of the GI tract. In addition, the microbiota provides a protective barrier against pathogens by competing for nutrients and space and through the production of antimicrobial compounds. The microbiota also plays a critical role in the metabolism of dietary nutrients and xenobiotics, such as phytochemicals and drugs (Sekirov and others 2010). Consequently, an imbalance between putative ‘protective’ versus ‘harmful’ intestinal bacteria, also known as dysbiosis, is associated with diseased states (Ley and others 2006; Turnbaugh and others 2006; Castellarin and others 2012; Kostic and others 2012; Larsen and others 2010).

The gut microbiota of humans and mice are considered to share broad similarities as the Firmicutes and Bacteroidetes phyla are the dominant populations in both, with low abundances of phyla such as Actinobacteria and Proteobacteria (Eckburg and others 2005; Ley and others 2005). Alterations in the composition of intestinal bacterial communities, such as reduced abundance of Firmicutes and Bacteroidetes and increased Proteobacteria, are present in individuals with inflammatory bowel diseases (IBD) (Frank and others 2007; Ott and others 2004; Lupp and others 2007). Changes at the phylum level have been observed in obese humans and mice with an increased relative abundance of Firmicutes (Castellarin and others 2012) and decreased relative abundance of
Bacteroidetes (Ley and others 2005; Ley and others 2006). Interestingly, an increased capacity for harvesting energy from the diet is associated with this ‘obese microbiome’ (Turnbaugh and others 2006). Furthermore, a single member of the gut microbiome, *Fusobacterium nucleatum*, has been correlated with colorectal cancer and is directly associated with increased risk of metastasis (Castellarin and others 2012). In addition, decreased abundance of Firmicutes and Bacteroidetes is also found in colorectal tumors (Kostic and others 2012). Reduced Firmicutes and increased Betaproteobacteria were also detected in adults with Type 2 diabetes compared to non-diabetic subjects. (Larsen and others 2010). Whether these changes are the cause or the result of the disease remains elusive.

1.5.1 The effect of diet on the gut microbiota

Dietary factors have been shown to affect the composition of the gut microbiota with changes detected as soon as within 24h of dietary intervention (Turnbaugh and others 2009; David and others 2014). Studies in germ-free mice colonized with human fecal microbiota show that switching from a low fat/high fiber diet to a ‘Western’ diet high in fat and sugar alters the composition of the microbiome by increasing the abundance of members of the Firmicutes, such as Erysipelotrichi and Bacilli, while decreasing the abundance of Bacteroidetes (Turnbaugh and others 2009). In humans, ingestion of an animal-based diet (high in fat and protein) increases the abundance of bile-tolerant bacteria such as *Bacteroides* and *Alistipes*, and decreases that of polysaccharide-metabolizing members of the Firmicutes such as *Ruminococcus bromii*, in comparison to
consumption of a plant-based (high in fiber and low in protein/fat) diet (David and others 2014).

The influence of diet on the composition of the microbiome is perhaps best exemplified by the effects of prebiotics, i.e., selectively fermented dietary ingredients with the ability to change the activity and composition of the microbiota and promote health (Gibson and others 2004). Ingestion of prebiotics, such as low digestible carbohydrates like inulin and fructo-oligosaccharides, has been associated with an increase in bifidobacteria in humans (Ramirez-Farias and others 2009). Fermentation of soluble dietary fiber by the colonic microflora stimulates the synthesis of short chain fatty acids (SCFA) such as acetate, propionate, and butyrate, which serve as a source of energy for colonocytes, lower colonic pH, and exert anti-inflammatory activities (Cummings 1981). Like prebiotics, other dietary components not absorbed in the small intestine are potential substrates for bacterial metabolism. These bacterial metabolites, however, may not only exert beneficial health effects but can potentially have adverse consequences (Blaut and Clavel 2007).

Diets rich in fruits and vegetables, for instance, have been associated with a healthy gut. This effect has been attributed in part to polyphenols. These plant-derived secondary metabolites have antioxidant and antimicrobial activities in the plant (Bravo 1998). Recent evidence suggests that dietary polyphenols have health-promoting activities that include anti-oxidant, anti-apoptotic, anti-carcinogenic, anti-inflammatory, and anti-angiogenic properties (Del Rio and others 2013). Although many polyphenols are poorly absorbed, their concentrations in the lumen of the GI tract can be as high as several
hundred micromolar (Scalbert and Williamson 2000). Polyphenols are therefore potential substrates of the colonic microbiota, and in fact, some bacterial metabolites of polyphenols have been found to exert bioactivities originally attributed to the parent compound (Espin and others 2013; Possemiers and others 2006; Bolca, Van de Wiele, Possemiers 2013). Because polyphenols also exert inhibitory effects on the growth of pathogenic bacteria and fungi, as well as viruses (Daglia 2012; Taguri, Tanaka, Kouno 2006), they can also adversely affect the composition of the non-pathogenic gut microbiota. *In vitro* screening of pure bacterial cultures revealed that quercetin and naringenin exhibited a dose dependent inhibitory effect of the growth of *Bacteroides galacturonicus, Lactobacillus spp.*, *Enterococcus ceccae, Bifidobacterium catenulatum, Ruminococcus gauvreauii*, and *Escherichia coli*. In the same study, hesperetin affected the growth of *Bifidobacterium catenulatum, R. gauvreauii*, and *Bacteroides galacturonicus* (Duda-Chodak 2012). In another study using a simulator of the intestinal microbial ecosystem (SHIME), a black tea extract exerted antimicrobial activity against bifidobacteria, *Blautia coccoides, Anaeroglobus*, and *Victivallis*, while stimulating the growth of *Klebsiella spp.*, Enterococcii and *Akkermansia*. This study also reported that a red wine grape extract inhibited bifidobacteria, *Bacteroides, Blautia coccoides, Anaeroglobus*, and *Subdoligranulum*, and promoted the growth of *Klebsiella spp.*, *Alistipes, Cloacibacillus, Victivallis*, and *Akkermansia*. (Kemperman and others 2013). Of note is the stimulation of the Proteobacteria member, *Klebsiella*, a commensal opportunistic pathogen, by both polyphenolic-rich extracts. The mechanisms by which
polyphenols influence the composition of non-pathogenic microbial communities in the gut remain elusive.

1.6 Dissertation Research

1.6.1 Overview and main research objectives

The health-promoting properties of mangosteen pericarp have been associated with a family of compounds referred to as xanthones. The use of supplements containing mangosteen has increased greatly in recent years as a result of aggressive marketing of their proposed health benefits. Mangosteen juice, for instance, has been promoted as beneficial for gastrointestinal health and immune system function. However, scientific evidence supporting these and other claims is lacking. In addition, delivery of xanthones and/or their metabolites to target tissues is necessary for bioactivity. α-MG is selected as the xanthone for investigation as it is the most abundant (>70%) and best characterized of these compounds in mangosteen pericarp. There is limited data on the metabolism and anti-inflammatory activity of α-MG in human cells. Available data are currently limited to anti-inflammatory effects of α-MG on human adipocytes and the U937 macrophage-like cell line (Bumrungpert and others 2009b; Bumrungpert and others 2010). Assessment of the possible anti-inflammatory activity on human cells of other tissue origins has not been performed. In addition, no information exists on the in vivo effect of α-MG in the context of UC. Other than a previous single report from our lab (Bumrungpert and others 2009a), there is no information on the metabolism of this
xanthone by human cells. Whether the metabolism of α-MG differs between normal and transformed cells, or the possible effect that a pro-inflammatory condition may have on the metabolism of this xanthone has not been examined to the best of my knowledge. The impact of the antimicrobial activities of α-MG (Pedraza-Chaverri and others 2008) on the gut microbiota has not been previously considered. My dissertation research provides novel insights into the metabolism of α-MG by human cells, as well as its role in modulating the response to an inflammatory insult by transformed versus normal primary human cells. It is also the first study to evaluate the effect of dietary α-MG in a well-established mouse mode of UC and investigate the impact of this xanthone on the gut microbiota. This information is expected to be of marked interest to researchers developing functional foods, physicians caring for UC patients, consumers concerned with prevention of inflammation, and individuals currently using complementary and alternative medicines for the prevention and treatment of inflammatory disorders.

1.6.2 Central hypothesis

The central hypothesis is that α-MG and/or its metabolites exert anti-inflammatory activity.

1.6.3 Overview of specific aims

Aim 1: To evaluate the metabolism and anti-inflammatory activity of α-MG on primary and transformed human cells. I treated cultures of prototypic and non-professional human cell lines with phenotypes that engage in host defense, as well as
primary human monocyte-derived macrophages, with α-MG prior to activating with pro-inflammatory mediators. I collected spent media and cells after 24h to analyze concentrations of inflammatory mediators and the extent of xanthone metabolism. Analyses were also performed in cultures treated with α-MG in the absence of pro-inflammatory conditions.

**Aim 2:** To determine the *in vivo* anti-inflammatory activity of α-MG using a mouse model of ulcerative colitis. I tested the effect of dietary α-MG on chemically-induced colitic C57BL/6 mice. The effects of the dietary intervention on gross pathology, markers of systemic inflammation, and epithelial injury and immune cell infiltration in the colon were assessed. Potential effects of dietary α-MG on the cecum and colon of healthy mice were also evaluated.

**Aim 3:** To determine the effect of dietary α-MG on gut microbiota and histology of colon and cecum in four strains of mice. The gut microbiota in cecum and colon of C57BL/6, Balb/c, C3H and FoxN1
 strains of mice was analyzed after feeding diet with α-MG or control diet for four weeks. Histological profiles of the colon and cecum were also studied and included epithelial cell proliferation and immune cell infiltration.
CHAPTER 2

α-MANGOSTIN: ANTI-INFLAMMATORY ACTIVITY AND METABOLISM
BY HUMAN CELLS*


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2.1 Abstract

Information about the anti-inflammatory activity and metabolism of α-mangostin (α-MG), the most abundant xanthone in mangosteen fruit, in human cells is limited. Based on available literature, we hypothesized that α-MG will inhibit the secretion of pro-inflammatory mediators by control and activated macrophage-like THP-1, hepatic HepG2, enterocyte-like Caco-2, and colon HT-29 human cell lines, as well as primary human monocyte-derived macrophages (MDM), and that such activity would be influenced by the extent of metabolism of the xanthone. α-MG attenuated TNF-α and IL-8 secretion by the various cell lines, but increased TNF-α output by both quiescent and LPS-treated MDM. The relative amounts of free and phase II metabolites of α-MG and other xanthones present in media 24h after addition of α-MG was shown to vary by cell type and inflammatory insult. Increased transport of xanthones and their metabolites across Caco-2 cell monolayers suggests enhanced absorption during an inflammatory episode. The anti-inflammatory activities of xanthones and their metabolites in different tissues merit consideration.

2.2 Introduction

Garcinia mangostana is a tree native to Southeast Asia that produces a fruit referred to as mangosteen. The aril portion of mangosteen fruit has an acidic sweet taste that is enjoyed by many, whereas extracts of the pericarp have been used in traditional medicine. The proposed health promoting properties of pericarp from mangosteen have been associated with a family of compounds referred to as xanthones (Pedraza-Chaverri
and others 2008). These hydrophobic compounds have a tricyclic aromatic ring system possessing various mixtures of isoprenyl, hydroxyl and methoxyl substitutions (Obolskiy and others 2009). α-Mangostin (α-MG, Figure 2.1) and γ-mangostin (γ-MG) are the most abundant xanthones in the pericarp of mangosteen fruit (Chaivisuthangkura and others 2009; Walker 2007). *In vitro* studies have consistently shown xanthones to possess antioxidant (Jung and others 2006; Williams and others 1995), anti-proliferative (Akao and others 2008), pro-apoptotic (Matsumoto and others 2003; Nakagawa and others 2007), antimicrobial (Suksamrarn and others 2003), anti-inflammatory (Chen, Yang, Wang 2008; Tewtrakul, Wattanapiromsakul, Mahabusarakam 2009), and anticarcinogenic activities (Lee and others 2010; Matsumoto and others 2003; Nakagawa and others 2007). Anti-inflammatory (Chen, Yang, Wang 2008; Jang and others 2012; Nakatani and others 2004; Shankaranarayan, Gopalak, Kameswaran 1979) and anticarcinogenic (Chitchumroonchokchai and others 2013; Johnson and others 2012; Shan and others 2011) activities have also been demonstrated in rodents. As a result of the aggressive marketing of health-promoting activities observed in cellular and rodent models, numerous supplements, beverages and food products containing mangosteen fruit have become available with sales of beverages alone in 2008 exceeding $200 million in the United States (Sloan 2010).
Figure 2.1. Structure of α-mangostin.
In order for xanthones to exert their proposed health-promoting activities these compounds or their active metabolites must be delivered to target tissues. We previously reported that α-MG and its phase II metabolites were transported across the basolateral membrane of Caco-2 human intestinal cells suggesting that a portion of xanthones in mangosteen products likely were bioavailable (Bumrungpert and others 2009a). Indeed, low concentrations of xanthones and their phase II metabolites have been identified in the plasma and urine of healthy adults after consumption of mangosteen juice (Chitchumroonchokchai and others 2012; Kondo and others 2009). Xanthones and phase II metabolites also have been detected in the plasma and liver of athymic Balb/c nu/nu mice fed an AIN-93G diet containing 900 mg xanthones/kg (Chitchumroonchokchai and others 2013), as well as in plasma from C57BL/6J mice orally dosed with α-MG (Ramaiya, Petiwala, Johnson 2012). Moreover, the presence of α-MG and other xanthones in the HT-29 human colon cell xenografts in mice fed the diet containing α-mangostin was associated with decreased tumor growth and reduced tumor expression of the mitogenic Wnt protein and anti-apoptotic bcl-2 protein (Chitchumroonchokchai and others 2013). These data suggest that xanthones and/or their metabolites are absorbed and delivered to various tissues where they may be accumulated, further metabolized and modulate cellular processes.

The anti-proliferative and pro-apoptotic activities of xanthones have been demonstrated in numerous in vitro studies using rodent cell lines (Akao and others 2008; Matsumoto and others 2003; Nakagawa and others 2007; Chen, Yang, Wang 2008; Tewtrakul, Wattanapiromsakul, Mahabusarakam 2009). However, the reported anti-
inflammatory activity of xanthones in cells of human origin has been limited to primary cultures of adipocytes (Bumrungpert 2009b) and the U937 macrophage-like cell line (Bumrungpert 2010). To the best of our knowledge, the uptake and metabolism of these compounds by cells of animal or human origin have not been examined with the exception of differentiated cultures of Caco-2 human intestinal cells (Bumrungpert 2009a). Similarly, the effect that the pro-inflammatory condition may have on the metabolism of α-MG has not been addressed.

Although it has been generally assumed that xanthones are stable in cell culture media, many polyphenols degrade spontaneously in vitro and generate products such as hydrogen peroxide that is known to induce transcriptional activity associated with the anti-inflammatory response (Halliwell 2009; Long, Hoi, Halliwell 2010). The first objective of the present study was to investigate the anti-inflammatory activity of α-MG in prototypical human immune cell types and in other human cells originating from tissues responsive to inflammatory insult. This initially required developing appropriate delivery systems to ensure the stability of the xanthone in the different culture media used for the proliferation and activities of the tested cell types. The second objective was to study the metabolism of this xanthone by these cell types maintained in normal and pro-inflammatory environments. To the best of our knowledge, this work is the first to systematically compare the anti-inflammatory activity of α-MG for human cells with various tissue origins, as well as the metabolism of this xanthone under normal and inflammatory conditions.
2.3 Materials and methods

2.3.1 Chemicals and reagents

α-, γ-, and β-mangostins, 9-hydroxycalabaxanthone, gartanin, and garcinones D and E were purified (>98% as assessed by NMR spectroscopy and ESIMS) as described previously (Chaivisuthangkura and others 2009; Jun and others 2006). All solvents (acetonitrile, ethyl acetate, acetic acid, n-butanol, isopropanol) and water were HPLC grade from Fisher Scientific (Pittsburgh, PA). Cell culture reagents were from Sigma-Aldrich (St. Louis, MO) and Gibco (Grand Island, NY). IL-8 and TNF-α ELISA DuoSet Kits were purchased from R&D Systems (Minneapolis, MN). MCS-F was obtained from Peprotech, Inc. (Rocky Hill, NJ). Fluorochrome-conjugated anti-CD14 and anti-CD11b were purchased from BD Biosciences (San Jose, CA). Ficoll-Paque PLUS® was obtained from GE Healthcare (Uppsala, Sweden). Transwell® inserts and filters were from Millipore (Billerica, MA). All other reagents and materials were from Sigma-Aldrich.

2.3.2 Cell cultures

The following human cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) for use in this study: THP-1 (monocyte-like leukemia); HepG2 (hepatocellular carcinoma); Caco-2 HTB-37 (colorectal adenocarcinoma cells that spontaneously differentiate to enterocyte-like phenotype); and HT-29 (colorectal adenocarcinoma). Since α-MG has been reported to exert anti-inflammatory activity in murine RAW 264.7 macrophage-like cells (Chen, Yang, Wang 2008; Tewtrakul, Wattanapiromsakul, Mahabusarakam 2009), this cell line was included
for comparison with the responses with the human cell types. HepG2 cells (passages 5-15), HT-29 cells (passages 135-139) and RAW 264.7 (unknown passage) were maintained as per ATCC recommendations and used for experiments two days after monolayers reached confluency. THP-1 monocyctic cells (unknown passage) were differentiated to macrophage-like cells by treatment with 100 nM phorbol myristate acetate (PMA) for 48h before initiating experiments. Caco-2 cells (passages 20-40) spontaneously differentiated to a phenotype resembling the intestinal epithelium by culturing by 21–25 day post-confluency as previously described (Chitchumroonchokchhai, Schwartz, Failla 2004). Primary cultures of monocytes were prepared from buffy coats obtained from the American Red Cross (Columbus, OH). Mononuclear cells were separated by centrifugation through a Ficoll-Paque PLUS and further purified by the clumping method (Shen and others 1986). Monocytes were differentiated to macrophages by treatment with 50 ng/mL M-CSF for 6 days. Monocyte-derived macrophages (MDM) were 97% pure as estimated by flow cytometry after staining cells with anti-CD14 and anti-CD11b as cellular markers. All cultures were maintained at 37 °C in humidified atmosphere of 95% air/5% CO₂.

