Functional Food-Related Bioactive Compounds: Effect of Sorghum Phenolics on Cancer Cells in vivo and Conversion of Short- to Long-Chain Omega-3 Polyunsaturated Fatty Acids in Duck Liver in vivo

by

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B.S., Nanchang University, 2010
M.S., Nanjing University of Economics and Finance, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Food Nutrition Dietetics and Health
College of Human Ecology

KANSAS STATE UNIVERSITY
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Abstract

Many functional food related bioactive compounds have been discovered and draw the attention of scientists. This dissertation focused on sorghum phenolic compounds and omega-3 polyunsaturated fatty acids.

Study 1: phenolic agents in plant foods have been associated with chronic disease prevention, especially cancer. However, a direct evidence and the underlying mechanisms are mostly unknown. This study selected 13 sorghum accessions and was aim to investigate: (1) the effect of extracted sorghum phenolics on inhibiting cancer cell growth using hepatocarcinoma HepG2 and colorectal adenocarcinoma Caco-2 cell lines; (2) and the underlying mechanisms regarding cytotoxicity, cell cycle interruption, and apoptosis induction. Treatment of HepG2 and Caco-2 cells with the extracted phenolics at 0-200 μM GAE (Gallic acid equivalent) up to 72 hrs resulted in a dose- and time-dependent reduction in cell number. The underlying mechanism of cell growth inhibition was examined by flow cytometry, significant inverse correlations were observed between the decreased cell number and increased cell cycle arrest at G2/M or induced apoptosis cells in both HepG2 and Caco-2 cells. The cytotoxic assay showed that the sorghum phenolic extracts were non-toxic. Although it was less sensitive, a similar inhibitory impact and underlying mechanisms were found in Caco-2 cells. These results indicated for the 1st time that a direct inhibition of either HepG2 or Caco-2 cell growth by phenolic extracts from 13 selected sorghum accessions was due to cytostatic and apoptotic but not cytotoxic mechanisms. In addition, these findings suggested that sorghum be a valuable functional food by providing sustainable phenolics for potential cancer prevention.

Study 2: omega-3 polyunsaturated fatty acids (ω-3 PUFAs) especially long-chain ω-3 PUFAs, have been associated with potential health benefits in chronic disease prevention.
However, the conversion rate from short- to long-chain ω-3 PUFAs is limited in human body. This study was aim to assess the modification of fatty acid profiles as well as investigate the conversion of short- to long-chain ω-3 PUFAs in the liver of Shan Partridge duck after feeding various dietary fats. The experimental diets substituted the basal diet by 2% of flaxseed oil, rapeseed oil, beef tallow, or fish oil, respectively. As expected, the total ω-3 fatty acids and the ratio of total ω-3/ ω-6 significantly increased in both flaxseed and fish oil groups when compared with the control diet. No significant change of total saturated fatty acids or ω-3 fatty acids was found in both rapeseed and beef tallow groups. Short-chain ω-3 α-linolenic acid (ALA) in flaxseed oil-fed group was efficiently converted to long-chain ω-3 docosahexaenoic acid (DHA) in the duck liver. This study showed the fatty acid profiling in the duck liver after various dietary fat consumption, provided insight into a dose response change of ω-3 fatty acids, indicated an efficient conversion of short- to long-chain ω-3 fatty acid, and suggested alternative long-chain ω-3 fatty acid-enriched duck products for human health benefits.

In conclusion, the two studies in this dissertation provided a fundamental understanding of anti-cancer activity by sorghum phenolic extracts and the conversion of short- to long-chain ω-3 PUFAs in duck liver, contribute to a long term goal of promoting sorghum and duck as sustainable phenolic and ω-3 PUFAs sources as well as healthy food products for human beings.
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Preface

This dissertation is original work completed by the author, Xi Chen. Chapter 2 was formatted for publication according to required standards of the journal: Nutrition Research. Chapter 3 was formatted for publication according to the required standards of the journal: Experimental Biology and Medicine.
Chapter 1 - Literature review

Summary

Functional food related bioactive compounds such as omega-3 polyunsaturated fatty acids (PUFAs) and phenolic compounds are reported to have the potential to provide health benefits to human beings. Compelling data shows they are associated with chronic diseases including cardiovascular disease, inflammatory diseases, type-2 diabetes, and cancers etc. However, humans cannot synthesis omega-3 polyunsaturated fatty acids or phenolic compounds in their body, these compounds must be obtained from foods. This review falls into two parts: 1) the first part reviews the background information of sorghum phenolic compounds, associated cancer prevention mechanisms, and effect on cancer cells in vitro; 2) the second part introduces some background information of omega-3 polyunsaturated fatty acids, their health benefits and the mechanism of the conversion from short- to long- chain omega-3 PUFAs followed by their application in animal models in vivo especially duck model.

Part 1 Sorghum phenolic compounds, their anti-cancer mechanisms and effect of on cancer cells in vitro

Sorghum phenolic compounds

Phenolic compounds are phytochemicals possessing one or more aromatic rings with one or more hydroxyl groups. They are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8,000 phenolic structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins. Plant phenolics include phenolic acids, flavonoids, tannins (Figure 1-1) and the less common stilbenes and lignans (Figure 1-2).¹
Sorghum is a major cereal food crop used in many parts of the world. Sorghum caryopsis consists of three distinct anatomical components (Figure 1-3): pericarp (outer layer), endosperm (storage tissue), and germ (embryo). All sorghums contain phenolic compounds, the amount present in any particular cultivar is influenced by its genotype and the environment in which it is grown. In addition, these same factors affect the color, appearance, and nutritional quality of the grain and its products. Among cereals, sorghum has the highest content of phenolic compounds reaching up to 6% (w/w) in some varieties. Sorghums with a pigmented testa and spreader genes (B1B2S) or with purple/red plants and thick pericarp genes have the highest levels of phenolic compounds. The main classes of sorghum phenolic compounds are simple phenols, hydroxybenzoic acids, hydroxycinnamic acids, flavonoids (flavanols, flavones, flavanones, isoflavones and anthocyanins), chalcones, aurones (hispidol), hydroxycoumarins, lignans, hydroxystilbenes and polyflavans (proanthocyanidins and pro-deoxyanthocyanidins). These compounds are soluble in water or organic solvents (methanol, HCl-methanol, acetone, dimethylformamide, etc.). Lignans and hydroxystilbenes have not been detected in sorghum grain.

**Simple phenols and phenolic acids**

Simple phenols are relatively rare in plants. Catechol and resorcinol were reported in sorghum grain, these compounds are undesired in food products because they are carcinogenic, hepatotoxic and goitrogenic. The phenolic acids of sorghum are largely exist as benzoic or cinnamic acid derivatives (hydroxybenzoates and hydroxycinnamates) (Figure 1-4), they are mostly found in free form or bound as esters, and are concentrated in the bran (outer covering of grain), and mostly in bound forms (esterified to cell wall polymers), with ferulic acid being the most abundant bound phenolic acid in sorghum. Several other phenolic acids also have been
identified in sorghum including syringic, protocatechuic, caffeic, p-coumaric, and sinapic.$^{12}$

**Flavonoids**

Flavonoids constitute the largest class of phenolic compounds with more than 3000 structures, possessing in common a flavylium unit (C$_6$-C$_3$-C$_6$).$^{13}$ Sorghum contains flavonoids such as flavanols (flavan-3-ols, flavan-4-ols, etc., Figure 1-5), flavanones, flavones and anthocyanins.$^{14}$ The flavan-4-ols apiforol (pro-apigeninidin or leuco-apigeninidin) and tuteoforol (proluteolinidin or leuco-luteolinidin) are abundant in sorghum,$^{15}$ and precursors of apigeninidin and luteolinidin, respectively.$^{16}$ Sorghums with a black pericarp have higher levels flavan-4-ols and anthocyanins than the other varieties.$^{3,17}$ The most common anthocyanins in sorghum are the 3-deoxyanthocyanidins, which include apigeninidin and luteolinidin (Figure 1-6).$^{18}$ The red color of the grain’s pericarp is essentially due to the presence of 3-deoxyanthocyanidins.$^{19}$ The 3-deoxyanthocyanidins (3-DAs), are particularly abundant in notably red sorghum grain,$^{15}$ but rare or absent in other plants.$^{14,20,21}$ 3-DAs are of interest because they are more stable in organic solvents as well as in acidic solutions than anthocyanidins commonly found in fruits, vegetables and other cereals.$^{20,21}$ This has been suggested as a potential advantage of sorghum as a viable commercial source of anthocyanins.$^{14}$

**Condensed tannins**

Tannins of sorghum are almost exclusively of the “condensed” type. They are mainly polymerized products of flavan-3-ols (proanthocyanidins) and/or flavan-3,4-diols (pro-3-deoxyanthocyanidins, Figure 1-7).$^{14}$ No sorghum varieties contain tannic acid and hydrolysable tannins.$^{5}$ Sorghum proanthocyanidins consist of flavan-3-ol units linked by C-C (type B proanthocyanidins) and occasionally C-O-C (type A proanthocyanidins) bonds ranging from one to fifteen.$^{7,14}$ The most abundant condensed tannins in sorghum are homopolymers of
catechin/epicatechin with uniform B-type interflavan bonds,\textsuperscript{7} however, not all sorghum varieties contain these because their content is genetically governed by B1-B2 genes.\textsuperscript{22} In general, varieties with pigmented testa layers contain proanthocyanidins.\textsuperscript{15,23} The main pro-deoxyanthocyanidins found in sorghum are pro-apigeninidins and pro-luteolinidins (Figure 1-7). Although present in sorghum, these compounds are very rare in other plants. Hydrolysis of pro-apigeninidins and pro-luteolinidins yields apigeninidins and luteolinidins, respectively.\textsuperscript{24}

**Cancer prevention mechanism of phenolic compounds**

Phenolic agents in plant foods have been associated with chronic disease prevention, especially cancer. It was found that in addition to their antioxidant activity, this group of compounds displays a wide variety of biological functions which are mainly related to modulation of carcinogenesis. To understand the mechanisms of action of phenolic compounds in preventing cancer, first we need to know how cancer arises.

**Causes of cancer**

Cancer development is a multistage process that involves a series of individual steps including initiation, promotion, progression, invasion and metastasis. Tumor initiation begins when DNA, in a cell or population of cells, is damaged by exposure to carcinogens, which are derived from three major sources: cigarette smoking, infection/inflammation, and nutrition/diet.\textsuperscript{25}

The process of carcinogenesis can be broadly categorized into three distinct phases: tumor initiation, promotion and progression. Genetic mutation take place when DNA damage escapes repairing. The resulting somatic mutation in a damaged cell can be reproduced during mitosis, which given rise to a clone of mutated cells. Tumor promotion is a selective clonal expansion of the initiated cells to form an actively proliferating multi-cellular premalignant tumor cell population. It is an interruptible or reversible and long term process. During progression,
premalignant cells developed into tumors through a process of clonal expansion. In the late stages of cancer development, invasion and metastasis happens, where tumor cells detach from the primary tumor mass, migrate through surrounding tissues toward blood vessels or lymphatic vessels, and create a second lesion. Metastasis is the major cause of cancer mortality. It is widely accepted that human cancer development does not occur through these discrete phases in a predictable manner, rather it is best characterized as an accumulation of alteration in cancer regulating genes, such as oncogenes, tumor suppressor genes, resulting in altered cellular processes, namely, decreased apoptosis, increased proliferation, and cell maturation and differentiation.

Phenolic compounds, appear to play a significant role in suppressing all three stages of tumor formation and metastasis, including the transformative, hyper-proliferative and inflammatory processes involved in initiation, the angiogenic processes required for tumor growth as well as the vascular adhesive properties necessary for metastasis or tumor dispersion.

**Mechanisms of phenolic compounds in cancer prevention**

*Alteration of pro-carcinogenic metabolism by phenolics*

In the initiation stage, phenolics may inhibit activation of procarcinogens by inhibiting phase I metabolizing enzymes, such as cytochrome P450 and also facilitate detoxifying and elimination of the carcinogens by induction of phase II metabolizing enzymes such as glutathione S-transferase (GST), NAD(P)H quinine oxidoreductase (NQO), and UDP-glucuronyl- transferase (UGT). They may also limit the formation of the initiated cells by stimulating DNA repair.

*Phenolics as potent antioxidants in oxidation*

ROS/RNS are constantly produced during normal cellular metabolism or by other exogenous means including the metabolism of environmental toxins or carcinogens, by ionizing
radiation and by phagocytic cells involved in the inflammatory response. When the cellular concentration of oxidant species is increased to an extent that overcome the endogenous antioxidant defense system, oxidative stress occurs, leading to lipid, protein, and DNA damage. In addition, ROS, particularly \( \text{H}_2\text{O}_2 \), are potent regulators of cell replication and play an important role in signal transduction.\(^{31}\) Hence, oxidative damage is considered a main factor contributing to carcinogenesis and evolution of cancer. Numerous dietary phenolics are potent antioxidants, capable of scavenging deleterious reactive species such as superoxide anions, singlet oxygen hydroxy radicals, nitric oxide and peroxynitrite.\(^{32-36}\) Various phenolics are also able to attenuate ROS generation through inhibition of redox sensitive transcription factors such as NF-kB and AP-1 responsible for the expression of the ROS-induced inflammatory enzyme cascade. Xanthine oxidase, COX-II and LOX were shown to be reduced by dietary phenolics like curcumin, silymarin and resveratrol. Polyphenols can also induce detoxifying enzymes such as glutathione-S-transferase (GST) and quinone reductase (QR) which can protect cells from carcinogenic intermediates, exogenous or endogenous.\(^{32-34,37-40}\)

Historically, the anti-cancer effects of phenolic compounds have been mostly explained through their antioxidant potentials, but this seems very complicated. There is evidence manifesting risky effects of antioxidants rather than their benefits in patients suffering from cancer.\(^{41,42}\) Therefore, it is essential to differentiate and clarify antioxidant and anti-cancer actions of these compounds in order to help clinicians to establish new strategies for prevention and treatment of cancer.