2.3.3 Stability of α-MG during incubation in the cell culture environment

As many polyphenolic compounds spontaneously degrade in cell culture media (Halliwell 2009; Long, Hoi, Halliwell 2010), we first examined the stability of α-MG solubilized in DMSO and added to the various media lacking FBS. Stability was assessed by incubation in a cell free system for 24h. Aliquots were collected at 0, 4, 8, and 24h
before extraction and analyzed as described below. Recovery of α-MG from all basal media decreased in proportion to duration of incubation in the absence of cells (Figure 2.2A). The extent of loss after 24h ranged from 55-97% depending on the composition of the medium. The calculated rate of α-MG loss per hour during the 24h incubation was 2.7, 4.0, 3.2, and 2.3% in RPMI, MEM, DMEM, and McCoy’s 5A, respectively.

Based on the above observation, the influence of delivery vehicle and the presence of FBS in media were tested as possible stabilizers of α-MG during incubation in cell-free media. Dimethyl sulfoxide (DMSO), Tween 40 and FBS were used to prepare stock solutions of the xanthone for addition to media. A stock solution of α-MG was prepared in DMSO before dilution in MEM, DMEM, and RPMI media containing 10% FBS and filter sterilized (0.22 µm pores). The final amount of DMSO in the test media was <0.03%. To prepare Tween 40 micelles containing α-MG, the compound was solubilized in acetonitrile and mixed with 20% Tween 40:80% acetone before evaporating the solvent under a stream of nitrogen gas. After addition of basal media (either DMEM or McCoy’s 5A), the mixture was sonicated for 30 min to facilitate incorporation of the xanthone in the Tween micelles. Media were filtered (0.22 µm pores) and further diluted to obtain indicated concentrations of the xanthone. The final content of Tween 40 in test media was <0.02%. FBS was also used as delivery vehicle by incubating pure α-MG with 100% FBS for 48h at 37 °C in a shaking water bath (85 rpm). This stock solution was then filtered (0.22 µm pores) and diluted 1:10 with RPMI 1640. The stability of α-MG during the 24h incubation period was increased to 101±6% (p<0.05) when DMSO containing the xanthone was added to media (RPMI 1640, MEM and DMEM) with 10%
(v/v) FBS. Similarly, recovery of α-MG from DMEM and McCoy’s media without FBS after 24h incubation was 110±3 and 108±4, respectively when the xanthone was incorporated in Tween 40 micelles. Stability of α-MG was 95±8% after 24h when the xanthone was added to 100% FBS that was diluted 1:10 (v:v) into RPMI 1640 (Figure 2.2B). Subsequently, α-MG solubilized in DMSO was delivered to medium containing 10% FBS for macrophage-like THP-1, macrophage-like RAW 264.7 and HepG2 cells, and α-MG was solubilized in Tween 40 micelles added to medium for enterocyte-like Caco-2 cells and HT-29 colonic cells. For MDM cells, α-MG was delivered in 100% FBS to a final concentration of 10% FBS in RPMI 1640 medium.
Figure 2.2. Stabilization of α-MG in basal media. Lack of stability of α-MG in basal media in cell-free dishes (panel A) was offset by addition of the xanthone solubilized in DMSO to media containing 10% FBS or by addition of the xanthone incorporated in Tween 40 to basal media (panel B). Data are mean ± SD for n=3 dishes with media.
2.3.4 Cytotoxicity of α-MG

Cell cultures were exposed to increasing concentrations of α-MG to determine cytotoxicity. Viability of RAW 264.7, MDM, HepG2 and HT-29 was assessed after 24h, whereas that of THP-1 and Caco-2 cells was determined after 8h and 4h, respectively. Times of exposure matched those used in experiments addressing anti-inflammatory activity and metabolism described below. Examination of cell morphology by phase contrast microscopy and reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan were used as indicators of cytotoxicity as this reaction is dependent on mitochondrial respiration (Mosmann 1983).

2.3.5 Anti-inflammatory activity of α-MG

The anti-inflammatory activity of α-MG was studied by measuring its inhibitory effects on the secretion of pro-inflammatory mediators. Preliminary studies were performed for each cell type to establish non-toxic concentrations and appropriate periods for pre-incubation with α-MG, as well as the concentrations and duration of exposure to pro-inflammatory stimuli. Cells were incubated in media containing concentrations of α-MG well below the IC₅₀ to determine the effect, if any, on responsiveness to pro-inflammatory insult. Experimental conditions for determining the anti-inflammatory activity of α-MG are presented in Table 2.1. As Caco-2 cells were grown on Transwell® membrane inserts, α-MG was added to the apical compartment before addition of IL-1β to the basolateral compartment. IL-8 was selected as biomarker of inflammation in Caco-2 and HT-29 cells given its essential role as a chemoattractant during intestinal
inflammation (Schuerer-Maly and others 1994). IL-8 also served as the marker for THP-1 macrophage-like cells as it is the predominant chemokine in supernatants of LPS-treated cells (Chanput and others 2010). TNF-α is a key mediator of hepatic physiology and pathology and its secretion was selected as the marker for PMA-stimulated HepG2 cells (Bauer and others 2007). Because TNF-α secretion by tissue macrophages has been proposed as a key mediator of LPS-triggered septic shock, this inflammatory cytokine was selected as a marker in MDM cells (Xaus and others 2000). Finally, medium nitrite concentration was used as a surrogate for the induction of iNOS (inducible nitric oxide synthase) activity in RAW 264.7 cells. IL-8 and TNF-α were quantified by ELISA as instructed by the manufacturer. Nitrite concentration in medium was measured by the Griess reaction (Wang and others 2000).
Table 2.1. Experimental conditions for assessing the anti-inflammatory activity of α-mangostin.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Delivery vehicle</th>
<th>IC₅₀ (µM)</th>
<th>α-MG pretreatment (µM)</th>
<th>Pro-inflammatory stimulus (ng/mL)</th>
<th>Inflammatory marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW 264.7</td>
<td>10% FBS</td>
<td>23</td>
<td>10</td>
<td>LPSα (5)</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>THP-1</td>
<td>10% FBS</td>
<td>23</td>
<td>10</td>
<td>LPS (0.1)</td>
<td>IL-8</td>
</tr>
<tr>
<td>MDM</td>
<td>10% FBS</td>
<td>&gt;12</td>
<td>4.5</td>
<td>LPS (100)</td>
<td>TNF-α</td>
</tr>
<tr>
<td>HepG2</td>
<td>10% FBS</td>
<td>25</td>
<td>7</td>
<td>PMAβ (50)</td>
<td>TNF-α</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Tween 40</td>
<td>&gt;80</td>
<td>15</td>
<td>IL-1β (5)</td>
<td>IL-8</td>
</tr>
<tr>
<td>HT-29</td>
<td>Tween 40</td>
<td>180</td>
<td>10</td>
<td>LPS (100)</td>
<td>IL-8</td>
</tr>
</tbody>
</table>

αLipopolysaccharide (LPS) from *E.coli* 0111:B4. βPhorbol 12-myristate 13-acetate. γMeasured in basolateral medium.
2.3.6 **Cellular uptake and metabolism of α-MG**

Cells were exposed to non-toxic concentrations of α-MG (5.2, 3.3, 7.7, 9.0, 12.0 and 8.5μM, for THP-1, MDM and RAW 264.7, HepG2, Caco-2 and HT-29 cells, respectively), using previously established delivery systems (Figure 2.2B) to ensure stability of the xanthone in culture. After 24h, spent medium and cells were collected separately for analysis, except for RAW 264.7 cells where both cells and medium were collected in the same tube as some cells were non-adherent at confluency. For cultures with Caco-2 cells, medium with α-MG was added to the apical compartment and medium from both the basolateral and apical compartments along with cell monolayer were collected separately for analysis. Spent medium and cells were extracted as previously described (Bumrungpert and others 2009) prior to reverse phase high performance liquid chromatography (RP-HPLC) (Chaivisuthangkura and others 2009). Glucuronidated/sulfated metabolites of α-MG were determined as previously described (Bumrungpert and others 2009). Identification and quantification of xanthones was based on retention time, UV spectrum, and 5 point standard curves using pure (>98%) α-, γ-, and β-mangostins, 9-hydroxycalabaxanthone, gartanin, and garcinones D and E. When pure compounds were not available, identification was made by comparison with the retention time and UV spectrum reported in the literature (Chaivisuthangkura and others 2009; Walker 2007) and the concentration estimated as α-MG equivalents. Peaks with a xanthone-like spectra (λ_{max}: 240-300 and 310-370 nm) (Walker 2007) that could not be matched to one of the standards were labeled as unknown.
2.3.7  **Effect of inflammation on uptake and metabolism**

To study the effect of inflammatory conditions on the metabolism of α-MG, cells were exposed to the following inflammatory insults: 5ng/mL LPS for THP-1 and RAW 264.7 cells; 100ng/mL LPS for MDM; 10ng/mL TNF-α for HepG2 cells; 5ng/mL IL-1β for Caco-2 cells; and, 800ng/mL LPS for HT-29. LPS was used at higher concentrations in cultures with THP-1 and HT-29 cells than those used for the anti-inflammatory experiments in order to ensure a robust pro-inflammatory state. Since HepG2 cells secrete TNF-α in response to PMA treatment, we used this cytokine to induce a pro-inflammatory state. After 4h, media containing inflammatory stimuli were aspirated and cells washed with sterile PBS. Cultures were then incubated in medium containing α-MG at concentrations similar to those used in cultures under control conditions. Spent media and washed cells were collected after 24h for analysis as above.

2.3.8  **Statistical analysis**

The number of independent cultures tested per cell line for each experiment was ≥ 5 and each experiment was repeated at least twice. Data are reported as mean ± standard deviation (SD). For analysis, one-way ANOVA was performed followed by Tukey *post hoc* in order to determine differences between treatments. A *p*-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using Minitab ® 16.2.2.
2.4 Results

2.4.1 Anti-inflammatory activity of α-MG

α-MG was previously reported to exert an anti-inflammatory effect on murine RAW 264.7 macrophage-like cells (Chen, Yang, Wang 2008; Tewtrakul, Wattanapiromsakul, Mahabusarakam 2009). We initially monitored the effect of treatment with α-MG on LPS-induced generation of nitric oxide (NO) in RAW 264.7 cultures for comparison with its effect on the response of various cell lines to pro-inflammatory insult. Exposure to LPS increased NO production 36-fold above that in control cultures. Concentrations of 1, 3 and 10 µM α-MG inhibited LPS-induced production of NO by 50, 61 and 78%, respectively, significantly (p<0.05) exceeding the extent of inhibition (30%) mediated by 200µM L-NAME, a widely used nonspecific inhibitor of iNOS (data not shown).

The effect of α-MG on the secretion of inflammatory mediators was dependent on human cell type and state of cellular activation (Figure 2.3). Pre-treatment with α-MG significantly (p<0.05) attenuated IL-8 secretion by activated macrophage-like THP-1 (Figure 2.3A), enterocyte-like Caco-2 (Figure 2.3D) and colonic HT-29 (Figure 2.3E) cells, and TNF-α secretion by PMA-activated HepG2 cells (Figure 2.3C). In contrast, pre-treatment of MDM cells with α-MG increased LPS-stimulated secretion of TNF-α more than 50% (Figure 2.3B). When added to non-activated cultures, α-MG also more than doubled basal secretion of TNF-α by MDM cells (Figure 2.3B) and IL-8 by HT-29 cells (Figure 2.3E), suggesting pro-inflammatory activity. Concentrations of α-MG as low as 0.7µM also significantly (p<0.05) increased TNF-α secretion by non-activated MDM cells, although secretion of this cytokine was similar (p>0.05) in LPS-treated
cultures in the absence and presence of 0.7µM α-MG (data not shown). Secretion of TNF-α in non-activated cultures of HepG2 (Figure 2.3C) and secretion of IL-8 by non-activated cultures of THP-1 (Figure 2.3A) and Caco-2 cells (Figure 2.3D) was not altered by the presence of α-MG in medium.

2.4.2 Cell uptake and metabolism of α-MG in non-activated cultures

The reported anti-inflammatory activities of α-mangostin have assumed that the compound added to cell culture media or administered either by diet, gastric gavage or injection in pre-clinical models is responsible for the observed effects. Although limited, recent data suggests that dietary xanthones are poorly absorbed (Chitchumroonchokchhai and others 2012; Kondo and others 2009; Li and others 2011; Ramaiya, Petiwala, Johnson 2012). There is increased awareness that metabolites of “bioactive” dietary compounds often are the modulators of observed biological effects (Li and others 2007; Terao, Murota, Kawai 2011; Williamson and others 2005). Thus, we tested whether the observed anti-inflammatory activity of α-MG with the human cell lines was associated with the extent of its metabolism and profile of metabolites. Analysis of both media and cells indicated that α-MG was taken up by all tested cell lines. Glucuronide/sulfate conjugates of α-MG were identified in either both medium and cells or only in cells as described below. Xanthones other than α-MG also were present despite their absence in medium added to cultures or in medium incubated for the same period of time in the absence of cells. These xanthones were detected in cultures of RAW 264.7, THP-1, HepG2, Caco-2, and HT-29 cells, but not in MDM.
Figure 2.3. \(\alpha\)-MG attenuates secretion of inflammatory mediators in activated human cell lines, but exacerbates TNF-\(\alpha\) secretion by LPS-treated cultures of human monocyte-derived macrophages (MDM). THP-1 and MDM cells were differentiated to macrophage-like phenotype and Caco-2 cells differentiated to an enterocyte-like phenotype. Data are mean ± SD, for n ≥5. Different letters above bars in a panel indicate significant differences at \(p<0.05\).
Analysis of combined medium and cells revealed that RAW 264.7 murine macrophage-like cultures, our reference cell line, internalized and metabolized α-MG. Glucuronidated/sulfated conjugates (61±6%) were the primary metabolites with free α-MG accounting for 18±3% of the initial concentration present in cultures. In addition, relatively low amounts of garcinone C and an unknown xanthone were detected. Recovery of α-MG was 80±6% after 24h incubation.
Table 2.2. Profile of xanthones in media of human cell cultures 24h after addition of α-MG.

<table>
<thead>
<tr>
<th>Xanthone</th>
<th>THP-1</th>
<th>HepG2</th>
<th>Caco-2</th>
<th>HT-29</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pmol/dish)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-MG (0h)</td>
<td>33710±161</td>
<td>17834±115</td>
<td>20741±261</td>
<td>16517±373</td>
</tr>
<tr>
<td>α-MG (24h)</td>
<td>13215±212</td>
<td>4688±354</td>
<td>9366±510</td>
<td>7512±410</td>
</tr>
<tr>
<td>garcinone C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
<td>339±41</td>
<td>273±80</td>
</tr>
<tr>
<td>garcinone D</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>461±115</td>
</tr>
<tr>
<td>calabaxanthone derivative&lt;sup&gt;c&lt;/sup&gt;</td>
<td>nd</td>
<td>173±17</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>9-hydroxy calabaxanthone</td>
<td>nd</td>
<td>44±10</td>
<td>80±7</td>
<td>nd</td>
</tr>
<tr>
<td>unknowns&lt;sup&gt;d&lt;/sup&gt;</td>
<td>nd</td>
<td>1190±127</td>
<td>407±18</td>
<td>502±170</td>
</tr>
</tbody>
</table>

<sup>a</sup>Quantity estimated using garcinone D calibration curve; <sup>b</sup>nd: not detected <sup>c</sup>Quantity estimated using 9-hydroxy calabaxanthone calibration curve; <sup>d</sup>Quantities estimated as α-MG equivalents. Data are mean ± SD) for n ≥5 cultures per cell type.
Table 2.3. Intracellular profile of xanthones 24h after addition of α-MG to control cultures of human cell types.

<table>
<thead>
<tr>
<th>Xanthone</th>
<th>THP-1</th>
<th>HepG2</th>
<th>Caco-2</th>
<th>HT-29</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MG, 0h</td>
<td>33710±161</td>
<td>17834±115</td>
<td>20741±261</td>
<td>16517±373</td>
</tr>
<tr>
<td>α-MG, 24h</td>
<td>11439±995</td>
<td>9344±298</td>
<td>3000±551</td>
<td>807±178</td>
</tr>
<tr>
<td>garcinone D</td>
<td>66±2</td>
<td>nd(^a)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>calabaxanthone derivative(^b)</td>
<td>nd</td>
<td>56±10</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>9-hydroxy-calabaxanthone</td>
<td>nd</td>
<td>44±5</td>
<td>100±17</td>
<td>nd</td>
</tr>
<tr>
<td>unknowns(^c)</td>
<td>355±57</td>
<td>732±174</td>
<td>170±12</td>
<td>nd</td>
</tr>
</tbody>
</table>

\(^a\) nd: not detected; \(^b\) Quantity estimated using 9-hydroxy-calabaxanthone calibration curve; \(^c\) Quantity estimated as α-MG equivalents. Data are mean ± SD for n ≥5 cultures per cell type.
Free xanthones (20±4% of initial α-MG) and phase II conjugates (14±3%) were present in THP-1 cells, whereas only free xanthones (40±1%) were present in spent medium (Figure 2.4A). Garcinone D and three unknown xanthones also were detected in the cells (Table 2.3). Recovery of xanthones after 24h incubation was 75±6% of the amount of α-MG added to cultures. Only free xanthones also were detected in spent medium from MDM and HepG2 cultures, and accounted for 57±4% and 36±2% of initial amounts of α-MG, respectively. Both free and glucuronidated/sulfated conjugates of α-MG were present in MDM cells (22±5 and 20±10% of total, respectively) (Figure 2.4B) and HepG2 cells (27±3 and 29±3% of total, respectively) (Figure 2.4C). Other xanthones including 9-hydroxycalabaxanthone, a calabaxanthone-like compound and several unknown xanthones also were detected in spent medium and cells in HepG2 cultures (Tables 2.2 and 2.3), but not in cultures of MDM cells. Recovery of α-MG equivalents added to cultures of MDM and HepG2 was 99±12% and 91±4%, respectively.