Besides antioxidant activity, several other important mechanisms as follows also contribute to anti-cancer effect of phenolic compounds.

*Phenolics as potent inhibitors of Growth Factors and Receptors (GFRs)*
Plant phenolics such as curcumin, genistein, resveratrol and catechins are potent inhibitors of a number of growth factors binding and signaling pathways implicated in cancer. Growth factors are proteins that bind to specific receptors on cell surfaces to elicit a signaling cascade responsible for the normal activation of cell proliferation/differentiation required for tissue growth and repair. Aberrant growth factor expression/availability results in a signaling cascade leading to uncontrolled cell proliferation and differentiation, suppression of apoptotic signals and ultimately in carcinogenesis, tumor growth/progression and metastasis.\textsuperscript{32-35} Major growth factors implicated in carcinogenesis are epidermal growth factor (EGF), plate-derived growth factor (PDGF), fibroblast growth factors (FGFs), transforming growth factors-\(\alpha\) and -\(\beta\) (TGFs-\(\alpha\) and -\(\beta\)), insulin-like growth factor (IGF), erythropoietin (EPO), as well as the inflammation-related cytokines interleukin1,2,-6,-8 (IL-1,2,-6,-8), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interferon-\(\gamma\) (IFN-\(\gamma\)) and colony stimulating factors (CSFs).\textsuperscript{33-35} The binding of these factors to their specific receptors elicits powerful cell proliferation signals through the activation of signal cascades involving various receptor-regulated and cytosolic kinases and transcription factors which forms the basis of growth factor/receptor driven carcinogenesis and tumor progression.\textsuperscript{33-35} Compounds such as phenolic compounds that can attenuate GF binding and the attendant signal cascade are generally regarded as excellent chemo-preventive agents.

\textit{Regulation of cell cycle and apoptosis by phenolics}

Disruption of the normal regulation of cell-cycle progression and division are important events in the development of cancer. The cell cycle is the recurring sequence of events that includes the duplication of cell contents and subsequent cell division. For eukaryotic cells, the cell cycle has been defined as the interval between the completion of mitosis in a cell and the completion of mitosis by one or both of its daughter cells.\textsuperscript{43} Traditionally the cell cycle in eukaryotic cell has
been divided into four phases: Gap phase 1 (G1); DNA synthesis (S); Gap phase 2 (G2), during which the cell prepares itself for division; and mitosis (M) during which the chromosomes separate and the cell divides. The progression of cell cycle from one phase to the next is regulated by sequential activation and inactivation of many “check points” that monitor the status of the cell as well as environmental cues. Cyclin:cyclin-dependent kinase complexes control the two critical checkpoints in the cell cycle at the G1/S and G2/M transitions by phosphorylating a variety of proteins such as nuclear lamins and histones for nuclear membrane breakdown and chromosome condensation, as well as proteins leading to the transcription of genes required for proliferation. Different cell-cycle phases are associated with production of distinct types of cyclins. In contrast to CDKs, having constant level during cell cycle, the level of regulatory subunits and cyclins rises and falls in different cell-cycle phases (Figure 1-8). Passage through the cell cycle is governed by cyclin-dependent kinases (cdks) which complex with their "partner" cyclins. The G1 cyclins and their associated cdks regulate the G1/S transition. Cyclins D1, D2 and/or D3 are expressed in early G1 phase, and cyclin E is expressed in late G1 phase. D-type cyclins complexed with cdk2, 4 or 6 promote cell cycle progression by phosphorylating a variety of substrates such as the retinoblastoma (Rb) protein, which is required for entry into S phase. The mitotic cyclins, first discovered as proteins with abundant oscillation in early invertebrate embryonic cell cycles, form complexes with cdks and regulate the G2/M transition. In most organisms, B-type cyclins function as a subunit of the major mitotic protein kinase.

The chemical structures of phenolic compounds are widely diverse from simple phenols to tannins. Presence of phenolic hydroxyl groups allows them to cause some interactions with proteins changing their functions. They share a phenolic proton in hydrogen bonds with a special functional group of proteins such as amide, guanidine, peptide amino or carboxyl groups.
ability provides several mechanisms for phenolic compounds to prevent or cure cancer. They interact with specific proteins to regulate cell cycle and consequently modulate cellular proliferation. Therefore, they have potential to control cell cycle progression in cancer cells via targeting various components in the cell cycle regulatory network.\textsuperscript{46,48} For instance, Treatment of various cancer cells (prostate, lung and skin) with EGCG altered the pattern of cell cycle proteins; specifically the inhibition of CDKs. EGCG also enhances the expression of CDKI proteins, such as Cip1/p21 and Kip1/p27 while reducing the expression of cyclin D1 and the phosphorylation of retinoblastoma protein. EGCG causes cell cycle arrest and promotes apoptosis via a dose- and time-dependent upregulation of Cip1/p21, Kip1/p27, and p16/INK4A and down-regulation of proteins such as cyclin D1, cyclin E, CDK2, and CDK4.\textsuperscript{49} EGCG caused growth arrest at G1 stage of cell cycle through regulation of cyclin D1, CDK4, CDK6, Cip1/p21 and Kip1/p27, and induced apoptosis through generation of reactive oxygen species and activation of caspase-3 and caspase-9.\textsuperscript{50} Apoptosis, or programmed cell death, is an important mechanism in normal development and in anticancer surveillance. The process is regulated by various oncogenes/proteins, including the important pro-apoptotic p53, the anti-apoptotic and cell survival Bcl-2 and the caspase cascade.\textsuperscript{33–35,43} The regulation of cell cycle and apoptosis by other natural phenolic compounds such as gallic acid, coumarin, genistein etc. are summarized in Figure 1-9.\textsuperscript{46}

\textbf{Mediation of inflammatory process by phenolics}

One important aspect of carcinogenesis is recognized to be the involvement of inflammation. For instance, prostaglandins are mediators of inflammation and chronic inflammation predisposes to carcinogenesis. The over-expression of inducible cyclooxygenases (COX-2), the enzyme which catalyzes a critical step in the conversion of arachidonic acid to prostaglandins and is induced by pro-inflammatory stimuli, including mitogens, cytokines and
bacterial lipopolysaccharide (LPS), is believed to be associated with colon, lung, breast and prostate carcinogenesis. Natural phenolics have been reported to inhibit transcription factors closely linked to inflammation (e.g., NF-κB), pro-inflammatory cytokines release and enzymes such as COX-2, lipoygenases (LOX), inducible nitric oxide synthase (iNOS) that mediate inflammatory processes, both in vitro and in vivo. In many cases, polyphenols exhibit anti-inflammatory properties through blocking MAPK-mediated pathway. Furthermore, a few structure-activity studies have been conducted. For example, Hou et al. examined the inhibitory effects of five kinds of green tea proanthocyanidins on cyclooxygenase-2 (COX-2) expression and PGE-2 release in LPS-activated murine macrophage RAW-264 cells. It was revealed that the galloyl moiety of proanthocyanidins appeared important to their inhibitory actions. Another study by Herath et al. suggested that the double bond between carbon 2 and 3 and the ketone group at position 4 of flavonoids are necessary for potent inhibitory effects on LPS-induced tumor necrosis factor-alpha (TNF-α) production in mouse macrophages (J774.1).

**Suppression of angiogenesis by phenolics**

As stated previously, angiogenesis, or new blood vessel formation, is vital for supplying nutrients and oxygen to the tumor and ensuring its growth and progression, its invasiveness and spread to other tissues. Prevention of angiogenesis would “starve” the tumor and prevent metastasis. Consequently, angiogenesis inhibition is an active objective in current anticancer research. Quercetin, genistein, resveratrol and various phenolic acids are capable of inhibiting angiogenesis in in vitro cell-based systems as well as in animal models. These effects appear to be due to attenuation of matrixmetalloproteinases (MMPs) inhibition of VEGF and their Src kinases. These phenolics have also been shown to attenuate the expression of vascular adhesion molecules which would reduce the metastatic process.
Anti-cancer effect of sorghum phenolic compounds in vitro

Phenolic extracts or isolated phenolics from different plant food have been studied in many cell lines. For example, berry extracts prepared from blackberry, raspberry, blueberry, cranberry, and strawberry including anthocyanins, kaempferol, quercetin, esters of coumaric acid and ellagic acid, were shown to inhibit the growth of human oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT-116), and prostate (LNCaP, DU-145) tumor cell lines in a dose-dependent manner with different sensitivity between cell lines. Similar results have also been reported with wine extracts, tea extracts and major green tea polyphenols. Other phenolic extracts from legumes, citrus and apples were also studies.

However, studies regarding the anti-cancer effect of sorghum phenolic compounds in vitro are scarce. A few studies published recently used sorghum extracts to treat various cancer cells. Suganya Devi used breast cancer cell line (MCF-7) to evaluate the anti-proliferative activity of red sorghum bran anthocyanin, and found that the red sorghum anthocyanin induced growth inhibition of MCF-7 cells at significance level. Similar results were also found using other cancer cell lines including leukemia (HL-60), breast (MDA-MB 231), colon (HT-29) and liver (HepG2) cells as inducing cell apoptosis, inhibiting cell proliferation, promotion the expression of cell cycle regulators and p53 gene by sorghum extracts. In vitro data is the first and necessary step to understand the underlying mechanisms of anti-cancer effect of sorghum phenolic compounds. The lag of sorghum phenolic compounds behind similar research on other plant food drives us to investigate the effect of sorghum phenolic compounds in cancer cell growth inhibition and the underlying mechanisms.
Part 2 Omega-3 polyunsaturated fatty acids and their health benefit as well as metabolism, conversion in vivo especially in duck model

Introduction to omega-3 PUFAs

Fatty acids with more than one double bond are classified as polyunsaturated fatty acids (PUFAs). Omega-3 PUFAs are fatty acids that have a double bond at the third position from the methyl end of carbon chain (Figure 1-10). There are two types of omega-3 PUFAs, known by short-chain omega-3 PUFAs which have less than 20 carbons such as α-linolenic acid (ALA, C18:3n-3) and long-chain omega-3 PUFAs which have more than or equal to 20 carbons such as eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). Other naturally occurring omega-3 PUFAs including stearidonic acid (C18:4n-3), eicosatrienoic acid (C20:3n-3), eicosapentaenoic acid (C20:4n-3), docosapentaenoic acid (C22:5n-3), etc.

Our diet contains a complex mixture of fats and oils whose basic structural components are fatty acids. Omega-3 PUFAs generally account for a small fraction of the total daily consumption of fatty acids in Western societies. Usually, short-chain omega-3 PUFAs are presented in plant seeds and oil (such as canola oil, flaxseed oil, soybean oil, walnut oil, etc.), green leafy vegetables, and nuts and beans (such as walnuts and soybeans), while long-chain omega-3 PUFAs are found in marine products such as fish and algae. ALA is the major n-3 PUFA in the diet, with daily mean intakes estimated to be 1.6 g and 1.1 g per day for men and women, respectively; while EPA and DHA intakes are estimated to be between 100–200 mg/d in the United States.

Omega-3 PUFAs must be obtained from diet, because humans do not have the enzymatic machinery required to synthesize omega-3 PUFAs, which makes them essential. Through an inefficient enzymatic process of desaturation, ALA produce Arachidonic acid, precursors to a group of eicosanoids (prostaglandins, thromboxanes, and leukotrienes) that are anti-inflammatory,
antithrombotic, antiarrhythmic and vasodilatory.\textsuperscript{75} Compelling data from epidemiological and interventional studies demonstrated that it is long-chain but not short-chain omega-3 PUFAs have inverse correlation with the risk of some chronic disease.\textsuperscript{76–84} Thus, the 2015 Dietary Guidelines for Americans recommends the consumption of 8 oz. of seafood per week to provide an average of 250 mg/day of long-chain omega-3 fatty acids for health benefits\textsuperscript{85}. Moreover, The American Heart Association’s Strategic Impact Goal Through 2020 and Beyond recommends at least two servings with 3.5 oz. fish every week to increase EPA and DHA intakes\textsuperscript{86}.