Apical medium containing 12µM α-MG was added to cultures of differentiated Caco-2 cells on Transwell inserts and incubated for 24h. Xanthones in apical medium, basolateral medium and cells accounted for 47±3% (35±1% free and 11±2% conjugates), 3±0.3% (0.8±0.1% free and 1.7±0.3% conjugated), and 16±3% (6±0.6% free and 10±3% conjugated) of the initial amount of α-mangostin, respectively. In addition, garcinone C, 9-hydroxycalabaxanthone and two unknown xanthones were detected in Caco-2 cultures (Tables 2.2 and 2.3). Recovery of xanthones represented approximately 65% of the amount of α-MG added to the cultures, suggesting the presence of other unknown metabolites (Figure 2.4D).
Figure 2.4. Metabolism of α-MG in control and activated cell cultures. Data (mean±SD, n ≥5) represent the amounts of free xanthones and their glucuronidated/sulfated metabolites in medium and cells as a percentage of the initial amount of α-MG added to each culture. Dark bars: free xanthones; open bars: glucuronidated/sulfated metabolites. M: medium, C: cells, AM: apical medium, BM: basolateral medium. * p<0.05 (free:conjugated ratio in activated cultures when compared to control cultures).
HT-29 cells metabolized the majority of α-MG to phase II conjugates which were present in both medium and cells, accounting for 42±3% and 5±1%, respectively, of α-MG added to the cultures. Garcinones C and D and an unknown xanthone also were detected in spent medium (Table 2.2). Free xanthones accounted for 7±2% of initial amount of α-MG in the medium, but only traces of these non-conjugated xanthones were detected in cells (Figure 2.4E). Recovery of α-MG after incubation in cultures of HT-29 cells was only 54±4%.

The above data generally show that α-MG was transported and metabolized to phase II compounds by all human cell types and converted into other xanthones by the transformed human cell lines but not by the primary cultures of MDM cells.

2.4.3 Uptake and metabolism of α-MG in response to inflammatory insult

As α-MG was shown to attenuate secretion of IL-8 and TNF-α by the activated cell lines, we next examined whether the metabolism of this xanthone was altered in a pro-inflammatory environment. Cultures were exposed to cell-specific inflammatory stimuli before addition of α-MG to media. After 24h, media and cells were analyzed for xanthones and their metabolites. The total amounts in xanthones in media and cells were similar to that in non-activated cultures (Figure 2.4). However, the inflammatory environment was associated with an increased intracellular ratio of free to conjugated xanthones in THP-1 (Figure 2.4A), HepG2 (Figure 2.4C) and Caco-2 cells (Figure 2.4D), as well as in medium of HT-29 cultures (Figure 2.4E) and apical medium in Caco-2 cell cultures (p<0.05). In contrast, the amount of xanthones and the ratio of
conjugated to free xanthones transported into the basolateral compartment by Caco-2 cells were increased by treatment with IL-1β (p<0.05) (Figure 2.4D). The ratio of free to conjugated xanthones in RAW 264.7 cultures also was increased by LPS treatment (data not shown). Treatment of MDM cells with LPS did not significantly alter the relative amounts of free xanthones and phase II metabolites of xanthones either in medium or intracellularly (Figure 2.4B).

2.5 Discussion

The goal of this study was to examine the in vitro anti-inflammatory activity and metabolism of α-MG in cells of human origin. Stability of α-MG was maintained by delivering the xanthone into media containing FBS or by solubilizing the xanthone in Tween 40 micelles for addition to serum-free media. Non-cytotoxic concentrations of α-MG attenuated secretion of IL-8 and TNF-α by the various human cell lines. Influx and metabolism of α-MG to phase II metabolites and/or other xanthone products occurred with all cell lines. The extent of metabolism and types of products depended on cell type. In marked contrast with the results from the various cell lines, α-MG stimulated TNF-α secretion in both control and activated primary cultures of human MDM cells and only glucuronidated/sulfated metabolites were detected in these cells. The results suggest that metabolic conversion of α-MG to other xanthones may be necessary for anti-inflammatory activity. As Caco-2 cells have been shown to take up and transport metabolites of α-MG across the basolateral membrane (Bumrungpert 2009a) and metabolites of xanthones were present in sera soon after ingesting mangosteen juice
(Chitchumroonchokchai and others 2012), it is expected that peripheral tissues are exposed to both free and conjugated xanthones.

Epidemiological evidence suggests that a diet rich in fruit and vegetables prevents or may delay onset of cardiovascular disease, diabetes and cancer (Carter and others 2010; Dauchet and others 2006; Hung and others 2004; Riboli and Norat 2003). These effects have been attributed, in part to plant-derived polyphenolic compounds (Soto-Vaca and others 2012). Xanthones from mangosteen fruit, for instance, exhibit anti-inflammatory (Chen, Yang, Wang 2008; Tewtrakul, Wattanapiromsakul, Mahabusarakam 2009) and anti-carcinogenic (Lee and others 2010; Matsumoto and others 2003; Nakagawa and others 2007) activities in vitro. Consideration of the in vitro stability of phytochemicals when assessing bioactivities is often neglected despite the fact that many polyphenolic compounds readily react with components of cell culture media to generate H₂O₂, quinones and semiquinones capable of inducing alterations in cellular activities (Halliwell 2009; Long, Hoi, Halliwell 2010). As part of our preliminary analysis, we found that α-MG was unstable when introduced into different basal media in the commonly used DMSO vehicle. Previous cellular studies reporting the in vitro anti-inflammatory activity of α-MG bioactivities used DMSO to deliver the xanthone to medium (Chen, Yang, Wang 2008; Tewtrakul, Wattanapiromsakul, Mahabusarakam 2009). It is unclear if FBS was present and stability of α-MG in these studies was not reported. The report that addition of catalase to medium inhibited γ-mangostin mediated apoptosis in human malignant glioma cells supports the likelihood that spontaneous degradation of xanthones in medium can induce alterations in cellular activities (Chang
and others 2010). Thus, our first goal was to stabilize α-MG in cell culture in order to test its in vitro anti-inflammatory activity and cellular metabolism. Previous investigators have used Tween 40 to solubilize and stabilize β-carotene (During and others 2002) and fetal bovine serum for delivery of lycopene (Lin, Huang, Hu 2007) to cultured cells. We found that degradation of α-MG in media was prevented by incorporating α-MG in Tween 40 micelles, in media containing FBS, and by direct addition to FBS prior to its dilution in media (Figure 2.2). As lipophilic compounds are incorporated into micelles during digestion before being transported into enterocytes, Tween 40 micelles were used to deliver α-MG to enterocyte-like Caco-2 cells and colonic HT-29 cells. Because liver and immune cells are exposed to plasma components in vivo, fetal bovine serum was selected as the delivery vehicle for HepG2, THP-1, MDM and RAW 264.7 cells.

Previous studies examining the anti-inflammatory activity of α-MG in human cells have been limited to primary adipocytes (Bumrungpert and others 2009b) and macrophage-like U937 lymphoma cells (Bumrungpert and others 2010) where α-MG decreased LPS-induced expression of inflammatory genes. As the focus of our study was the anti-inflammatory activity of α-MG, we selected the human immune macrophage-like THP-1 cell line and primary monocyte-derived macrophages (MDM) from human peripheral blood for investigation. HepG2 cells were included in our study because hepatic epithelial cells synthesize and secrete greater amounts of cytokines and acute phase proteins in response to pro-inflammatory signals and our lab has identified xanthones and their metabolites in hepatic tissue of mice fed diet containing 900 mg α-MG/kg (Chitchumroonchokchai and others 2013). Differentiated cultures of Caco-2
human intestinal cells possess enterocyte-like characteristics including a variety of host defense activities such as antigen-processing and presentation and secretion of a battery of cytokines and chemokines in response to pro-inflammatory insult (Van de Walle and others 2010). Furthermore, we recently reported that α-MG inhibits colon HT-29 tumorigenicity in vitro and in vivo (Chitchumroonchokchai and others 2013). Thus, these two colonic cell lines also were included in the study. Finally, because α-MG has been reported to inhibit the LPS-induced synthesis of NO by murine RAW 264.7 macrophage-like cells (Chen, Yang, Wang 2008; Tewtrakul, Wattanapiromsakul, Mahabusarakam 2009), the metabolism of the xanthone by these cells was considered for comparison with that by the human cells.

Our results showed that the impact of α-MG on the human cell lines differed from that on the primary MDM. Treatment of activated THP-1, Caco-2 and HT-29 cells with α-MG inhibited IL-8 chemokine secretion by 30-40%. Similarly, α-MG attenuated TNF-α secretion by 22% in PMA-activated cultures of HepG2 cells. In contrast, α-MG enhanced TNF-α secretion by both quiescent and activated primary cultures of MDM. This stimulatory effect with quiescent cultures was observed even when the concentration of the xanthone was less than 1µM. This lower concentration is more in line with those we found in serum of healthy adults following ingestion of a mangosteen juice product (Chitchumroonchokchai and others 2012) and in mice after oral administration α-MG in cottonseed oil (Ramaiya, Petiwala, Johnson 2012). Possible explanations for different responses of the cell lines and the primary MDM cells to α-MG may include differences in phenotype, tissue origin, and metabolism by transformed versus normal cells. Our
experimental design is limited by the selection of only two inflammatory markers, viz.,
secretion of IL-8 and TNF-α, to assess the activity of α-MG. More detailed studies, and
particularly in vivo studies, are needed to further characterize the pro- and anti-
inflammatory effects of xanthones. In this regard, increased concentrations of
inflammatory cytokines IL-1α and IL-1β, as well as higher T helper cell frequency and
increased levels of complement C3 and C4, were found after healthy human subjects
consumed 59mL of a blended juice product containing mangosteen for 30 days (Tang and
others 2009). The possibility that the proposed health-promoting effects of dietary
xanthones are mediated by induction of an adaptive stress or “hormetic” response in non-
transformed cells merits investigation (Siow and Mann 2010; Speciale and others 2011),
as does the possibility that xanthones may be detrimental for some inflammatory
disorders.

Although there is extensive literature demonstrating the health-promoting properties of
phytochemicals in vitro, many of these compounds undergo extensive first-pass
metabolism to glucuronidated, sulfated and methylated conjugates in vivo. Although such
metabolites are generally assumed to be inactive (Lotito and others 2011; Spencer, Abd-
el-Mohsen, Rice-Evans 2004), recent evidence suggests phase II metabolites exert some
of the activities attributed to their parent compounds (Larrosa and others 2010; Li and
others 2007; Terao, Murota, Kawai 2011; Williamson and others 2005). We previously
reported the presence of glucuronidated/sulfated metabolites of α-MG as early as 1h after
incubation of cells with the xanthone (Bumrungpert and others 2009a). We also observed
these metabolites in cultures of RAW 264.7 cells 8h after addition of α-MG (data not
shown). These observations suggest that metabolites and/or the parent compound α-MG may affect early events in signaling pathways involved in synthesis of pro-inflammatory mediators. The present results agree with our recent reports that Caco-2 cells (Bumrungpert and others 2009a), mice (Chitchumroonchokchai and others 2013; Ramaiya, Petiwala, Johnson 2012), and human subjects (Chitchumroonchokchai and others 2012) metabolize α-MG to phase II products. In addition to phase II metabolites, other xanthones such as garcinones C and D, 9-hydroxycalabaxanthone, a calabaxanthone derivative and several unknown xanthone compounds were detected in our analysis with the human cell lines. Many of these identified metabolites of α-MG are also present in mangosteen pericarp, suggesting that mixtures of xanthones may have greater efficacy than individual compounds. Furthermore, recovery of α-MG in cultures for several of the human cell types was incomplete, suggesting the presence of additional metabolites. It also is possible that we underestimated xanthone conjugates due to incomplete enzymatic hydrolysis by the mollusc preparation due to the nature and position of substitutions on the tricyclic aromatic compounds, binding of conjugated xanthones to proteins, localization within organelles and the presence of natural inhibitors (Gu and others 2005). More robust analyses are required to further characterize xanthone metabolism and the bioactivities of the various metabolites.

During an inflammatory insult, the ratio of free to conjugated xanthones increased in THP-1, HepG2 and Caco-2 cells, and in the media of HT-29 and Caco-2 cell cultures. LPS has been reported to increase intracellular and secreted β-glucuronidase activity in RAW 267.4 cells causing enhanced hydrolysis of quercetin glucuronides (Kawai and
others 2008). Similarly, increased serum β-glucuronidase activity in LPS-treated rats was associated with an increase in deconjugation of luteolin glucuronide (Shimoi and others 2001). Glucuronidase activity was increased in media of LPS-activated THP-1 and RAW 264.7 cells (data not shown). These observations may explain the greater concentration of free xanthones in cells and media when α-MG was added to activated cultures. Also, transport of xanthones (mainly conjugates) across the Caco-2 monolayer was increased when cells were pre-treated with IL-1β, supporting the possibility that xanthones may be more bioavailable during inflammatory episodes. The possible use of xanthones as co-adjuvants for the treatment of chronic inflammatory conditions merits consideration.

The results for the present investigation show that α-MG attenuates the secretion of pro-inflammatory mediators by activated human cell lines of diverse tissue origin. However, this xanthone stimulates the secretion of TNF-α by primary cultures of quiescent and activated monocyte-derived human macrophages. We also show that α-MG was transported into cells where it undergoes phase II metabolism and other metabolic processes in a manner that is dependent on cell type and inflammatory status. We speculate that these metabolites may be immunomodulatory either by stabilizing the parent compound or by directly exerting anti- or pro-inflammatory activity. Finally, we also show that inflammatory conditions may alter cellular metabolism of α-MG and possibly increase its bioavailability.
CHAPTER 3

DIETARY α-MANGOSTIN, A XANTHONE FROM MANGOSTEEN FRUIT, EXACERBATES EXPERIMENTAL COLITIS AND PROMOTES DYSBIOSIS IN MICE*


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3.1 Abstract

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon. α-Mangostin (α-MG), the most abundant xanthone in mangosteen fruit, exerts anti-inflammatory and anti-bacterial activities in vitro. We evaluated the impact of dietary α-MG on murine experimental colitis and on the gut microbiota of healthy mice. Colitis was induced in C57BL/6J mice by administration of dextran sulfate sodium (DSS). Mice were fed control diet or diet with α-MG (0.1%). α-MG exacerbated the pathology of DSS-induced colitis. Mice fed diet with α-MG had greater colonic inflammation and injury, as well as greater infiltration of CD3+ and F4/80+ cells, and colonic myeloperoxidase, than controls. Serum levels of granulocyte colony stimulating factor, IL-6, and serum amyloid A were also greater in α-MG-fed animals than in controls. The colonic and cecal microbiota of healthy mice fed α-MG but no DSS shifted to an increased abundance of Proteobacteria and decreased abundance of Firmicutes and Bacteroidetes, a profile similar to that found in human UC. α-MG exacerbated colonic pathology during DSS-induced colitis. These effects may be associated with an induction of intestinal dysbiosis by α-MG. Our results suggest that the use of α-MG-containing supplements by patients with UC may have unintentional risk.
3.2 Introduction

Ulcerative colitis (UC) is a chronic disease characterized by mucosal and sub-mucosal inflammation limited to the colon with cryptitis and crypt abscesses. Persistent progressive or relapsing inflammation results in bloody diarrhea and abdominal distress that are hallmarks of UC (Khor, Gardet, Xavier 2011). In addition, the relative risk of UC patients developing colorectal cancer is substantially increased with duration and severity of the disease (Clapper, Cooper, Chang 2007). The etiology of UC remains only partially understood, but it is thought to result from a dysregulated immune response to the gut microbiota in a genetically susceptible host (Ordás and others 2012). Dysbiosis, an imbalance between putative species of “protective” versus “harmful” intestinal bacteria, has been implicated in UC (Tamboli and others 2004).

The chemically induced dextran sulfate sodium (DSS) colitis model has been shown to mimic human UC pathology, and recent preclinical studies have supported its use as a system to evaluate the role of anti-inflammatory agents (Clapper, Cooper, Chang 2007). Oral ingestion of DSS induces diarrhea, rectal bleeding, ulceration of the colonic epithelium, loss of goblet cells, and immune cell infiltration, similar to the phenotypic changes observed in human UC (Melgar, Karlsson, Michaëlsson 2005). Attenuation of the synthesis and secretion of pro-inflammatory mediators is expected to be beneficial during chronic inflammatory conditions such as inflammatory bowel disease (IBD). Medical therapy for the treatment of IBD has only modest success and is associated with adverse side effects (Lanzoni and others 2008). This likely contributes to the use of complementary and alternative medicine (CAM), such as herbal preparations, by as many
as 50% of IBD patients (Rahimi, Mozaffari, Abdollahi 2009). However, clinical evidence supporting the use of such products in UC management is limited or absent.

In vitro and in vivo studies have shown that dietary components can protect against inflammation (Wu and Schauss 2012). Plant-derived phytochemicals, such as specific polyphenols, have been shown to inhibit cell signaling processes that are involved in the inflammatory response, thus attenuating synthesis of pro-inflammatory cytokines and cell adhesion molecules (Pan, Lai, Ho 2010). *Garcinia mangostana* is a tree native to Southeast Asia that produces a fruit known as mangosteen, which has been used in traditional medicine to treat inflammation, infections, wounds, and diarrhea. The bioactivities of mangosteen have been associated with a family of polyphenolic compounds referred to as xanthones (Pedraza-Chaverri and others 2008). α-Mangostin (α-MG; Figure 2.1), the most abundant xanthone in the pericarp of mangosteen fruit (Walker 2007), attenuates secretion of pro-inflammatory cytokines in colonic and immune human cell lines (Gutierrez-Orozco and others 2013, Chapter 2) and reduces the inflammatory response by human and rodent macrophages, as well as primary human adipocytes (Bumrungpert and others 2010). In vivo, α-MG attenuates paw edema and airway inflammation in rodents (Chen, Yang, Wang 2008; Jang and others 2012). However, α-MG stimulates tumor necrosis factor-α (TNF-α) secretion in primary human blood monocyte derived macrophages (Gutierrez-Orozco and others 2013, Chapter 2), and ingestion of a mangosteen juice supplement by healthy individuals is associated with elevated serum levels of IL-1 and complement components (Tang and others 2009). α-MG also exerts antibacterial, antifungal, and antiviral activities (Pedraza-Chaverri and
others 2008). For instance, α-MG inhibits pathogenic bacteria such as S. aureus and B. subtilis, but has no effect on E. coli and C. albicans (Pedraza-Chaverri and others 2008), which suggests low selectivity against these pathogens. As a result of the aggressive marketing of purported health-promoting activities, numerous supplements, beverages, and food products containing mangosteen are now available. Mangosteen juice, for instance, has been promoted as beneficial for gastrointestinal and immune health. Although objective scientific data supporting these and other claims is lacking, sales of mangosteen-containing beverages alone exceeded $200 million in the United States in 2008 (Sloan 2010). Indeed, many individuals suffering illness consume these products without the knowledge of their medical team. The potential for both adverse interactions with conventional medications and unintended effects on health outcomes is often overlooked.

The modulatory effects of α-MG in the context of intestinal inflammation remain unknown. Because greater concentrations of dietary bioactive components, such as α-MG, are found in the gastrointestinal tract than in peripheral tissues (Chitchumroonchokchai and others 2013), this xanthone may exert protective effects in conditions such as UC. Thus, we hypothesized that α-MG would ameliorate colonic inflammation and injury during experimental colitis. The chemically induced DSS colitis model was used in the present study. C57BL/6 mice were fed standard diet or diet containing α-MG and disease severity was assessed based on body weight loss, diarrhea, and rectal or occult bleeding. Colonic infiltration of immune cells and epithelial cell proliferation, as well as systemic and colonic inflammation, were evaluated. Finally,
because α-MG has been reported to exert antibacterial activities, its impact on the gut microbiota of healthy, non-colitic mice was also studied.

3.3 Materials and Methods

3.3.1 Mice

For colitis studies, 10-week-old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were housed in the animal facilities at The Ohio State University (OSU) under conventional conditions with controlled temperature at 23 °C and a 12-h light/dark cycle. Mice were acclimatized for 1 week before entering the study and had free access to water and standard AIN93G diet. All procedures were approved under Protocol #2011A00000006 and followed the guidelines by the Institutional Animal Care and Use Committee (IACUC) at OSU.

3.3.2 Diet

α-MG was >98% pure (Chaivisuthangkura and others 2009; Walker 2007). Gamma-irradiated AIN93G diet (control) and AIN93G diet containing 900 mg/kg α-MG and FDA approved dyes E102 and E133 for green color were prepared by Research Diets (New Brunswick, NJ). This dose has been reported to be safe and effective in reducing tumor mass in xenograft models of colon and prostate cancer. For a mouse weighing 20 g and ingesting 2.5 g diet/day, this dose equates to 112 mg/kg body weight. The human equivalent dose (mg/kg) is calculated as animal dose (mg/kg) x (mouse Km/human Km), where Km is a correction factor reflecting the relationship between body weight and body
surface area (Reagan-Shaw, Nihal, Ahmad 2008). Thus, the human equivalent dose \([(112 \text{ mg/kg})/(3/37) = 9.12 \text{ mg/kg}]\) for an adult weighing 60 kg equates to 547 mg/day. Intake of this amount could be achieved by ingesting 1-2 capsules of mangosteen extract (Johnson and others 2012), or 1 cup of 100% mangosteen juice (Chitchumroonchokchai and others 2012).

3.3.3 Induction of colitis and experimental groups

DSS-induced colitis is a widely used model to evaluate the role of anti-inflammatory agents (Clapper, Cooper, Chang 2007). Murine strain, gender, microbiota, the molecular weight, concentration and batch of DSS, and the duration of administration of the chemical insult are known to affect susceptibility to DSS-induced colitis. We selected the C57BL/6J mouse strain as it has been shown that DSS-induced colitis in this strain mimics the chronicity observed in human ulcerative colitis (Melgar, Karlsson, Michaëlsson 2005). Furthermore, female mice were selected as their response to DSS-induced inflammation has been reported to be less severe than males (Ding and others 2012). In addition, two pilot studies were performed to optimize the dose of DSS and duration of administration necessary to induce colitis. Accordingly, the dose of DSS in water was decreased from 3% to 2%, and DSS was administered for four rather than five days. These conditions were found to induce colitis without significant loss of body weight. The experimental design is presented in Figure 3.1. Colitis was chemically induced with DSS (MP Biomedicals; mol wt 36,000-50,000) dissolved in the drinking water (2% wt/vol) and provided ad libitum for 4 days. One week prior to the induction of
colitis, mice were randomized to be fed either the standard AIN93G diet or the AIN93G diet with α-MG. Mice continued to be fed their respective diets until the end of the study. We followed this approach to mimic chronic use of mangosteen-containing products during both inflammatory flare ups and remission periods. To induce colitis, mice in each diet group were provided ad libitum access to either tap water [control groups: control (n=20) and α-MG (n=20)], or water containing 2% DSS [DSS groups: DSS (n=20) and DSS+α-MG (n=20)] for 4 days. After DSS administration, all mice were given tap water without DSS and allowed to recover for either 1 or 2 weeks before euthanasia. Ten mice per group were used at each end point.

3.3.4 Disease activity index (DAI)

The DAI was calculated as previously described (Murthy and others 1993). The percentage of body weight (BW) lost over the course of the study was calculated by comparing daily BW to that at the initiation of the study. BW loss was scored as follows: 0 = none; 1 = 1-5%; 2 = 5-10%; 3 = 10-15%; 4 = >15%. Stool consistency was scored as follows: 0 = normal; 2 = loose stools; 4 = diarrhea. Rectal or occult bleeding (Hemoccult slides, Beckman Coulter, Fullerton, CA) were scored as 0 = negative, 2 = Hemoccult positive, and 4 = gross bleeding. The three scores were determined daily for each animal receiving DSS starting 1 day after DSS administration and continued until one day before euthanasia (n=20/dietary group), and summed to calculate the DAI as previously described (Murthy and others 1993).
Figure 3.1. Experimental design for colitis study. Clinical assessment and body weight were taken throughout the study. Dextran sulfate sodium (DSS) was provided in the drinking water for 4 days and mice were allowed to recover for 1 or 2 weeks. Ten mice per group were used at each time point.
3.3.5 **Histopathological evaluation of colitis**

At necropsy, the colon was excised from the ileocecal junction to the anal verge and its length was measured before opening longitudinally to remove luminal contents. The colon was gently rinsed by swirling in cold PBS and weighed. Colons were divided into three segments of equal length (proximal, middle, and distal). Each segment was further divided for biochemical analysis or histological evaluation. Sections from each segment were fixed in 10% neutral buffered formalin, paraffin embedded, and stained with hematoxylin and eosin (H&E) for examination by light microscopy (n=9-10/group). Histological evaluation was performed in a blinded manner by a board certified veterinary anatomic pathologist (LDBB). The severity of colonic inflammation was assigned a numerical grade between 0 and 3 as originally described (Cooper and others 1993) with 0 indicating no significant inflammation, 1 being mild inflammation restricted to the lamina propria, 2 indicating moderate inflammation that extended to the submucosa, and 3 being severe transmural inflammation or inflammation associated with an ulcer greater than 1 mm in length. Fibrosis of the lamina propria was included in the characterization of inflammation (Mähler and others 1998). Crypt lesions and loss were graded as 0 (no lesion) to 4 (erosion or ulceration) (Cooper and others 1993). The presence or absence of an ulcer was assigned either a 0 if there was no ulcer or a 1 if an ulcer was present (Mähler and others 1998). Hyperplasia was scored as 0 (no hyperplasia), 1 (mild hyperplasia), 2 (moderate hyperplasia), or 3 (severe hyperplasia) (Cooper and others 1993; Mähler and others 1998). All lesions (inflammation, crypt lesions, ulceration, and hyperplasia) were also assigned the following distribution grade
that reflected the percentage of colon involved: 1 (1-25%); 2 (26-50%); 3 (51-75%); and, 4 (76-100%) (Cooper and others 1993; Mähler and others 1998). The numerical lesion grade for each lesion type was then multiplied by the distribution of that lesion, resulting in the lesion score for that segment of colon (Cooper and others 1993). Inflammation, crypt lesions, ulceration, and hyperplasia, as well as lesion distribution, in each colon section were graded. A combination of the grading and scoring schemes established previously was used (Cooper and others 1993; Mähler and others 1998).

3.3.6 Tissue analysis

At necropsy, liver, kidney, and spleen were excised and weighed (n=10/group). A section of each was fixed in 10% neutral buffered formaldehyde and processed for histologic evaluation. Tissue sections (5 µm) were stained with H&E for light microscopy examination for lesions consistent with toxicity (n=4-8 mice/group). Histological evaluation was performed in a blinded manner by a board certified veterinary anatomic pathologist (LDBB).

3.3.7 Biochemical Analyses

Distal colonic tissue was lysed (n=8-10 mice/group) as previously described (Melgar, Karlsson, Michaëlsson 2005) and colonic myeloperoxidase (MPO) protein was measured by ELISA according to the manufacturer’s instructions (Hyccult biotech, Plymouth Meeting, PA). Serum amyloid A (SAA) levels were quantified by ELISA (n=6-8 mice/group) (Tridelta Development Ltd., Ireland). To assess possible liver toxicity, serum
(n=3-6 mice/group) was analyzed for aspartate (AST) and alanine (ALT) aminotransferase enzyme activities using commercial assays (Pointe Scientific Inc., Canton MI). A panel of 15 cytokines (IL-12p40, IL-12p70, M-CSF, G-CSF, GM-CSF, TNF-α, IL-6, IL-1β, IFN-gamma, IL-17A, IL-23, IL-10, IL-4, and IL-5, IL-13) were analyzed in plasma from each mouse at study endpoint using the commercially available, high-throughput Luminex Multplex Cytokine Kits (ProcartaCytokine Assay Kit, Affymetrix, Santa Clara, CA). All samples were batch run in duplicate and quantified based on a unique standard curve for each analyte.

### 3.3.8 Immunohistochemistry (IHC)

For IHC analysis, 5-μm-thick, paraffin-embedded sections of the distal colon from 3 mice per group were randomly selected. T cells and macrophages were identified using CD3 and F4/80, respectively, as markers. For identification of CD3+, and F4/80+ cells, sections were treated with Target retrieval solution (Dako) using a decloaking chamber. Slides were blocked in 3% hydrogen peroxide and Dako serum-free protein block. CD3 immunostain was done using a rabbit anti-human polyclonal antibody diluted 1:200 (Dako #A0452, Dako, Carpinteria, CA). F4/80 detection was performed using a rat anti-mouse monoclonal antibody diluted 1:100 (Serotec #MCA497G, Bio-Rad, Raleigh, NC). ABC was used for detection (Vector), followed by DAB chromogen, and hematoxylin as counterstain. To quantify CD3+ and F4/80+ cells, three randomly selected 200X fields for each sample were scanned to obtain pixel count using Aperio Smage Scope (v11.2). Data (expressed as percentage) represent the average number of weak positive, positive, and
strong positive pixels per total pixels. To evaluate cell proliferation, tissue sections were immunostained for Ki67. Tissue sections were immunostained using a commercially available rat anti-mouse monoclonal antibody diluted 1:50 (clone TEC-3; #M7249; Dako, Carpinteria, CA). Citra Plus antigen retrieval solution (Biogenex, Fremont, CA) was used followed by the application of a protein block (peroxidase block, Dako). Detection was done using the streptavidin/HRP method, DAB as chromogen followed by hematoxylin counterstain. For each colonic section, the percentage of Ki67-immunopositive cells in the crypts in three randomly selected 200X fields was calculated using Image-Pro Plus software.

3.3.9 Bacterial analyses

To study the effect of dietary α-MG on the gut microbiota of healthy mice (i.e. non-DSS treated), 10-week-old female C57BL/6 mice (Jackson Laboratories) were housed under sterile conditions with controlled temperature at 23 °C and a 12-h light/dark cycle. Mice were acclimatized for 1 week before entering the study with free access to water and AIN93G diet. Mice were randomly assigned to AIN93G diet (control group, n=5 mice) or the AIN93G diet with α-MG (α-MG group, n=5 mice) for 4 weeks ad libitum. At necropsy, cecum and distal colon were excised under aseptic conditions, gently rinsed in sterile cold PBS and collected in sterile tubes. Tissue was frozen in liquid nitrogen and stored at -80 °C until analysis. Bacterial analyses were performed using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) at the Research and Testing Laboratory (Lubbock, TX).
The 16S rRNA gene sequence was amplified for pyrosequencing using a forward and reverse fusion primer. The forward primer was constructed with (5’-3’) the Roche A linker (CCATCTCATCCCTGCGTGTCTCCGACTCAG), an 8-10bp barcode, and the forward specific primers GGCGVACGGGTGAGTAA (Proteobacteria), GGCAGCAGTRGGGAATCTTC (Firmicutes), and AACGCTAGCTACAGGCTT (Bacteroidetes). The reverse fusion primer was constructed with (5’-3’) a biotin molecule, the Roche B linker (CCTATCCCTGTGTGCCTTGGCAGTCTCAG), and the reverse specific primers CCGCNGCNGCTGGCAC (Proteobacteria), ACACYTAGYACTCATCGT TT (Firmicutes), and CAATCGGAGTTCTTCGTG (Bacteroidetes). Amplifications were performed in 25 µL reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, CA), 1 µL of each 5 µM primer, and 1 µL of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, CA) using the following thermal profile: 95 °C for 5 min, then 35 cycles of 94 °C for 30 sec, 54 °C for 40 sec, 72 °C for 1 min, followed by one cycle of 72 °C for 10 min and 4 °C hold. Amplification products were visualized with eGels (Life Technologies, Grand Island, NY). Products were then pooled equimolar and each pool was cleaned with Diffinity RapidTip (Diffinity Genomics, West Henrietta, NY), and size selected using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, IN) following Roche 454 protocols (454 Life Sciences, Branford, CT). Size selected pools were then quantified and 150 ng of DNA were hybridized to Dynabeads M-270 (Life Technologies, Grand Island NY) to create single stranded DNA following Roche 454 protocols (454 Life Sciences, Grand Island, NY). Single stranded DNA was diluted and used in emPCR
reactions, which were performed and subsequently enriched. Sequencing was done following established manufacture protocols (454 Life Sciences).

Although three individual primers for the Firmicutes, Bacteroidetes and Proteobacteria phyla were used, bacterial sequences from other phyla were also detected as the Proteobacteria primer was less specific. Sequences obtained from the 3 individual assays were collated for analysis. Fasta and qual files obtained from pyrosequencing were uploaded into Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso and others 2010a). The minimum and maximum sequence lengths were 200 and 1000, respectively. The minimum qual score was 25. The maximum number of ambiguous bases was 6 and no primer mismatch was allowed. 89.1% of sequences were retained after quality trimming. Clustering of sequencing reads into operational taxonomic units (OTUs) was achieved at 97% identity. OTU picking was performed in the QIIME pipeline using the Uclust algorithm (Edgar 2010). Taxonomic assignment was achieved using the Ribosomal Database Project (RDP) classifier (Wang and others 2007), employing the GreenGenes database (McDonald and others 2012). Sequences PyNAST (Caporaso and others 2010b) aligned by QIIME were used to calculate α-diversity (Shannon index) using a cutoff depth of 5490 and 4534 sequences for colon and cecum samples, respectively. Unifrac analysis (Lozupone and Knight 2005) followed by principal coordinate analysis (PCoA, based on unweighted unifrac distance) was used to characterize the diversity in the bacterial populations and done by using 6102 and 4553 sequences for colon and cecum, respectively, and jackknifed data (4577 and 3414 sequences for colon and cecum, respectively).
3.3.10 Statistical analysis

All data are expressed as mean ± SD. For parametric data, statistical differences were determined by one-way analysis of variances followed by Tukey’s test. Nonparametric data were analyzed by Kruskal-Wallis test followed by selected mean comparisons with Bonferroni correction. Differences were considered statistically significant at $p < 0.05$. Analyses were performed using SPSS v. 20 (IBM, Armonk, NY). For pyrosequencing data, statistical differences in unweighted Unifrac distance were investigated by Analysis of Similarities (ANOSIM), provided by R’s vegan package implemented into QIIME.