**Health benefit of long-chain omega-3 PUFAs**

It has been reported that long-chain but not short-chain omega-3 PUFAs possess the physiological properties of regulating blood pressure\textsuperscript{87}, platelet reactivity and thrombosis\textsuperscript{88}, plasma TG concentrations\textsuperscript{89}, vascular function\textsuperscript{90}, cardiac arrhythmias\textsuperscript{91}, heart rate variability\textsuperscript{91}, and inflammation\textsuperscript{92}. These physiological properties are associated with some chronic diseases such as cardiovascular disease (CVD)\textsuperscript{76,77}, psoriasis\textsuperscript{80}, mental illnesses\textsuperscript{81}, type-2 diabetes, cancers\textsuperscript{82,83}, asthma\textsuperscript{84} etc. Several studies also revealed that in those chronic conditions, essential fatty acids metabolism is abnormal such that plasma and tissue concentrations of arachidonic acid (AA), EPA and DHA in the phospholipid fraction are low.\textsuperscript{93–96}

Given to a long history of studying cancer prevention, our lab has special interest in the mechanism of dietary compounds in preventing cancer. Increasing evidence from animal and in vitro studies indicates that omega-3 PUFAs, especially their long-chain members such as EPA and DHA could inhibit carcinogenesis.\textsuperscript{97} Several molecular mechanisms whereby omega-3 PUFAs potentially affect carcinogenesis have been proposed. These mechanism include: (1) suppression of AA derived eicosanoid biosynthesis, which results in altered immune response to cancer cells and modulation of inflammation, cell proliferation, apoptosis, metastasis, and angiogenesis; (2)
influences on transcription factor activity, gene expression, and signal transduction, which leads to changes in metabolism, cell growth, and differentiation; (3) alteration of estrogen metabolism, which leads to reduced estrogen-stimulated cell growth; (4) increased or decreased production of free radicals and reactive oxygen species; and (5) mechanisms involving insulin sensitivity and membrane fluidity. However, the epidemiological studies on the association between long-chain omega-3 PUFAs intake and cancer risk remains controversial. The possible causes of the absence of an association between dietary long-chain omega-3 PUFAs and cancer risk in some epidemiological studies may falls into two aspects: (1) possible beneficial effect of marine omega-3 PUFAs may be offset by potential carcinogenic substances, such as some pesticides and heavy metals (e.g., mercury), that accumulate in fatty fish. Furthermore, heterocyclic amines formed during the cooking of fish at high temperatures have been shown to produce cancer in various organs in animals (2) the discrepancy between animal and epidemiologic studies involves differences in doses and the stage of tumor development. In animal studies, large doses of omega-3 PUFAs were usually used, and tumors were artificially induced. High doses of omega-3 PUFAs applied during the promotion and progression stages of tumor development may indeed inhibit carcinogenesis in animal models, whereas long-term exposures to relatively low doses of long-chain omega-3 PUFAs may not be as effective against cancer development in humans.

Metabolism of omega-3 fatty acids

While mammals cannot synthesize omega-3 PUFAs de novo, mammalian cells can interconvert omega-3 PUFAs within each series by elongation, desaturation, and retroconversion. The simplest omega-3 PUFA, α-linolenic acid (ALA, 18:3n-3), is synthesized from linoleic acid (LA, 18:2n-6) by desaturation, catalyzed by Δ-15 desaturase in plants. Animals, including humans, do not possess the Δ-15 desaturase enzyme and so cannot
synthesize ALA. Thus, ALA is a classically essential fatty acid, along with LA.

**Interconversion of omega-3 PUFAs**

After ingestion, ALA is converted to EPA, occurring primarily in the endoplasmic reticulum of liver cells and involves a series of elongation enzymes that sequentially add 2-carbon units to the fatty acid backbone and desaturation enzymes that insert double bonds into the molecules (Figure 1-2). EPA is elongated to docosapentaenoic acid (DPA) by elongase, the final step from DPA converting to DHA is controversial, there’re two possible pathways: (1) The Δ4 pathway: DPA may be converted to DHA by Δ-4 desaturase however, the existence of Δ-4 desaturation in the biosynthesis of DHA had been questioned for years, until a Δ-4 fatty acid desaturase was characterized in vegetative organisms. Nevertheless, the existence of such conversion for DHA synthesis in mammals is still unclear; (2) The Sprecher pathway: DPA is elongated to tetracosahexaenoic acid (THA) and translocation to peroxisomes, a 2-carbon removal by β-oxidation, and translocation of DHA back to the endoplasmic reticulum.

**The conversion from short-chain ALA to long-chain EPA and DHA**

ALA from plant oils is a metabolic precursor of the long-chain omega-3 PUFAs found in fish and fish oil, but the conversion is not efficient in humans, usually below 5% in adults or even less than 1% in infants and aging people. This is because: (1) humans are lacking the key enzyme required to convert short-chain ALA to long-chain EPA and DHA; (2) the background diet may also have a significant impact on the conversion from ALA to DHA since the same enzymes are involved in omega-3 and -6 pathways; (3) EPA and DHA themselves may downregulating the conversion of ALA to long-chain PUFAs.

Several investigators have performed detailed studies of the in vivo conversion of ALA to its long-chain omega-3 derivatives EPA, DPA and DHA in humans by using uniformly labeled
[\textsuperscript{13}C]- or [\textsuperscript{2}H] ALA as a tracer.\textsuperscript{106,108} These studies have consistently shown that over 15-35\% of dietary ALA is rapidly catabolized to carbon dioxide for energy,\textsuperscript{109-112} and that only a small proportion, estimated by using compartment models to be less than 1\% is converted to DHA.\textsuperscript{113} The fractional conversion of ALA to EPA, estimated by measuring peak or area under the curve plasma contents of the labeled fatty acids, varies between 0.3\% and 8\% in men, and the conversion of ALA to DHA is 4\% and often undetectable in males.\textsuperscript{110,112,114} Conversion of ALA to long-chain omega-3 fatty acids appears to be more efficient in women: up to 21\% is converted to EPA and up to 9\% is converted to DHA\textsuperscript{111}, with a concomitant reduction in the rate of ALA oxidation (22\% compared with 33\% in men).\textsuperscript{110-112}

Background diet may have a significant impact the conversion from ALA to EPA and DHA. Diets high in LA may influence the metabolism of the omega-3 fatty acids through substrate competition and inhibition of \(\Delta\)6 desaturase enzyme. Emken et al\textsuperscript{115} showed that a diet high in LA reduces the conversion of ALA to its long-chain derivatives by 40\%, with a net reduction in long-chain n 3 fatty acid accumulation of 70\%. Diets high in ALA appear to increase the rate of ALA oxidation, limiting its accumulation in plasma and reducing its conversion rate to EPA and DHA.\textsuperscript{109}

The conversion of DPA to DHA is the rate-limiting step in the conversion of ALA to DHA, and dietary DHA and EPA down-regulate this step by 70\%.\textsuperscript{116} It is also reported that dietary EPA and DHA reduce the conversion of ALA to long-chain omega-3 fatty acids.\textsuperscript{109,110} DHA itself can serves as a substrate for metabolic retro-conversion to EPA and DPA through a \(\beta\)-oxidation reaction. In studies in which \(\textsuperscript{13}C\)-labeled DHA was fed, Brossard et al\textsuperscript{117} calculated the retro-conversion rate of DHA to EPA in humans receiving normal dietary amounts of DHA to be 1.4\%. Human clinical data have suggested rates as high as 12\% with high chronic DHA
consumption.\textsuperscript{118,119} Retro-conversion of DHA to EPA is hormonally regulated and decreases in women receiving hormone replacement therapy.\textsuperscript{120}