3.4 Results

3.4.1 Dietary $\alpha$-MG exacerbates disease activity in DSS-induced colitis

The DAI was calculated by adding the scores from the percentage of body weight (BW) lost from baseline, stool consistency, and rectal bleeding (Murthy and others 1993). As expected, DSS treatment increased DAI. However, DAI was significantly greater in mice that received DSS and were fed the AIN93G diet with $\alpha$-MG (DSS+$\alpha$-MG group) than in mice that received DSS while being fed a standard AIN93G diet (DSS group) ($p < 0.01$). While DAI in the DSS group steadily declined to baseline 5 days after cessation of DSS treatment, increased severity of symptoms persisted in the DSS+$\alpha$-MG group and remained significantly greater 8 days post-DSS administration ($p < 0.01$) (Figure 3.2B). Significant loss of BW (17% from baseline) only occurred in the DSS+$\alpha$-MG group following DSS treatment but was recovered 1 week post-DSS (data not shown). Diarrhea and rectal bleeding were also more severe in the DSS+$\alpha$-MG group.
Food intake was temporarily decreased in the DSS+α-MG group after cessation of DSS treatment but reverted to that of all other groups 5 days later. No alterations in liver AST and ALT enzyme activities or histological evidence of hepatic toxicity were detected in any experimental group (data not shown).

3.4.2 DSS-induced colonic inflammation and injury are aggravated by α-MG

Microscopic assessment of H&E stained colon (Figure 3.2A) confirmed induction of colitis by DSS, as mice in the DSS group had significantly greater inflammation (Figure 3.2D) and crypt injury (Figure 3.2E) scores in the mid colon 1 week after DSS administration compared to mice receiving tap water ($p < 0.05$). Although there was no significant difference in the scores for inflammation and crypt injury in the distal colon of mice in the DSS group after recovering for 1 week, these scores were significantly greater compared to the control group after 2 weeks of recovery (Figures 3.2D-E, $p < 0.05$). Mice in the DSS+α-MG group had more severe inflammation in the mid and distal colon compared to DSS group 1 and 2 weeks after cessation of DSS treatment (Figure 3.2D, $p < 0.05$). Crypt injury scores were also significantly higher in the mid and distal colon of mice in the DSS+α-MG group compared to DSS group after 1 week of recovery from DSS (Figure 3.2E, $p < 0.05$). Ulcers were only present in the mid and distal colon of animals receiving DSS+α-MG (Table 3.1).

Although administration of 3% DSS to C57BL/6 mice for 5 days has been reported to shorten the colon (Melgar, Karlsson, Michaëlsson 2005), colonic length of mice treated with 2% DSS was not altered in this study (data not shown). However, the ratio of colon
weight (after removal of luminal contents) to colon length was significantly greater in non-DSS treated animals receiving α-MG in the diet compared to mice fed standard diet after 1 and 2 weeks of recovery ($p < 0.05$). This ratio was also greater in the DSS+α-MG group than in the DSS group at 1 and 2 weeks post-DSS treatment ($p < 0.05$) (Table 3.2). Increased fluid volume in the colonic lumen of mice fed diet with α-MG was evident especially in the proximal and mid colon, independently of DSS-treatment (Figure 3.2C).
Table 3.1. Effect of dietary α-mangostin (α-MG) on colonic ulceration and epithelial hyperplasia.

<table>
<thead>
<tr>
<th></th>
<th>1 week-recovery</th>
<th>2 week-recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mid</td>
<td>Distal</td>
</tr>
<tr>
<td>Ulceration score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSS</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>DSS+α-MG</td>
<td>1.4±1.0 (100%)*</td>
<td>1.4±1.0 (90%)*</td>
</tr>
<tr>
<td>Hyperplasia score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSS</td>
<td>0.1±0.3 (10%)</td>
<td>0.1±0.3 (10%)</td>
</tr>
<tr>
<td>DSS+α-MG</td>
<td>2.3±0.9 (100%)*</td>
<td>1.4±1.1 (90%)*</td>
</tr>
</tbody>
</table>

*Number in parenthesis indicates percentage of mice displaying ulceration or hyperplasia. *p < 0.05 against same colon section and recovery time in DSS group. The data points represent the mean (± SD) of values from 9-10 mice/group.
Figure 3.2. Histological changes and gross pathology associated with colitis. (A) H&E staining in distal colon from mice following 1 week recovery after administering dextran sulfate sodium (DSS). Magnification: 20x. (B) Dietary α-mangostin (α-MG) exacerbates disease activity index (DAI) (*p < 0.05). (C) Increased fluid content in colonic lumen of mice fed AIN93G diet with α-MG; experimental groups: 1, control; 2, α-MG; 3, DSS; 4, DSS+α-MG. The green pigmentation in the colonic lumen of mice fed diet with α-MG is the dye added to the diet (see Methods). (D) Inflammation and (E) crypt injury scores in the mid and distal colon are greater in the DSS+α-MG group compared to DSS group after 1 and 2 weeks of recovery (*p < 0.05 against control; #p < 0.05 against DSS group). The data points represent the mean (± SD) of values from 9-10 mice/group.
Table 3.2. Liver and spleen weight and ratio of colon weight to colon length.

<table>
<thead>
<tr>
<th></th>
<th>1-week recovery</th>
<th>2-week recovery</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>α-MG</td>
</tr>
<tr>
<td>Liver&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0±0.3</td>
<td>3.9±0.5</td>
</tr>
<tr>
<td>Spleen&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3±0.1</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Colon&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.5±2.8</td>
<td>28.5±2.9*</td>
</tr>
</tbody>
</table>

<sup>a</sup> % body weight; <sup>b</sup> colon weigh to length ratio in mg/cm; *p < 0.05 against control group; <sup>##</sup>p < 0.05 against DSS group. The data points represent the mean (± SD) of values from 10 mice/group.
3.4.3 Dietary α-MG stimulates colonic epithelial cell proliferation

Crypt distortion, increased epithelial cell proliferation, and dysplasia have been described in the DSS colitis model (Cooper and others 1993). Mild hyperplasia was observed in both the mid and distal colon of mice in the DSS group 1 week after cessation of DSS treatment (Figure 3.2A). Hyperplasia was reversible, as it was not observed in this group at 2 weeks after DSS treatment. In contrast, moderate hyperplasia in the mid and distal colon of mice in the DSS+α-MG group was present at 1 week after DSS treatment and mild hyperplasia remained evident following 2 weeks of recovery (Table 3.1).

Colonic epithelial cell proliferation, as determined by the percentage of Ki67+ cells in the crypts (Figure 3.3A), was significantly greater in the DSS+α-MG group compared to the DSS group after 1 and 2 weeks of recovery ($p < 0.05$). Interestingly, the percentage of Ki67 expressing cells was greater in the colonic epithelium of non-DSS treated mice fed diet with α-MG as compared to control mice fed standard diet at 1 ($p > 0.05$) and 2 weeks ($p < 0.05$) (Figure 3.3D).
Figure 3.3. Representative images of (A) Ki67, (B) CD3 and (C) F4/80 immunostaining in distal colon of mice 1 week after cessation of dextran sulfate sodium (DSS) treatment. Magnification: 20x. (D) Ki67+ immunostaining of colonic epithelial cells was increased in animals fed diet with α-mangostin (α-MG). (E) CD3+ stained tissue for T cells in the colonic lamina propria of mice fed diet with α-MG was greater than in the control group. (F) Significant macrophage infiltration, as determined by immunostaining for F4/80, in the DSS+α-MG 2 weeks after recovery. *p < 0.05 against control group; # p < 0.05 against DSS group). The data points represent the mean (± SD) of values from 3 mice/group.
3.4.4 Macrophage and T cell infiltration in the colon are exacerbated by α-MG

Increased colonic infiltration of T cells and macrophages in response to DSS ingestion has been reported (Ramirez-Carrozzi and others 2011; Sund and others 2005). Thus, we examined the expression of CD3$^+$ and F4/80$^+$ in the distal colonic lamina propria as markers of T cell and macrophage infiltration, respectively (Figures 3.3B-C). The area of positively stained tissue for CD3$^+$ cells was greater in mice fed the diet with α-MG compared to control mice at 1 week, independently of DSS treatment. At 2 weeks, only the DSS+α-MG group had significantly greater infiltration of CD3$^+$ cells compared to control group ($p < 0.05$) (Figure 3.3E). Infiltration of F4/80$^+$ macrophages, as determined by the area of F4/80 staining in the tissue section, also was significantly greater in the DSS+α-MG group compared to DSS group after 2 weeks of recovery ($p < 0.05$) (Figure 3.3F).

3.4.5 Dietary α-MG exacerbates DSS-induced colonic and systemic inflammation

We examined MPO protein expression in the distal colon as a surrogate indicator of neutrophil infiltration and activity (Ren and others 2011). Colonic MPO expression was significantly greater in the DSS+α-MG group compared to the DSS group after both 1 and 2 weeks post-DSS treatment ($p < 0.05$). MPO was also significantly greater in non-DSS treated mice fed diet with α-MG compared to control mice fed standard diet at 1 and 2 weeks ($p < 0.05$) (Table 3.3).
Table 3.3. Colonic myeloperoxidase (MPO) protein and serum amyloid A levels (SAA) at 1 and 2 weeks post-DSS administration.

<table>
<thead>
<tr>
<th></th>
<th>1-week recovery</th>
<th>2-week recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>α-MG</td>
</tr>
<tr>
<td><strong>MPO</strong></td>
<td>6.1±2.7</td>
<td>25.2±12.5*</td>
</tr>
<tr>
<td><strong>SAA</strong></td>
<td>35.5±3.8</td>
<td>30.5±4.4</td>
</tr>
</tbody>
</table>

*a Colonic myeloperoxidase in ng/mg protein. The data points represent the mean (± SD) of values from n=8-10 mice/group; b Serum amyloid A in µg/mL. The data points represent the mean (± SD) of values from 6-8 mice/group; *p < 0.05 against control group; "p < 0.05 against DSS group.
Splenomegaly and increased liver weight were observed in mice in the DSS+α-MG group (Table 3.2), thus we determined the effects of α-MG on systemic markers of inflammation by analyzing a panel of soluble inflammatory factors in serum. SAA has been shown to be well correlated with disease activity in the DSS-induced colitis model (Melgar, Karlsson, Michaëlsson 2005). Consistent with these data, SAA was significantly higher in the DSS group compared to the control group ($p < 0.05$). SAA in the DSS+α-MG group was significantly greater than in the DSS group after 1 and 2 weeks of recovery ($p < 0.05$) (Table 3.3). The acute inflammatory response in the DSS-induced colitis model also has been characterized by increased serum levels of TNF-α, IL-6, and IL-17, and elevated levels of IL-6, IFN-γ, IL-4, and IL-10 have been reported in chronic colitis (Alex and others 2009). Multiplex analysis of 15 cytokines involved in inflammatory and immune responses revealed that IL-6 and granulocyte colony stimulating factor (G-CSF) were significantly increased in mice receiving DSS+α-MG treatment compared to other groups after 1 week of recovery. Following 2 weeks of recovery, G-CSF and IL-12p40 levels were significantly elevated in the DSS+α-MG group ($p < 0.05$) (Table 3.4).

3.4.6 Dietary α-MG induces changes in tissue-associated colonic and cecal microbiota

Because alterations in the gut microbiota have been implicated in the pathogenesis of UC (Tamboli and others 2004) and α-MG has been reported to exert anti-microbial activities (Pedraza-Chaverri and others 2008), we examined if this xanthone was capable
of inducing changes in the abundance of tissue-associated bacterial communities in the colon and cecum of C57BL/6 mice in the absence of DSS-induced colonic inflammation. Consistent with our initial findings (Figure 3.2C), there was an increase in fluid content in the of colonic lumen and stool of non-DSS treated mice fed diet with α-MG (α-MG group) compared to mice fed standard diet (control group), while BW and food intake did not differ between the groups (data not shown).

There was a change in the microbiota profile in the colon and cecum of mice fed diet with α-MG but no DSS. The Firmicutes and Bacteroidetes phyla comprised approximately 95-97% of the identified sequences in the colonic and cecal tissue of mice in the control group. Changes at the phylum taxonomic level were identified in mice in the α-MG group compared to animals in the control group (Figure 3.4A). The relative abundances of Firmicutes and Bacteroidetes in the colon were significantly reduced in mice fed diet with α-MG ($p < 0.01$), as was the relative abundance of Bacteroidetes in cecum ($p < 0.01$). Conversely, there was a significant increase in the relative abundance of Proteobacteria in the colon and cecum of mice fed diet with α-MG ($p < 0.01$).

To investigate which bacterial populations accounted for the changes induced by dietary α-MG at the phylum level, the relative abundances of the top 10 Orders were analyzed (Figure 3.4B). α-MG elicited a significant reduction in the abundance of Lactobacillales and Bacteroidales in the colon ($p < 0.05$). Only Bacteroidales abundance was significantly reduced in the cecum ($p < 0.01$). Within Lactobacillales, there was a significant increase in the genus Enterococcus and a decrease in the genus Lactobacillus in both colon and cecum of mice fed α-MG ($p < 0.01$, data not shown). Furthermore,
significantly lower abundances of Erysipelotrichales, Clostridiales, and Bifidobacteriales were also found in colon and cecum of these mice ($p < 0.05$). In contrast, there was a significant increase in the relative abundance of an unclassified genus of bacteria in the family Enterobacteriaceae (order Enterobacteriales) in the colon and cecum of mice in the group fed diet with $\alpha$-MG ($p < 0.01$). The Shannon index was calculated to describe within-sample diversity. Although no significant changes were identified in the colon (Figure 3.4C), a significant reduction in bacterial diversity was observed in the cecum of mice in the $\alpha$-MG group compared to mice fed standard diet ($p < 0.05$) (Figure 3.4D). PCoA provided additional evidence of changes in colonic and cecal bacterial populations induced by dietary $\alpha$-MG. Data corresponding to mice in the $\alpha$-MG group (red) clustered together and away from those corresponding to control group (blue) (Figures 3.4E-F). There were significant differences in the $\beta$-diversity of the colonic and cecal microbiota between $\alpha$-MG and control groups ($p < 0.01$).
Figure 3.4. Dietary α-mangostin (α-MG) changes the relative abundance of colonic and cecal bacterial communities at the (A) phylum and (B) order levels. Shannon Index (α-diversity) in (C) colon and (D) cecum. Principal coordinate analysis (PCoA) in (E) colon and (F) cecum. Samples in the α-MG group (red) cluster together and away from those in control group (blue). Data are from 5 mice/group.
Table 3.4. Serum cytokine profiles 1 and 2 weeks post-DSS administration.

<table>
<thead>
<tr>
<th>Cytokine&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1 week recovery</th>
<th>2 week recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>α-MG</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>37.6±16.3</td>
<td>34.3±6.4</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>11.5±17.0</td>
<td>12.5±15.5</td>
</tr>
<tr>
<td>M-CSF</td>
<td>1.6±2.2</td>
<td>1.6±1.7</td>
</tr>
<tr>
<td>G-CSF</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>8.9±10.7</td>
<td>10.5±8.5</td>
</tr>
<tr>
<td>TNF-α</td>
<td>7.5±6.7</td>
<td>6.0±3.7</td>
</tr>
<tr>
<td>IL-6</td>
<td>9.0±12.6</td>
<td>8.4±8.5</td>
</tr>
<tr>
<td>IL-1β</td>
<td>58.9±86.9</td>
<td>79.3±69.1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.8±1.3</td>
<td>0.6±0.8</td>
</tr>
<tr>
<td>IL-17A</td>
<td>4.3±4.2</td>
<td>4.1±3.1</td>
</tr>
<tr>
<td>IL-23</td>
<td>20.1±31.9</td>
<td>18.9±22.3</td>
</tr>
<tr>
<td>IL-10</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.1±0.2</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>IL-5</td>
<td>7.7±11.7</td>
<td>7.5±8.3</td>
</tr>
<tr>
<td>IL-13</td>
<td>6.6±9.9</td>
<td>7.9±8.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> cytokines in pg/mL *p < 0.05 against all other groups; nd, not detected
3.5 Discussion

CAM has become increasingly popular in the last few years with nearly 40% of American adults reporting its use (Barnes, Bloom, Nahin 2007). Given the marginal success and adverse side effects of conventional therapies, CAM is used by up to 50% of UC patients (Lanzoni and others 2008; Rahimi, Mozaffari, Abdollahi 2009). Mangosteen-containing supplements are promoted as beneficial for gut health and the immune system. α-MG, the most abundant xanthone in mangosteen, has been shown to exert anti-inflammatory activities in vitro and in vivo (Gutierrez-Orozco and Failla 2013) but its effect on experimental colitis has not been previously examined. In addition, antimicrobial activities of α-MG against pathogenic bacteria, fungi, and virus have been reported (Pedraza-Chaverri and others 2008). The aim of this study was to characterize the effects of dietary α-MG in the DSS-induced mouse model of colitis. The dose of α-MG was selected based on recent studies that showed decreased tumor growth in xenograft models of colon and prostate cancer (Chitchumroonchokchai and others 2013; Johnson and others 2012). Surprisingly, dietary α-MG exacerbated, rather than attenuated, DSS-induced inflammation and injury in the colon of C57BL/6 mice. Increased disease severity in animals receiving DSS and fed diet with α-MG included loss of body weight, greater inflammatory and crypt injury scores, immune cell infiltration, ulceration, an increased degree of hyperplasia and epithelial cell proliferation in the colon, and greater systemic and colonic inflammation. Interestingly, increased colonic MPO protein levels, epithelial cell proliferation, and immune cell infiltration were also observed in non-DSS treated mice fed diet with α-MG. Dietary α-MG also
elicited a shift in the mucosa-associated microbiota profile in the colon and cecum of non-DSS treated mice. This shift was similar to that seen in human UC (Frank and others 2007). We speculate that this alteration may be associated with the exacerbation of DSS-induced pathology.

We first observed that dietary α-MG exacerbated the pathology of DSS-induced colitis in two pilot studies. Because previous studies with transformed cells and rodents suggested that the compound has anti-inflammatory activity (Gutierrez-Orozco and Failla 2013, Chapter 2), the possibilities that the xanthone may have been degraded to toxic products or that there may have been other adverse changes in dietary quality were considered. Analyses showed that α-MG was stable in the diet during storage for at least 6 months. Also, fresh diet was prepared for use in the more comprehensive present study. The dose and time of exposure to DSS were adjusted to induce colitis without significant loss of body weight in control mice. Despite such changes, the adverse effect of dietary α-MG on colonic pathology in mice administered DSS was replicated. Contamination of diets with microbial pathogens also was unlikely because they were irradiated and handled aseptically until placed in cages. The above considerations led us to consider several reports that α-MG also has pro-inflammatory activity. This xanthone stimulated TNF-α output by normal human monocyte-derived macrophages (Gutierrez-Orozco and others 2013, Chapter 2) and increased serum levels of IL-1α, IL-1β, and complement components C3 and C4 in human subjects ingesting a mangosteen supplement (Tang and others 2009). Also, several other phytochemicals and plant extracts generally assumed to be health-promoting have been reported to exacerbate DSS-induced colitis in mice. These
included dietary luteolin, tomato lycopene, and green tea polyphenol extracts (Joo and others 2009; Karrasch and others 2007; Kim and others 2010).

Mangosteen has been used as an anti-diarrheal agent in traditional medicine for centuries (Pedraza-Chaverri and others 2008). However, mice fed diet with α-MG developed loose stools shortly after initiating feeding and independent of the administration of DSS. Preparation of the anti-diarrheal liquid involves boiling mangosteen pericarp in water (Yapwattanaphun and others 2002). The differential effect of the hot water extract and dietary α-MG is likely due to the absence of lipophilic xanthones in the former. The effect of α-MG on consistency of stool in mice aligns with the gentle laxative activity advertised online as a purported health benefit of ingested mangosteen products. It is interesting that mangosteen xanthones are structurally similar to anthraquinones, which are widely used as laxatives and have been shown to induce apoptosis of colonic epithelial cells (Walker, Bennett, Axelsen 1988). α-MG also has anti-microbial activity. For example, this xanthone inhibited pathogenic bacteria, such as *B. subtilis* (IC$_{50}$ 3.9 µM), *S. aureus* (IC$_{50}$ 7.8 µM), with no effects against other pathogens, such as *E. coli* and *C. albicans* (IC$_{50}$ >200 µM) (Al-Massarani and others 2013). In addition, α-MG has been shown to inhibit bacterial species such as methicillin-resistant *S. aureus*, anti-vancomycin-resistant *Enterococci*, *Mycobacterium tuberculosis*, and *Helicobacter pylori* at concentrations in the range of 1.6-12.5 µg/mL (Chin and Kinghorn 2008). Although these studies have been done using isolated species, they suggest that α-MG, at the levels found in the gut lumen after oral ingestion (Chitchumroonchokchai and others 2013) may affect the balance between commensal
and pathogenic bacterial communities. More importantly, the non-selective anti-microbial activities of α-MG could potentially impact commensal bacteria (Sindermsuk and Deekijserumphong 1989). Altered consistency of stool and exacerbation of DSS-induced colitis by α-MG, as well as reported antimicrobial activities of α-MG (Pedraza-Chaverri and others 2008), led us to investigate the possible effect of α-MG on the gut microbiota of healthy mice (i.e., non-DSS treated). Consistent with previous reports in both humans and mice (Eckburg and others 2005; Ley and others 2005), 95-97% of tissue-associated colonic and cecal bacteria of mice fed the control diet belonged to the Firmicutes and Bacteroidetes phyla. In contrast, mice fed the diet with α-MG had reductions in the relative abundance of Firmicutes and Bacteroidetes and increased Proteobacteria, a shift similar to that seen in UC and CD (Frank and others 2007; Lupp and others 2007; Ott and others 2004).

The change in the gut microbiota may have enhanced colonic inflammation in response to the chemical insult. Bacteria in the Bacteroidetes and Firmicutes phyla ferment dietary fiber, generating SCFA that exert anti-inflammatory activity (Macfarlane and Macfarlane 2003; Maslowski and others 2009). It is possible that the lower abundance of Bacteroidetes and Firmicutes associated with ingestion of α-MG resulted in reduced levels of colonic SCFA, thus exacerbating inflammation. In addition, some members of the microbiota, such as bacteria of the genus Lactobacillus, have immunoregulatory effects (Christensen and others 2002). Ingestion of α-MG led to a decrease in the relative abundance of Lactobacillus in the colon and cecum, and a concomitant increase in the Enterobacteriaceae, which have known pro-inflammatory
effects. This profile is often evident in various mouse models of colitis and in IBD patients (Fite and others 2013; Ley and others 2005; Pathmakanthan, Thornley, Hawkey 1999). Moreover, the increase in Enterococcus in the colon and cecum in the present study is similar to that seen during DSS-induced colitis (Heimesaat and others 2007). The reduced microbial diversity in α-MG-fed animals is similar to reports of patients with active UC (Frank and others 2007; Ott and others 2004). The possibility that the overgrowth of pathogenic bacteria associated with administration of α-MG, coupled with the DSS-induced injury in the gut epithelial barrier, may contribute to the exacerbating effects of the xanthone merits investigation. However, because host-mediated inflammation is known to alter colonic microbial populations (Lupp and others 2007), secondary changes in the gut microbiota resulting from the host’s immune response to the dietary xanthone also need to be considered.

Despite similarities at the phylum level in the gut microbiota of mice and humans, it is known that there are differences at other taxonomic levels. Nevertheless, our finding that dietary α-MG induced changes in the microbiota at the phylum level is provocative. Because host genetics and environmental factors are known to affect the composition of the gut microbiota (Campbell and others 2012) and susceptibility to DSS-induced inflammation (Kitajima and others 2001), we have examined the effect of dietary α-MG on the microbiome of healthy Balb/c, C3H, and athymic FoxN1nu mice. Interestingly, we have found that induction of dysbiosis by dietary α-MG is not strain specific, but rather a more generalized effect in mice (Chapter 4).
Because we only evaluated colonic pathology at two times after DSS administration, and the cecal and colonic microbiota after feeding diet with α-MG to healthy mice for four weeks, the effect of α-MG on earlier changes in the colonic epithelium and gut microbiota, as well as its mechanisms of action, will be a focus of future investigation. Whether a similar shift in the gut microbiota in response to dietary α-MG also occurs when DSS is administered to mice remains elusive, as does the effect of the host inflammatory response to the xanthone on the gut microbiota. Similar to the previously described hormetic response by dietary flavonoids (Siow and Mann 2010), dietary xanthones may act as hormetic agents by exerting beneficial effects at low concentrations, but having detrimental activities at higher levels. The effect of lower doses of α-MG and feeding of ground pericarp rather than the purified compound on experimental colitis also merit consideration.

In summary, our results show that dietary α-MG exacerbates DSS-induced colitis and modifies the gut microbiota in non-DSS treated mice by shifting it to a profile resembling that found in UC. We suggest that chronic consumption of mangosteen supplements rich in pericarp, and therefore xanthones, should be considered with caution by those with inflammatory bowel disorders and perhaps even by healthy individuals.
CHAPTER 4

INDUCTION OF INTESTINAL DYSBIOSIS AND COLONIC EPITHELIAL HYPERPROLIFERATION BY DIETARY $\alpha$-MANGOSTIN IS INDEPENDENT OF MOUSE STRAIN
4.1 Abstract

The gastrointestinal tract harbors a complex collection of microorganisms known as the gut microbiota that is often altered in diseased states. Dietary factors such as polyphenols have been shown to modulate the composition of the gut microbiota and we recently reported that α-mangostin (α-MG), the most abundant xanthone in mangosteen fruit, promoted dysbiosis in healthy C57BL/6J mice and exacerbated chemically-induced colitis. Despite lacking scientific evidence, mangosteen supplements claim to support gut health and immunity. The objective of this study was to determine whether the induction of dysbiosis by dietary α-MG is strain-dependent or reflect a more general response of mice to the dietary xanthone. C3H, Balb/c, and Nude FoxN1nu, and C57BL/6J mice were fed standard diet or diet containing 0.1% α-MG for 4 weeks. Pyrosequencing analysis showed that dietary α-MG significantly altered the cecal and colonic microbiota in mice independent of the strain, promoting a reduction in generally assumed beneficial bacterial groups while increasing the abundance of pathogenic bacteria. Consumption of α-MG was associated with reduced abundance of Firmicutes and increased abundance of Proteobacteria. The abundance of Lachnospiraceae, Ruminococcaceae, and Lactobacillaceae was reduced in α-MG-fed mice, while that of Enterobacteriaceae and Enterococcaceae was increased. Dietary α-MG also promoted increased proliferation of colonic epithelial cells and infiltration of immune cells, as well as increased fluid content in stool. These results suggest that ingestion of mangosteen-containing supplements may have unintended risks on gut health and should be used with caution.
4.2 Introduction

The gastrointestinal (GI) tract is inhabited by a collection of bacteria, viruses, and single-cell eukaryotes commonly referred to as the gut microbiota. These microbes play an essential role in the development of immunity both systemically and in the intestinal mucosa, as well as in the functional and structural maturation of the GI tract. In addition, the microbiota provides a protective barrier against pathogens and has a critical role in the metabolism of dietary nutrients and xenobiotics, such as phytochemicals and drugs (Sekirov and others 2010). An imbalance between putative ‘protective’ versus ‘harmful’ intestinal bacteria, also known as dysbiosis, is often associated with diseased states (Ley and others 2006; Turnbaugh and others 2006; Castellarin and others 2012; Kostic and others 2012; Larsen and others 2010). For instance, the microbiota of individuals with inflammatory bowel diseases (IBD) shifts to a reduced abundance of Firmicutes and Bacteroidetes and increased Proteobacteria (Frank and others 2007; Ott and others 2004; Lupp and others 2007). Altered bacterial profiles are also found in the gut of obese humans and mice (Castellarin and others 2012) (Ley and others 2005; Ley and others 2006), and in individuals with colorectal tumors (Kostic and others 2012), and type 2 diabetes (Larsen and others 2010). Whether such alterations in the gut microbiota are the cause or the result of the disease remains elusive.

Dietary factors have been shown to affect the composition of the gut microbiota (Turnbaugh and others 2009; David and others 2014). For instance, switching from a low fat/high fiber diet to a ‘Western’ diet high in fat and sugar alters the composition of the microbiome (Turnbaugh and others 2009). Differences in microbiota composition are
also found between individuals on an animal-based diet versus a plant-based diet (David and others 2014). Ingestion of poorly digested carbohydrates, also known as prebiotics, has been associated with increased protective bifidobacteria in humans (Ramirez-Farias and others 2009). Like prebiotics, other dietary components not absorbed in the small intestine are potential substrates for bacterial metabolism. These bacterial metabolites have the potential to exert beneficial health effects but can also have adverse consequences (Blaut and Clavel 2007).

Diets rich in fruits and vegetables have been associated with a healthy gut. This effect has been attributed in part to polyphenols which possess anti-oxidant, anti-apoptotic, anti-carcinogenic, anti-inflammatory, and anti-angiogenic activities (Del Rio and others 2013). Although many polyphenols are poorly absorbed, their concentrations in the lumen of the GI tract can be several hundred micromolar (Scalbert and Williamson 2000). Because polyphenols also exert inhibitory effects on the growth of pathogenic bacteria, fungi, and viruses (Daglia 2012; Taguri, Tanaka, Kouno 2006), they can also adversely affect the composition of the non-pathogenic gut microbiota.

The pericarp of mangosteen (Garcinia mangostana), a fruit native to Southeast Asia, has been used in traditional medicine as an anti-inflammatory and wound disinfecting agent for centuries (Yapwattanaphun and others 2002). Xanthones, the most abundant polyphenolic compounds present in mangosteen pericarp, have been associated with these and other proposed health-promoting properties (Pedraza-Chaverri and others 2008). This has provided the basis for aggressive marketing of mangosteen-containing supplements and beverages as beneficial for gut health and immune function despite the
lack of supporting scientific evidence in humans. The anti-inflammatory activity of mangosteen xanthones has been addressed by various researchers (Gutierrez-Orozco and Failla 2013). We recently reported that α-MG, the most abundant xanthone in mangosteen pericarp, inhibits the secretion of pro-inflammatory cytokines in transformed human cells (Gutierrez-Orozco and others 2013). We therefore assumed that dietary α-MG would attenuate the severity of inflammation during chemically-induced colitis in mice. However, we found that dietary α-MG exacerbated colonic injury and inflammation in the DSS murine model in comparison to DSS-treated mice fed a standard diet (Gutierrez-Orozco and others 2014). Because of the relatively poor bioavailability of xanthones in humans (Chitchumroonchokchai and others 2012), the GI tract is exposed to high concentrations of these compounds (Chitchumroonchokchai and others 2013). This turned our attention to the relatively non-selective inhibitory activities of xanthones against bacteria, fungi, and viruses that have been previously reviewed (Pedraza-Chaverri and others 2008). These reports support the possibility that orally consumed α-MG may affect the gut microbial community. Indeed, we found that α-MG promoted dysbiosis in the colon and cecum of otherwise healthy C57BL/6 mice fed diet containing 0.1% α-MG for 4 weeks. This alteration was associated with loose stools, increased proliferation of colonic epithelial cell, and increased infiltration of immune cells in the colonic lamina propria, suggesting that α-MG-induced dysbiosis may have contributed to the exacerbation of inflammation occurring in response to the DSS insult. Because host genotype is one of many factors affecting the composition of the gut microbiota, the primary objective of the present study was to determine whether the induction of
dysbiosis by dietary α-MG is strain specific or represents a more general response to the xanthone. C3H, Balb/c, and Nude FoxN1\textsuperscript{nu}, and C57BL/6J mice were fed standard diet containing 0.1% α-MG for 4 weeks. Tissue-associated bacteria in the cecum and colon, as well as the histological profiles of the colon of these mice were also analyzed.

4.3 Materials and methods

4.3.1 Mice

10-week-old female C57BL/6 (Jackson Laboratories, Bar Harbor, ME), Balb/c (Jackson Laboratories), and C3H (Charles River, Wilmington, MA) mice, as well as 8-9 week old athymic FoxN1\textsuperscript{nu} (Harlan, Indianapolis, IN) were housed in the animal facilities at The Ohio State University (OSU) under sterile conditions with controlled temperature at 23 °C and a 12-h light/dark cycle. Female mice were used as in our previous study since their response to dextran sulfate sodium-induced inflammation is less severe than males (Ding and others 2012). Mice were acclimatized for 1 week before initiating the study and had free access to water and standard AIN93G diet. All procedures were approved under Protocol #2011A00000006 and followed the guidelines by the Institutional Animal Care and Use Committee (IACUC) at OSU.

4.3.2 Diet

Gamma-irradiated AIN93G diet (control) and AIN93G diet containing 900 mg/kg α-MG (>98% pure) and FDA approved dyes E102 and E133 for green color were prepared by Research Diets (New Brunswick, NJ). This dose of α-MG was selected in light of
recent reports indicating its safety and efficacy in reducing tumor mass in xenograft models of colon and prostate cancer (Chitchumroonchokchai and others 2013; Johnson and others 2012). For a mouse weighing 20 g and ingesting 2.5 g diet/day, this dose is equivalent to 112 mg/kg body weight. Thus, the equivalent dose \[ \frac{(112 \text{ mg/kg})}{(3/37)} = 9.12 \text{ mg/kg} \] for an adult weighing 60 kg equates to 547 mg/day (Reagan-Shaw, Nihal, Ahmad 2008). This dose is achievable for individuals ingesting 1-2 mangosteen supplement capsules (Johnson and others 2012) or 1 cup of 100% mangosteen juice (Chitchumroonchokchai and others 2012).

4.3.3 Experimental groups and tissue collection

After the one-week acclimation to the animal facility, mice of each strain were randomly assigned to be fed either the standard AIN93G diet (control group, n=5) or the AIN93G diet containing α-MG (α-MG group, n=5). Mice had ad libitum access to tap water and their respective diets for 4 weeks. Body weight (BW) was recorded throughout the experiment. Fresh stool was collected within a time frame of 10 min of expulsion and placed in sealed microtubes to prevent evaporation. Fresh weight was recorded soon after and stool was transferred to dishes and placed in an oven at 60 °C. Dry stool weight was determined to calculate water content. At necropsy, the colon and cecum were excised aseptically, opened and gently rinsed in sterile cold PBS. The cecum and the distal sections of the colon were transferred to sterile tubes, frozen in liquid nitrogen and stored at -80 °C until analysis of microbiota by pyrosequencing. In addition, the mid and
proximal sections of the colon were collected and fixed in 10% neutral buffered formalin for 24 h and processed for histologic evaluation and immunohistochemistry analysis.