**Omega-3 PUFAs in animal models**

In a typical Western diet, over 53\% of dietary fat is supplied through animal products. As mentioned above, long-chain omega-3 PUFAs possess the potential health benefits to lower the risk of some chronic diseases, but there’s still nutritional gap between the recommended and actual consumption levels of EPA and DHA\textsuperscript{121}. Therefore, feeding strategies have been adopted to increase the content of omega-3 fatty acids in animal food lipids like meat, eggs and milk.\textsuperscript{122–124}

It has been noted that supplemented diets with vegetable oil and fish oil effectively enhanced omega-3 PUFAs in the products of pork,\textsuperscript{125} broilers,\textsuperscript{126,127} and broiler eggs\textsuperscript{128,129}. Haak\textsuperscript{125} reported that linseed and fish oil supplementation could enhance the incorporation of omega-3 PUFAs in pork meat and also alter its fatty acid composition. Creps\textsuperscript{126} determined the effect of different dietary fatty acid profiles on nutrient and fatty acid balance in broiler chickens and found that chickens fed with linseed oil diet have higher amount of EPA and DHA in total body fat of chickens than other diets including tallow diet, olive oil diet and sunflower oil diet. This is in accordance with Lopez-Ferrer’s\textsuperscript{127} findings that supplemented linseed oil or fish oil in diet could significantly increase omega-3 PUFAs in chicken meat. Other animal product were also investigated, for example, Lemahieu’s\textsuperscript{128} and Antruejo\textsuperscript{129} found that feed supplemented with omega-3 PUFAs resulted in increased levels of these fatty acids in chicken product such as eggs.

Although there’re many studies investigated animal models including pigs and chickens, there’s limited research on using duck as a model to assess the effect of dietary fats on fatty acid profile in duck products.
Omega-3 PUFAs in duck model

When compared with humans, waterfowls such as duck and geese have been reported to convert short-chain to long-chain omega-3 fatty acids more efficiently by a series of linked desaturation and elongation reactions that mainly take place in the liver and subsequently excreted into blood circulation to other tissues. The diverse conversion rates between human and waterfowl have driven scientists to consider that waterfowls may provide an alternate and sustainable source of long-chain omega-3 PUFAs from plant-derived short-chain ALA.

Ducks are aquatic birds which have a high rate of lipogenesis to meet their energetic requirements during ancient migratory flight. It has been reported, for instance, the percent body fat could be reached as high as 37–42% in Peking ducks and 20–30% in Muscovy ducks. Although duck products are popular with its unique flavor and juicy texture, the high fat content has raised health concerns. While certain species of lean ducks, such as the Shan Partridge contains 7.5% of body fat only have been developed, modification of the fat composition in favor of higher omega-3 PUFAs in replace of omega-6 PUFAs and/or saturated fatty acids (SFAs) may provide promising healthy benefits. Chen and Hsu reported an increased trend of EPA, DHA, and total omega-3 PUFAs in duck egg yolks by feeding cod liver oil diet. Our previous study found that fish oil and sunflower oil could significantly enhance the levels of EPA and DHA in the leg and chest muscles as well as eggs of Shan Partridge duck.

In addition to storage and transportation of lipids, liver of birds has a very high capacity of lipogenesis. Fatty acids can be synthesis or converted in the liver, and then transported to other tissues such as adipose, cardiac, and skeletal muscle. The conversion of fatty acids in the liver and incorporation of them into various tissues are well-established in response to the observed change of fatty acid composition in the relative tissues. Therefore, fatty acid conversion in the liver may
provide impact not only on a varied fatty acid composition but also on the meat quality including flavor and muscle color. From an aspect of nutrition value, efficient conversion efficient conversion of short- to long-chain omega-3 PUFAs in the liver may boost the levels of long-chain omega-3 PUFAs and thus improve the meat quality.
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Figure 1-1 Structure of flavonoids, phenolic acids and tannins.¹
Figure 1-2 Structures of stilbene and lignan.\textsuperscript{1}

![Stilbene and Lignan Structures](image-url)
Figure 1-3 Diagram of sorghum caryopsis showing the pericarp [cutin, epicarp, mesocarp, tube cells, cross cells, testa, pedicel, and stylar area (SA)], endosperm (E) (aleurone layer, corneous, and floury), and germ [scutellum (S) and embryonic axis (EA)].
Figure 1-4 Structures of simple phenols and phenolic acids found in sorghum.\textsuperscript{9,10,14}

Simple phenols

- Phenol: $R_1 = R_2 = R_3 = H$
- Catechol: $R_1 = \text{OH}, R_2 = R_3 = H$
- Resorcinol: $R_2 = \text{OH}, R_3 = R_1 = H$
- Phloroglucinol: $R_2 = R_3 = \text{OH}, R_1 = H$

Hydroxybenzoic acids

- Gallic: $R_1 = H, R_2 = R_3 = R_4 = \text{OH}$
- Gentisic: $R_1 = R_2 = \text{OH}, R_3 = R_4 = \text{OH}$
- Salicylic: $R_1 = \text{OH}, R_2 = R_3 = R_4 = H$
- $p$-(OH)-Benzoic: $R_1 = R_2 = R_3 = H, R_4 = \text{OH}$
- Protocatechuic: $R_1 = R_2 = H, R_3 = R_4 = \text{OH}$
- Syringic: $R_1 = H, R_2 = R_4 = \text{OCH}_3, R_3 = \text{OH}$
- Vanillie: $R_1 = R_2 = H, R_3 = \text{OH}, R_4 = \text{OCH}_3$

Hydroxycinnamic acids

- $p$-Coumaric: $R_1 = R_2 = R_3 = H, R_4 = \text{OH}$
- Caffeic: $R_1 = R_2 = H, R_3 = R_4 = \text{OH}$
- Ferulic: $R_1 = R_2 = H, R_3 = \text{OCH}_3, R_4 = \text{OH}$
- Sinapic: $R_1 = H, R_2 = R_3 = \text{OCH}_3, R_4 = \text{OH}$
Figure 1-5 Structure of some sorghum flavanols.$^{14}$

**Flavan-3-ol**
- catechin

**Flavan-4-ols**
- Apiforol (leucoapigeninidin) : $R = H$
- Luteoforol (leucoluteolinidin) : $R = OH$
Figure 1-6  The main sorghum anthocyanidins and 3-deoxyanthocyanidin.$^{14,21}$