4.3.4 Histology and immunohistochemistry (IHC)

For IHC analysis, 5-μm-thick, paraffin-embedded sections of the mid colon from 5 mice per group were used. T cells and macrophages were identified using CD3 and F4/80, respectively, as markers. CD3 immunostain was performed using a rabbit anti-human polyclonal antibody diluted 1:200 (Dako #A0452, Dako, Carpinteria, CA). F4/80 detection was performed using a rat anti-mouse monoclonal antibody diluted 1:100 (Serotec #MCA497G, Bio-Rad, Raleigh, NC). Detection and quantification were performed as previously described (Gutierrez-Orozco and others 2014). To evaluate epithelial cell proliferation, tissue sections were immunostained for Ki67 using a commercially available rat anti-mouse monoclonal antibody diluted 1:50 (clone TEC-3; #M7249; Dako, Carpinteria, CA). Detection and quantification followed protocols previously described (Gutierrez-Orozco and others 2014).

4.3.5 Bacterial analyses

Bacterial analyses were performed using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) at the Research and Testing Laboratory (Lubbock, TX). The 16S rRNA gene sequence was amplified for pyrosequencing using a forward and reverse fusion primer. The forward primer was constructed with (5’-3’) the Roche A linker (CCATCTCATCCCTGCGTGTCTCCGACTCAG), an 8-10bp barcode, and the forward
specific universal primer 28F (5’-GAGTTTGATCNTGGCTCAG-3’). The reverse fusion primer was constructed with (5’-3’) a biotin molecule, the Roche B linker (CCTATCCCCTGTGTCCTGGCAGTCTCAG), and the reverse specific primer 519R (5’-GTNTTACNGCGGCKGCTG-3’). Amplifications were performed in 25 µL reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, CA), 1 µL of each 5 µM primer, and 1 µL of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, CA) using the following thermal treatment: 95 °C for 5 min, 35 cycles of 94 °C for 30 sec; 54 °C for 40 sec; 72 °C for 1 min, followed by one cycle of 72 °C for 10 min and 4 °C hold. Amplification products were visualized with eGels (Life Technologies, Grand Island, NY). Products were then pooled equimolar and each pool was cleaned with Diffinity RapidTip (Diffinity Genomics, West Henrietta, NY), and size selected using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, IN) following Roche 454 protocols (454 Life Sciences, Branford, CT). Size selected pools were then quantified and 150 ng of DNA were hybridized to Dynabeads M-270 (Life Technologies, Grand Island NY) to create single stranded DNA following Roche 454 protocols (454 Life Sciences, Grand Island, NY). Single stranded DNA was diluted and used in emPCR reactions, which were performed and subsequently enriched. Sequencing was done following established manufacturer protocols (454 Life Sciences). Fasta and qual files obtained from pyrosequencing were uploaded into Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso and others 2010b). Clustering of sequencing reads into operational taxonomic units (OTUs) was achieved at 97% identity. OTU picking was performed in the QIIME pipeline using the Uclust
algorithm (Edgar 2010). Taxonomic assignment was achieved using the Ribosomal Database Project (RDP) classifier (Wang and others 2007), employing the GreenGenes database (McDonald and others 2012). The minimum and maximum sequence lengths were 200 and 1000, respectively. The minimum qual score was 25. The maximum number of ambiguous bases was 6 and no primer mismatch was allowed. 86.1% of sequences were retained after quality trimming. Sequences PyNAST (Caporaso and others 2010a) aligned by QIIME were used the Shannon index (Shannon 1997) as a measurement of α-diversity using cutoff depths of 1370 and 1452, 1700 and 1912, 1774 and 1646, and 1112 and 902 sequences for cecum and colon samples, from Balb/c, C3H, C57BL/6, and Athymic FoxN1\textsuperscript{nu} strains, respectively. Unifrac analysis (Lozupone and Knight 2005) followed by principal coordinate analysis (PCoA, based on unweighted unifrac distance) were done to characterize the diversity in the bacterial populations by using 1639 and 1574, 1893 and 2074, 1858 and 1769, and 1140 and 1113 sequences for cecum and colon, from Balb/c, C3H, C57BL/6, and Athymic FoxN1\textsuperscript{nu} strains, respectively, and jackknifed data (1229 and 1180, 1419 and 1555, 1393 and 1326, and 855 and 834 sequences for cecum and colon, respectively) from Balb/c, C3H, C57BL/6, and Athymic FoxN1\textsuperscript{nu} strains.

4.3.6 Statistical analysis

All data are expressed as mean ± SD. For parametric data, statistical differences were determined by one-way analysis of variances followed by Tukey’s test. Nonparametric data were analyzed by Kruskal-Wallis test followed by selected mean comparisons with
Bonferroni correction. Differences were considered statistically significant at $p < 0.05$. Analyses were performed using SPSS v. 20 (IBM, Armonk, NY). For pyrosequencing data, statistically significant differences in unweighted Unifrac distance were investigated by Analysis of Similarities (ANOSIM), provided by R’s vegan package implemented into QIIME.

4.4 Results

4.4.1 Dietary $\alpha$-MG increases fluid content in stool

Ingestion of diet containing $\alpha$-MG did not result in significant differences in either food intake or body weight compared to ingestion of control diet for any of the four mouse strains (Table 4.1). However, consumption of $\alpha$-MG resulted in a rapid and significant increase in the fluid content of stools for all four strains (Figure 4.1). Fluid content of stool was significantly greater in mice fed $\alpha$-MG by 29%, 20%, 13%, and 8.5% five days after initiating the dietary intervention in C57BL/6J, Balb/c, C3H and athymic FoxN1$^{nu}$ mice, respectively, compared to control mice. Although this difference in stool fluid content between the dietary treatment groups tended to decrease for the C57BL/6J and Balb/c mice during the study, fluid content of stool from Balb/c and C3H mice fed diet with $\alpha$-MG for 26 days remained significantly elevated compared to mice fed control diet. Athymic FoxN1$^{nu}$ also had significantly greater water content in stool when fed $\alpha$-MG for 18 days, although there was no difference between control and $\alpha$-MG groups after 26 days.
4.4.2 Colonic epithelial cell proliferation and immune cell infiltration are stimulated by α-MG

To assess epithelial cell proliferation, Ki67+ cells were quantified in the mid colon of C3H, Balb/c, and athymic FoxN1<sup>nu</sup>, as well as in C57BL/6J mice to verify our previous observation with this strain (Gutierrez-Orozco and others 2014). There was a significant increase in Ki67+ cells in the mid colonic epithelium of all four strains of mice fed diet with α-MG compared to control animals (Figures 4.2A,D). Infiltration of CD3+ and F4/80+ cells, markers of T cells and macrophages, respectively, in mid colon also was significantly increased in all four strains of mice fed diet with α-MG compared to control diet (Figures 4.2B,E and 4.2C,F).
Table 4.1. Body weight (g) and average food intake (g/mouse/day) of mice fed control diet or diet with α-MG for 4 weeks.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Body Weight (g)/Experimental Day</th>
<th>Food intake (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>control</td>
<td>18.4±0.6</td>
<td>19.2±1.1</td>
</tr>
<tr>
<td></td>
<td>α-MG</td>
<td>18.7±1.9</td>
<td>19.0±1.6</td>
</tr>
<tr>
<td>Balb/c</td>
<td>control</td>
<td>19.9±1.1</td>
<td>20.6±1.0</td>
</tr>
<tr>
<td></td>
<td>α-MG</td>
<td>20.5±1.0</td>
<td>20.6±0.6</td>
</tr>
<tr>
<td>C3H</td>
<td>control</td>
<td>25.2±1.2</td>
<td>25.0±1.4</td>
</tr>
<tr>
<td></td>
<td>α-MG</td>
<td>25.6±1.1</td>
<td>25.4±1.6</td>
</tr>
<tr>
<td>Athymic Fox N1&lt;sup&gt;nu&lt;/sup&gt;</td>
<td>control</td>
<td>22.1±1.4</td>
<td>22.4±1.5</td>
</tr>
<tr>
<td></td>
<td>α-MG</td>
<td>23.0±0.9</td>
<td>23.6±1.0</td>
</tr>
</tbody>
</table>

Data are means ± standard deviation for n=5 mice/group
Figure 4.1. Water content in stool excreted by C57BL/6J, Balb/c, C3H, and Athymic FoxN1\textsuperscript{nu} mice during feeding of control diet or diet with \( \alpha \)-MG. Stools were collected at the times indicated after initiating dietary intervention. *\( p < 0.05 \) against control group. Data represent the mean (±SD) of values for 3-5 mice per group for each murine strain on indicated days of the study.
Figure 4.2. Dietary α-MG induces a significant increase in Ki67\(^+\) (A,D), CD3\(^+\) (B,E), and F4/80\(^+\) (C,F) immunostaining in the mid colon of C57/BL6J, Balb/c, C3H and Athymic FoxN1\(^{nu}\) mice. Representative images of Ki67\(^+\), CD3\(^+\), and F4/80\(^+\) staining are shown; magnification: 20X. *p<0.05 against control group. Data are mean (±SD) for 5 mice per group for each strain fed either control diet or diet containing α-MG.
4.4.3 Dietary α-MG induces dysbiosis in mice with different genetic backgrounds

Because host genetics are known to affect the composition of the gut microbiota (Campbell and others 2012), we sought to determine if diet with α-MG influenced the gut microbiota in a strain-specific or strain-independent manner. The tissue-associated bacterial communities in the colon and cecum of three additional mouse strains were determined after feeding either control diet or a diet with α-MG for 4 weeks. Rather than using individual primers as for our previous report (Gutierrez-Orozco and others 2014), a universal bacterial primer was used for the pyrosequencing analysis in the present study. Thus, samples from our previous study using the C57BL/6J strain were re-analyzed to verify our earlier observations.

4.4.3.1 C57BL/6J strain

The phyla Firmicutes, Bacteroidetes, and Tenericutes comprised 97% and 88% of the identified sequences in the cecal and colonic tissue of control C57BL/6J mice, respectively. The relative abundances of Firmicutes and Tenericutes were significantly reduced when C57BL/6J mice were fed diet with α-MG (p<0.01). This change was associated with a significant increase in the relative abundance of Proteobacteria in the cecum and colon of these mice (p<0.01), accounting for 86% and 82% of all sequences, respectively (Figure 4.3A). At the family level, dietary α-MG elicited a significant reduction in the relative abundance of Erysipelotrichaceae, Lactobacillaceae, Rikenellaceae, Rumminococcaceae and Lachnospiraceae (p<0.01), while inducing a
significant increase in the abundance of Enterobacteriaceae and Enterococcaceae in both cecum and colon (p<0.01) (Figure 4.3B). At the genus level, Allobaculum, Lactobacillus, Alistipes and an unclassified genus within Lachnospiraceae were reduced in mice fed α-MG, while increases in Enterobacter and Enterococcus were observed.

The within-sample diversity was calculated using the Shannon index. Dietary α-MG elicited a significant reduction in bacterial diversity in the cecum and colon of C57BL/6J mice (p<0.01) (Figures 4.3C and 4.3D). PCoA results indicated significantly different bacterial communities in the colon and cecum of these mice, as samples in the α-MG group clustered together and away from the control samples (Figures 4.3E and 4.3F). Accordingly, there were significant differences in the β-diversity between dietary groups (p<0.01).
Figure 4.3. α-mangostin (α-MG) changes the relative abundance of bacterial communities in the cecum and colon of C57BL/6J mice at the (A) phylum and (B) family levels. Mice fed α-MG had significantly lower α-diversity) in (C) cecum and (D) colon compared to mice fed control diet. Principal coordinate analysis (PCoA) in (E) cecum and (F) colon. Samples in the α-MG group (red) cluster together and away from those in control group (blue). Data are from 5 mice/group.
4.4.3.2 Balb/c strain

Firmicutes and Bacteroidetes accounted for 84% and 14%, respectively, of all sequences in the cecum of control Balb/c mice animals. These proportions were significantly altered by dietary α-MG to 59% and 31%, respectively (p<0.01), and accompanied by a significant increase in the abundance of Tenericutes (p<0.05). In the colon, only Firmicutes significantly decreased in α-MG-fed animals (p<0.01), with Bacteroidetes remaining unchanged. Greater relative abundance of Tenericutes and unclassified sequences at the phylum level was observed in colon of mice fed α-MG, although these changes failed to achieve statistical significance (Figure 4.4A). The abundance of the Firmicutes family members Lachnospiraceae and Ruminococcaceae in the cecum was significantly reduced (p<0.01). In addition to these changes, Lactobacillaceae was significantly decreased (p<0.01) in the colon of Balb/c mice fed diet containing α-MG. There was also a significantly greater abundance of an unclassified family within Bacteroidetes in the cecum of Balb/c mice fed α-MG (p<0.01). The relative abundance of Enterococcaceae was greatly increased in both cecum and colon of mice fed diet with α-MG (p<0.01) (Figure 4.4B). At the genus level, Lactobacillus and an unclassified genus within Lachnospiraceae were reduced in mice fed α-MG, while the relative abundance of Enterococcus was increased.

There was a significant reduction in the within-sample bacterial diversity in the cecum of Balb/c mice in the α-MG group (p<0.01) (Figure 4.4C). Reduced α-diversity was also observed in the colon (p=0.05) (Figure 4.4D). The bacterial communities in the colon and cecum of Balb/c mice analyzed by PCoA revealed marked separation of samples
according to the dietary treatment group (Figures 4.4E and 4.4F). Significant differences in the β-diversity between dietary groups also were observed (p<0.01).
Figure 4.4. The relative abundance of bacterial communities in the cecum and colon of Balb/c mice is altered by dietary α-mangostin. Relative abundance of bacteria at the (A) phylum and (B) family levels. Significantly lower α-diversity in (C) cecum and (D) colon of mice fed α-MG. Principal coordinate analysis (PCoA) in (E) cecum and (F) colon. Samples in the α-MG group (red) are well separated from those in control group (blue). Data are from 5 mice/group.
**4.4.3.3 C3H strain**

Firmicutes and Bacteroidetes accounted for 55% and 26% of all sequences in the cecum of C3H control mice. The relative abundance of Firmicutes was significantly reduced by dietary α-MG to 15% (p<0.05), whereas that of Bacteroidetes was not altered. There also was a significant increase in the abundances of Proteobacteria and Verrucomicrobia (p<0.01). While Firmicutes was significantly decreased in the colon of C3H mice in the α-MG group (p<0.01), the relative abundances of Verrucomicrobia, Bacteroidetes, and Proteobacteria significantly increased (p<0.05) (Figure 4.5A). Significantly reduced cecal abundance of Lachnospiraceae, Deferrribacteraceae, Ruminococcaceae, Lactobacillaceae, and an unclassified family within Bacteroides were observed in mice fed diet with α-MG (p<0.01). In addition to these changes, Lactobacillaceae significantly decreased in the colon in response to dietary α-MG (p<0.05). There also was a significantly greater abundance of Bacteroidaceae, Enterobacteriaceae, and Verrucomicrobiaceae in both cecum and colon of C3H mice fed α-MG (p<0.05). The abundance of Lactobacillaceae, Ruminococcaceae, and an unclassified family within Bacteroides in the colon of C3H mice fed α-MG also was decreased (Figure 4.5B). At the genus level, Lactobacillus, Mucispirillum, and an unclassified genus within Lachnospiraceae were reduced in mice fed α-MG, whereas there were increases in the abundance of Bacteroides, Enterobacter, and Akkermansia.

C3H mice fed α-MG had a significant reduction in the within-sample bacterial diversity in the cecum (p<0.01), but not in the colon (Figures 4.5C and 4.5D). Results from the PCoA revealed significantly different bacterial communities in the colon and
cecum of C3H mice fed diet with α-MG compared to those fed the control diet (Figures 4.5E and 4.5F). Significant differences in the β-diversity between dietary groups also were observed (p<0.01).
Figure 4.5. Dietary α-mangostin impacts the gut microbiota in the cecum and colon of C3H mice at the (A) phylum and (B) family levels. Mice fed α-MG had significantly lower α-diversity in (C) cecum but not in (D) colon. Principal coordinate analysis (PCoA) in (E) cecum and (F) colon. Samples in the α-MG group (red) cluster together and away from those in control group (blue). Data are from 5 mice/group.
4.4.3.4 Athymic FoxN1\textsuperscript{nu} strain

Firmicutes accounted for 99% and 61% of all sequences in the cecum and colon, respectively, of athymic FoxN1\textsuperscript{nu} mice fed the standard AIN-93G diet. The relative abundance of Firmicutes in the cecum and colon of mice fed diet with α-MG was significantly reduced to 62% and 19%, respectively (p<0.01). These changes were associated with a significant increase in Proteobacteria in cecum and colon (p<0.01) (Figure 4.6A). At the family level, Lachnospiracea and Turicibacteriacea were significantly reduced in cecum and colon of mice fed α-MG (p<0.01). A significant increase in the colonic and cecal abundance of Enterobacteriacea was also observed in this group (p<0.01) (Figure 4.6B). The relative abundance of two genera, Enterobacter and Anaerotruncus, significantly increased in the colon and cecum of mice fed the diet containing α-MG.

The α-diversity (as measured by the Shannon index) was significantly reduced in the cecum and colon of mice fed α-MG (p<0.01) (Figures 4.6C and 4.6D). These tissues also had significantly different microbial communities when compared to those from control animals, as seen in the PCoA analysis (Figures 4.6E and 4.6F). The β-diversity also was significantly different between dietary groups in both cecum and colon (p<0.01).
Figure 4.6. The gut microbiota in the cecum and colon of athymic FoxN1\textsuperscript{nu} mice is altered by dietary α-mangostin. Relative abundance of bacteria at the (A) phylum and (B) family levels. Mice fed α-MG had significantly lower α-diversity (C) cecum and (D) colon compared to mice fed control diet. Principal coordinate analysis (PCoA) in (E) cecum and (F) colon. Samples in the α-MG group (red) cluster together and away from those in control group (blue). Data are from 5 mice/group.
4.5 Discussion

Mangosteen is a fruit native to Southeast Asia where hot water extracts of pericarp have been used in traditional medicine to treat infected wounds, diarrhea and chronic ulcers (Yapwattanaphun and others 2002). In vitro and animal studies have suggested that α-MG has potential as an anti-inflammatory (Chen, Yang, Wang 2008; Jang and others 2012; Tewtrakul, Watanapiromsakul, Mahabusarakam 2009), anti-cancer (Shan and others 2011), and antimicrobial (Sindermsuk and Deekijsermphong 1989; Pedraza-Chaverri and others 2008) agent. Supplements containing mangosteen are advertised by the nutraceutical industry as beneficial for gut health and immunity, along with other claimed health promoting properties. However, prior studies by our group indicated that dietary α-MG, the most abundant xanthone in mangosteen pericarp, exacerbated colonic injury and inflammation in colitic C57BL/6J mice, and increased fluid content of stools and infiltration of immune cells in the colon of non-colitic animals. The dietary xanthone also induced dysbiosis in the cecum and colon of otherwise healthy C57BL/6J mice (Gutierrez-Orozco and others 2014).

The objective of the present study was to determine if the changes in the microbiota and colonic epithelium associated with ingestion of α-MG were specific to the C57BL/6J mouse strain or represented a more general response of mice to the xanthone. Host genetics affect both the response to chemical induction of colitis (Kitajima and others 2001) and the composition of the gut microbiota (Campbell and others 2012). The present results clearly show that feeding a diet containing 0.1% α-MG for 4 weeks to otherwise healthy C57BL/6J, C3H, Balb/c, and athymic FoxN1nu strains of mice induced an
increase in fluid content in stools without adversely affecting food intake or body weight. Moreover, chronic consumption of the diet with α-MG induced dysbiosis in the colon and cecum of these murine strains. These changes in the tissue-associated microbiota induced by α-MG resemble those reported in individuals with IBD (Fite and others 2013; Frank and others 2007; Pathmakanthan, Thornley, Hawkey 1999). Greater colonic epithelial cell proliferation and infiltration of T lymphocytes and macrophages were also observed in the colon of α-MG-fed mice. Our findings suggest that ingestion of mangosteen products may have unintended effects on health status, especially on individuals predisposed to or afflicted with inflammatory bowel disorders.

Inflammation has been associated with the development of many chronic diseases including cancer and cardiovascular disease (Calder and others 2009). Dietary polyphenols may modify inflammatory processes and have been reported as beneficial for the prevention of inflammation and the attenuation of the severity of pathology associated with excessive inflammation (Romier and others 2009). Various investigators have reported that α-MG also exerts anti-inflammatory activities in *in vitro* and animal models (Gutierrez-Orozco and Failla 2013). Less attention has been given to the well-known anti-microbial activities of these plant secondary metabolites (Daglia 2012), including α-MG and other xanthones (Pedraza-Chaverri and others 2008). As for other polyphenols, the majority of the studies addressing the anti-microbial activity of α-MG have used a single microorganism as the target rather than complex microbial communities such as those that exist in the large intestine. We are aware of a single report demonstrating the ability of α-MG to inhibit the growth of commensal bacteria
(Sindermsuk and Deekijsermphong 1989), supporting the possibility that dietary α-MG can alter the complex microbial communities in the cecum and colon. Our results clearly demonstrate that chronic ingestion of α-MG induced marked alterations in the colonic and cecal microbiota of all four murine strains selected for the present investigation. The extent and type of these disruptions differed among strains but shared several common features. For instance, α-MG decreased the relative abundance of the phylum Firmicutes and its families Lachnospiraceae, Ruminococcaceae, and Lactobacillaceae. Also, the relative abundance of Proteobacteria and its Enterobacteriaceae family and the Firmicutes family Enterococcaceae increased in response to dietary α-MG.

Firmicutes is the most abundant (64%) bacterial phylum in the human gut, while members from the Proteobacteria phylum are found in relatively lower abundance (8%) among the human gut microbiota (Frank and others 2007). This suggests that the alterations induced by α-MG may have important health implications. Reduced abundance of Firmicutes and increased abundance of Proteobacteria have been observed in individuals with IBD (Fite and others 2013; Pathmakanthan, Thornley, Hawkey 1999). Reduced abundance of the Firmicutes family members Rumminococcaea and Lachnospiraceae is likely to affect the production of short chain fatty acids which are known to have anti-inflammatory activities and serve as fuel for colonic epithelial cells (Macfarlane and Macfarlane 2003; Maslowski and others 2009). Similar to our results, the polyphenols quercetin and naringenin have been shown to inhibit the growth of Lactobacillus species in a dose dependent manner (Duda-Chodak 2012). This bacterial group is predominant in the gut and it is linked to beneficial effects in the colon, such as
the inhibition of growth of pathogenic bacterial species and the production of organic acids such as acetate and lactate that are utilized as fuel by colonic epithelial cells (Walter 2008).

The increase in Proteobacteria in the colon and cecum of mice fed α-MG was primarily associated with an increase in Enterobacteriaceae. A similar increase in Proteobacteria was caused by black tea and red wine grape polyphenols, although such change was largely due to greater abundance of Klebsiella sp., a commensal opportunistic pathogen (Kemperman and others 2013). The mechanisms by which polyphenols influence the composition of non-pathogenic microbial communities in the gut remain unknown. C3H mice fed diet containing α-MG also had increased abundance of Verrucomicrobia. Interestingly, this phylum is increased in the human gut after exposure to broad-spectrum antibiotics (Dubourg and others 2013). Dietary α-MG also increased the abundance of genera such as Alistipes and Akkermansia. Black tea and a red wine grape extract promoted the growth of Akkermansia, and the red wine grape extract also stimulated an increase in Alistipes (Kemperman and others 2013). Akkermansia participates in the degradation of mucin (Derrien and others 2008), and along with Alistipes, metabolize polyphenols (Li and others 2009; Kemperman and others 2013).

Because the absorption of most polyphenols is inefficient, these compounds transit into the colon in significant quantities (Scalbert and Williamson 2000) where they can exert effects on and be metabolized by the gut microbiota. The absorption of xanthones is relatively poor (Chitchumroonchokchai and others 2012) and their concentrations in plasma are much lower than those found in the gastrointestinal tract
(Chitchumroonchokchai and others 2013). It is possible that xanthones and/or their metabolites modulate crosstalk between the colonic epithelium, the gut microbiota and the gut immune system. Because balanced interactions among epithelium, microbiota, and immune system are crucial for maintaining health, disruption of the balance (dysbiosis) may predispose to a diseased state. More research is needed on the role of parent dietary bioactive compounds and/or their metabolites in modulating the gut microbiome. Whether α-MG is metabolized by gut microbes remains unknown, but the increased abundance of some groups of bacteria in response to the ingestion of the xanthone suggests that these groups may preferentially be able to catabolize the xanthone or its products for use as an energy source to support their growth. Elucidation of primary targets for the anti-bacterial activity of α-MG in the complex cecal and colonic environments also requires investigation, as does the reversibility of the changes on microbiota induced by α-MG. It is possible that the increased infiltration of pro-inflammatory immune cells in the colon could be a response of the host to the microbial changes. The gut microbiota, however, can also be altered in response to host mediated inflammation (Lupp and others 2007). The sequence of changes induced by α-MG requires further investigation to determine if dysbiosis is a direct effect of the xanthone or the result of a pro-inflammatory immune response of the host to α-MG that in turn mediates changes in the microbiota.

The present study was limited to examining the effect of a single dose (0.1% wt/wt) of α-MG. This dietary dose of α-MG decreased tumor growth in xenograft models of colon and prostate cancer (Chitchumroonchokchai and others 2013; Johnson and others 2012).
but exacerbated murine colitis in our recent study (Gutierrez-Orozco and others 2014). This dose was estimated to be equivalent to that delivered by ingesting 1-2 capsules of mangosteen pericarp supplement of unknown xanthone composition (Johnson and others 2012) or 1 cup of 100% mangosteen juice (Chitchumroonchokchai and others 2012). Studies are now needed to determine if the effects of dietary α-MG on the gut microbiota are dose dependent in mice and whether mangosteen-containing beverages and supplements also alter the microbiota in humans.

In summary, our results suggest that ingestion of α-MG may adversely impact the gut microbiota. These alterations resemble some of the changes present in the microbiota of individuals with IBD. The level of α-MG tested in our study can be achieved by ingesting available mangosteen-containing beverages and supplements. Thus, we suggest that consumption of such products may represent an unintended risk especially for those predisposed to or suffering from inflammatory bowel disorders and should be avoided pending further information.
CHAPTER 5

EPILOGUE

Xanthones are the major polyphenolic compounds found in mangosteen fruit. Using in vitro and animal models, several bioactivities have been attributed to xanthones such as anti-inflammatory properties. This led me to consider the possibility that α-MG could exert protective effects during chronic inflammation, such as that associated with ulcerative colitis. I also considered that studying the effects of α-MG on colonic inflammation was of physiological relevance given the low bioavailability of mangosteen xanthones. However, I found that dietary α-MG had detrimental effects on gut health (Chapter 3). My in vitro studies also supported the possibility that α-MG may have a pro-inflammatory effect on normal cells (Chapter 2). α-MG also modified the gut microbiota to a profile that is associated with diseased state (Chapter 4). My results have generated a number of questions that require further investigation.

More than 60 xanthones have been isolated from mangosteen pericarp, among which α-mangostin is the most abundant xanthone (Pedraza-Chaverri and others 2008; Walker
Therefore, $\alpha$-MG is by far the most studied xanthone regarding its bioactivities. Based on the limited scientific evidence currently available and the fact that mangosteen has been used in traditional Thai medicine for many years, nutraceutical companies have advertised numerous health benefits of mangosteen-containing supplements, such as gut health and immunity. However, I am not aware of any chemical characterization of the preparations of mangosteen used in traditional medicine to determine the relative concentrations of the different xanthones in these products and how those levels compared to the xanthone composition in mangosteen supplements. Because these preparations are usually made by boiling roasted pericarp in water, xanthone levels are expected to be very low as they are lipophilic compounds.

The relative bioactivities of the different xanthones present in mangosteen pericarp remain to be determined. Although some studies have been performed using crude extracts of mangosteen pericarp or pure xanthones, the relative contribution of each xanthone or their combinations to the observed biological activity remains unknown. Whether different xanthones interact in additive, synergistic, or antagonistic manners needs to be evaluated.

Phase I and II metabolism of xenobiotics results in the generation of metabolites that are more water soluble and available for efflux by phase III transporters. I found that phase II metabolites of $\alpha$-MG were absent in medium of HepG2, THP-1, and monocyte-derived macrophage cells, exposed to $\alpha$-MG for 24h. Rather, these metabolites were present in cell pellets (Chapter 2). Previous studies have reported that phytochemicals such as resveratrol, capsaicin, curcumin, and gingerol have inhibitory effects on efflux
transporters (Nabekura, Kamiyama, Kitagawa 2005; Lancon and others 2007). The potential inhibition of efflux transporters such as MRP2 (multidrug resistance protein 2) and P-gp (P-glycoprotein) by α-MG merits consideration. Mangosteen xanthones were also reported to be non-selective inhibitors of multiple P450 isoforms, and particularly the CYP2 family (Foti and others 2009). Inhibition of phase I and II enzymes, as well as efflux transporters, by mangosteen xanthones may have important implications, especially when mangosteen-containing supplements are co-administered with prescribed drugs. The interaction of xanthones with drug metabolism could result in increased absorption of drugs with potential adverse consequences. Although the bioavailability of xanthones is poor, the interaction of these compounds with drug efflux during first pass metabolism in the small intestine needs further investigation. Caco-2 human intestinal cells provide a useful model for investigating possible interactions between xanthones and efflux transporters. Cells would be treated with a single xanthone or mixture of xanthones in the apical compartment. Verapamil is an inhibitor of P-glycoprotein (Pgp), while MK571 is an inhibitor of multidrug resistance protein 2 (MRP2). Specific substrates for these efflux transporters, such as rhodamine 123 and carboxy-dichlorofluorescein diacetate (CDFA), respectively, would be added to the basolateral compartment and their efflux into the apical compartment measured by fluorescence.

Since recent evidence suggests that phase II metabolites, first assumed to be inactive, actually exert some of the bioactivities attributed to their parent compounds (Larrosa and others 2010; Lotito and others 2011; Williamson and others 2005), the biological activities of individual xanthone metabolites and/or their combinations also need to be
considered. Phase II metabolites could be generated using the Caco-2 small intestinal cell model. Cell cultures of macrophage-like cells, for instance, could then be pretreated with these metabolites before addition of a pro-inflammatory stimulus. The secretion of inflammatory mediators into medium would serve as a marker of activity of the metabolites.

The majority of studies addressing the biological activities of mangosteen xanthones have been performed in cultured cells of cancer origin. The effect of these compounds on normal cells has not been investigated. The results of my in vitro experiments suggested that α-MG affects the inflammatory response by normal cells differently when compared to transformed cells (Chapter 2). Whether this difference is limited to the inflammatory response in human cells is not clear. Two human studies also suggest that mangosteen xanthones may stimulate a pro-inflammatory response (Kondo and others 2009; Udani and others 2009). Food supplements are usually ingested not only for therapeutic purposes, but also for the prevention of disease. Thus, the possibility that mangosteen-containing supplements may have an adverse effect on otherwise healthy individuals needs further investigation. If resources were unlimited, a clinical study could be done in which healthy individuals would consume commercially available supplements containing mangosteen xanthones. Inflammatory markers in plasma would be taken at baseline and after chronic ingestion of the supplement. Ideally, and to eliminate confounding variables, subjects would consume a controlled diet throughout the study and the mangosteen supplement would be devoid of other components. In addition, the
potential long term toxicity of products containing mangosteen xanthones requires
assessment.

The effect of the food matrix on the release of xanthones during digestion has not been
evaluated. The concentrations of xanthones in the gut lumen that are available for
absorption by intestinal epithelial cells are expected to be different depending on the type
of ingested product, namely, milled pericarp, extracts, or pure xanthones. Because fiber is
present in products containing milled pericarp, fermentation of this fiber by the gut
microbiota may modify the levels of xanthones available in the colonic lumen.

I only tested the impact of α-MG on colitis using a single colitis model (Chapter 3).
Whether α-MG exacerbates colonic inflammation and injury in other colitis models needs
to be determined. For instance, a genetic model of colitis such as the IL10−/− KO model
could be used. Only one dose of α-MG was investigated in the animal studies. It is
possible that the bioactivities of xanthones and/or metabolites are mediated by inducing
an adaptive stress or “hormetic” response (Siow and Mann 2010; Speciale and others
2011). Therefore, higher concentrations of these compounds (such as those found in the
GI tract) may not necessarily be beneficial. Further investigations should evaluate the
lowest dose of α-MG or xanthone mixtures that induces changes in the gut microbiome
and the colonic epithelium. Also, milled pericarp rather than pure xanthones, should be
used in future dietary interventions in animals to determine if the presence of other
xanthones modifies the outcome.

I also found that α-MG stimulated proliferation of colonic epithelial cells in otherwise
healthy mice (Chapters 3 and 4). Although these observations contradict the findings
that dietary α-MG inhibited tumor growth in a xenograft model of colon cancer (Chitchumroonchokchai and others 2013), the levels of the xanthone in the tumor tissue are lower than those found in the gut lumen. Therefore, the implications of my findings regarding the development of pre-cancerous lesions in the colon after long term feeding of diet with α-MG, or other xanthones, warrants further investigation.

My results showed that mice that were fed diet with α-MG had increased infiltration of immune cells in the colon and altered composition of the gut microbiota (Chapters 3 and 4). However, it was unclear if α-MG directly affected the gut microbiota by promoting the overgrowth of pathogenic bacterial populations which could then stimulate a pro-inflammatory immune response. Alternatively, α-MG could have a direct effect on the gut-associated immune system, promoting host-mediated inflammatory conditions that benefit the overgrowth of pathogenic bacteria. To study this further, germ-free mice could be fed diet containing α-MG or milled pericarp to evaluate the effects on the immune cells in the gut wall independent of the gut microbiota. To study the effect of mangosteen xanthones on the gut microbiota independent of host-mediated inflammation, an ex vivo system such as SHIME, or simulator of the human intestinal microbial ecosystem, could be used. If resources are not a limiting factor, long term effects of a product containing mangosteen xanthones on specific bacterial species could be evaluated. It would also allow the study of metabolic products resulting from bacterial metabolism of α-MG. These metabolites could be isolated, identified, and tested for possible bioactivities.
Germ-free mice are a powerful tool to further investigate the interactions among mangosteen xanthones, the microbiota, and the immune system. Germ-free mice could be colonized with microbiota from healthy individuals and then fed diet containing xanthones for a set period of time. The microbiota of these animals could then be transplanted into germ-free mice while feeding them a standard diet. Assuming xanthones induce dysbiosis in the colon of the first set of mice, one could study if this dysbiosis is a reversible or a long-term change. In addition, this experiment would allow one to determine if the changes in microbiota are sufficient to stimulate changes in the colonic epithelium and an immune response by the host. The generation of microbial metabolites of xanthones could also be studied in this system.

Because I only evaluated the effects of α-MG on the gut microbiota at one time point and the effects on colitis at two time points (Chapters 3 and 4), further characterization of earlier changes mediated by mangosteen xanthones is needed. α-Mangostin and other xanthones have been shown to modulate signaling pathways in vitro, but whether similar events occur in vivo remains unknown. NanoString technology is a powerful tool to quantify RNA levels for hundreds of genes that could allow the identification of signaling pathways modulated by mangosteen xanthones and/or their metabolites in vivo.

In conclusion, more research is needed to evaluate the safety and health benefits of mangosteen xanthones before their use can be recommended for preventive or therapeutic purposes.
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