**Anthocyanidins**

Fisetinidin : $R_1 = R_2 = \text{OH}, R_3 = \text{H}$
Cyanidin : $R_1 = \text{OH}; R_2 = \text{H}, R_3 = \text{OH}$
Pelargonidin : $R_1 = R_2 = \text{H}, R_3 = \text{OH}$
Peonidin : $R_1 = \text{OCH}_3; R_2 = \text{H}, R_3 = \text{OH}$
Malvidin : $R_1 = R_2 = \text{OCH}_3, R_3 = \text{OH}$
Delfphinidin $R_1 = R_2 = R_3 = \text{OH}$
Petunidin $R_1 = \text{OCH}_3; R_2 = R_3 = \text{OH}$

**3-Deoxyanthocyanidins**

Apigeninidin : $R_1 = \text{H}$
Luteolinidin : $R_1 = \text{OH}$
Figure 1-7: Structure of condensed tannins in sorghum.\textsuperscript{7,14}

Proanthocyanidins

Pro-3-deoxyanthocyanidins

$R_1 = H$, pro-apigeninidins

$R_1 = OH$, pro-luteolinidins
Figure 1-8 Levels of different cyclins in different cell cycle phases and regulation of different cell cycle phases by different cyclin-CDK complexes.\cite{46}
Figure 1-9 Schematic diagram showing targets of same natural phenolic compounds in part of cell cycle regulatory network.\textsuperscript{46}

Red arrowhead or lines indicate activation or increasing of expression and green ones indicate inhibition or decreasing of expression. Quadrangular boxes contain phenolic compounds and round boxes contain components involving in cell cycle regulation.
Figure 1-10 Structure of representative omega-3 PUFAs.
(source: http://chemistry.tutorvista.com/biochemistry/omega-3-fatty-acids.html)

Alpha-linolenic acid (ALA, C18:3, omega-3)

Eicosapentaenoic acid (EPA, C20:5, omega-3)

Docosahexaenoic acid (DHA, C22:6, omega-3)
Figure 1-11 Conversion from ALA to EPA and DHA. ALA is converted to EPA through a sequential action of Δ-6 fatty acid desaturase, elongase, and Δ-5 desaturase.

Note: EPA may be elongated to docosapentaenoic acid (DPA) and then desaturated by Δ-4 desaturase to DHA. However, Sprecher et al.\textsuperscript{105} have demonstrated that EPA is likely elongated to DPA and to tetracosapentaenoic acid (TPA), desaturated to tetracosaheaxenoic acid (THA) by Δ-6 desaturase, translocated to peroxisomes, and β-oxidized to DHA.
Chapter 2 - Sorghum phenolic compounds are associated with cell growth inhibition through cell cycle arrest and apoptosis induction in human hepatocarcinoma HepG2 and colorectal adenocarcinoma Caco-2 cells
Abstract

Phenolic agents in sorghum have been associated with cancer prevention. However, direct evidence and the underlying mechanisms are mostly unknown. In this study, we selected 13 phenolic-enriched sorghum accessions and tested their cell growth inhibition in the hepatocarcinoma HepG2 and colorectal adenocarcinoma Caco-2 cell lines. Total phenolic contents, as determined by Folin-Ciocalteu, were 30-64 mg GAE/g DW for the phenolic extracts of various accessions with comparison of the control accession F10000 at 2 mg GAE/g DW. Treatment of HepG2 with the extracted phenolics at 0-200 μM GAE up to 72 hrs resulted in a dose- and time-dependent reduction in cell number. The IC₅₀ values were varied from 85 to 221 mg DW/mL when compared with the control F10000 at 1,275 mg DW/mL. The underlying mechanism was further examined in top two potential accessions (PI329694 and PI570481), significant inverse correlations were observed between the decreased cell number and increased cell cycle arrest at G2/M or induced cell death in both HepG2 and Caco-2 cells. The cytotoxic assay showed the sorghum phenolic extracts were non-toxic to the cells. The cytotoxic assay showed that the sorghum phenolic extracts were non-toxic. Although it was less sensitive, a similar inhibitory impact on cell growth and underlying mechanisms were found in colorectal adenocarcinoma Caco-2 cells. Taken together, these results indicated for the first time that a direct inhibition of either HepG2 or Caco-2 cell growth by phenolic extracts from the selected 13 sorghum accessions. These findings also suggested that sorghum could be a valuable functional food by providing sustainable phenolics for potential cancer prevention.

Keywords: sorghum, phenolic compounds, cell growth inhibition, cell cycle analysis, apoptosis, HepG2, Caco-2
1. Introduction

For many decades, the nutrition value of sorghum has considered to be less comparable with other cereals [1], even nowadays people still under-estimate the nutritional advantages of sorghum. In addition to gluten-free and suitable food for people with celiac disease, sorghum recently becomes of particular interests because of its high level and diversity of phenolics for potential health benefit, especially in cancer prevention [2]. Earlier investigation showed that an average of 35% of overall human cancer-related mortality was attributed to diet. [3] As a major component of healthy diets as well as the main bioactive compounds of sorghum, phenolic compounds are found rich in some sorghum accessions, whose profile is more diverse and higher than other crops such as wheat, barley, rice, maize, rye and oats. [4] Numerous studies reported cancer prevention effect of phenolic compounds in fruits and vegetables while few was focused on that of sorghum whole grain. [1,5]

Compelling data from epidemiological and animal studies have suggested that phenolic compounds could potentially contribute to anti-cancer effect through their biological properties including antioxidant activity, induction of cell cycle arrest and apoptosis, and promotion of tumor suppressor proteins, etc. [8-10] There’re plenty of literature available on properties of various phenolic-rich foods such as tea and red wine/grapes in relation to various types of cancer[5], while studies regarding the association between sorghum phenolic compounds and cancers are scarce. Epidemiological studies reported that sorghum consumption consistently correlated with low incidences of esophageal cancer in various parts of the world (including several parts of Africa, Russia, India, China, Iran, etc.) whereas wheat and corn consumption correlated with elevated incidences. [9–11] In vivo studies regarding sorghum phenolics’ anti-cancer effect are even less, Lewis et al [12] in 2008 reported that by feeding normolipidemic rats with diet containing sorghum
bran could significantly reduce the number of aberrant crypts in the rats. More recently, Park et al [13] found that the metastasis of breast cancer to the lungs was blocked by sorghum extracts in the metastatic animal model. However, the mechanisms by which sorghum reduced the risk of cancer are unclear. In vitro data is the first and necessary step to understand the underlying mechanisms of anti-cancer effect. A few in vitro studies published recently using sorghum extracts to treat several cancer cells including leukemia (HL-60)[14], breast (MCF-7, MDA-MB 231) [15-16], colon (HT-29) [16] and liver (HepG2) [16] cells and found consistent results such as induction of cell apoptosis, inhibition of cell proliferation, and promotion of expression of cell cycle regulators by sorghum extracts.

In our study, the human hepatocellular carcinoma HepG2 cells were selected since liver is the major site for the metabolism of dietary compounds including phenolic compounds. However, to use only one cell line seems not sufficient enough to get a comprehensive understanding of the anti-cancer effect of sorghum phenolics. Given the reason that intestine is the major site for the absorption of phenolic compounds, and colorectal cancer appears most relevant to dietary factors when comparing with other type of cancers, we also selected the colon adenocarcinoma Caco-2 cells, which is widely used as a valuable model for evaluation of anti-cancer effect of these compounds. Thus, our present study is the first time to use both HepG2 and Caco-2 cell lines for the effect on cell growth inhibition of sorghum phenolic extracts and meanwhile compare the underlying mechanisms including cell cycle arrest, apoptosis and cytotoxicity for both cell lines.

Given the high beneficial potential of sorghum phenolic compounds and the lag behind similar research on other plant foods, sorghum is a candidate food that deserves systematic investigation. Therefore, present study selected 13 sorghum accessions with high antioxidant activities tested by our previous studies, and was aimed to investigate: (1) the effect of extracted
sorghum phenolics on inhibiting cancer cell growth using HepG2 and Caco-2 cell lines; (2) and the underlying mechanisms regarding cytotoxicity and cell cycle interruption. The comparison between the two cell lines would contribute to a better understanding of anti-cancer activities by sorghum phenolics with a long-term goal to promote sorghum as a sustainable phenolics source as well as a healthy food product for humans in the western world.

2. Materials and methods

2.1. Sorghum accessions

Thirteen sorghum accessions that selected from previous studies as having high antioxidant activity were provided by Dr. Thomas Herald at USDA-ARS in Manhattan, KS. Accession numbers: PI152653, PI152687, PI193073, PI329694, PI559733, PI559855, PI568282, PI570366, PI570481, PI570484, PI570819, PI570889, PI570993. Another sorghum accession F1000 was selected as control as it has the least level of phenolics, while PI329694 and PI570481 together with the control were selected as representative sorghum accessions for the following assays in this study.

2.2. Reagents

Reagents including acetone, ethanol, Folin-Ciocalteu reagent, gallic acid, sodium carbonate, HyClone Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum, phosphate buffered saline (PBS), penicillin/streptomycin, trypsin-EDTA, propidium iodide (PI), RNase were purchased from Fisher Scientific Co. L.L.C (Pittsburgh, PA, 15275 USA). CytoSelect™ LDH Cytotoxicity Assay Kit was purchased from Cell Biolabs, Inc (San Diego, CA, 92126 USA).

2.3. Extraction
Sorghum flour (0.2-0.5g) were extracted in 10 mL 70% aqueous acetone (v/v) for 2 h while shaking at low speed using a 211DS shaking incubator (Labnet International, Inc., Edison, NJ USA), followed by storing at -20 °C in the dark overnight, allowing the phenolics to completely diffuse from the cellular matrix into the solvent and enhance extraction. Extracts were then equilibrated at room temperature, then centrifuged at 2970 × g for 10 min. The residue was rinsed with additional 10 mL solvents with 5 min shaking and centrifuged at 2970 × g for 10 min. The combined two aliquots were either used for total phenolic content determination or dried under a stream of Nitrogen followed by evaporation. The dried extracts were then dissolved in dimethyl sulfoxide (DMSO) to make stock solutions and stored at -20 °C for further analysis. Before each use, the stock solution was diluted with fresh medium to achieve the desired concentrations (0-200 μM GAE). The final DMSO concentration in all cultures was <0.1%, which did not alter cell growth and cell cycle measurements when compared with vehicle-free medium. All extractions and analysis were conducted in triplicate.

2.4. Total phenolic content

Determination of total phenolic contents was performed using the Folin-Ciocalteu assay [17], adapted to 96-well microplates. A stock solution of 1 mg/mL Gallic acid in distilled water was prepared, and concentrations ranging from 12.5-200 μg/mL in 70% acetone were made for a standard curve. A solution of Na₂CO₃ at 7.5% (w/v) was also prepared. To each of the 96 wells, 75 μL distilled water was added, followed by 25 μL either aliquot of extracts or gallic acid standard at various concentrations. Folin-Ciocalteu reagent diluted 1:1 with distilled water was then added to each well. The reaction was allowed to stand for 10 min at room temperature, and then 100 μL of Na₂CO₃ solution was added to each well. Plate was covered and stand in dark for 90 min before measuring. Absorbance were read in a microplate reader SynergyHT, Biotek (Winnoski, USA);
with Gen5™ 2.0 data analysis software. Results were expressed as mg gallic acid equivalent (GAE) per g dry weight (DW).

2.5. Cell culture

The human hepatocellular carcinoma cells HepG2 (HB-8065) and human colorectal adenocarcinoma cells Caco-2 (HTB-37) were purchased from American Type Culture Collection (ATCC), Manassas, VA, 20108 USA. Cells were cultured in DMEM supplemented by 10% FBS, 100 µg/mL streptomycin, and 100 units/mL penicillin at 37 °C in a 5% CO2 humidified atmosphere. Cells in the exponential growth phase were used for all experiments.

2.6. Cell growth inhibition assay

Two milliliter cell suspension (1 × 10^5 cells/mL) were seeded into 6-well plates and cultured in a humidified incubator to allow adhesion. Cells were then treated with extracted sorghum phenolics at various concentrations of 0-200 µM GAE for up to 72 hrs. After incubation with each treatment, cells at 24, 48 and 72 hrs were detached by 0.05% trypsin-EDTA solution at 37 °C and then suspended in PBS. The number of suspended cells was counted with a hemacytometer as described by our previous publication.[18] IC50 values for each accession at 72 hrs were calculated based on the cell growth inhibition data.

2.7. Cytotoxicity assay

Cytotoxicity was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. The activity of LDH in the medium was determined using a commercially available kit CytoSelect™ LDH Cytotoxicity Assay Kit from Cell Biolabs, Inc. Cell suspension containing 0.1-1.0 ×10^6 cells/mL were seeded into a 96-well plate and cultured at 37°C and 5% CO2 with sorghum extracts at 0-200 µM GAE for 24 hours. Negative (sterile water) control was then added to each well and positive (Triton X-100) control were added to wells without sorghum extracts at the same
time. Aliquots of media and reagents were mixed in a 96-well plate and incubated at 37 °C and 5% CO\textsubscript{2} for 0.5 hrs. Absorbance was recorded using a microplate reader SynergyHT, Biotek (Winnoski, USA), and analyzed with Gen5\textsuperscript{TM}2.0 data analysis software. The % Relative Cytotoxicity was calculated using the following equation:

\[
\frac{OD(\text{experiment sample}) - OD(\text{negative control})}{OD(\text{positive control}) - OD(\text{negative control})} \times 100 = \% \text{ Relative Cytotoxicity}
\]

2.8. Cell cycle analysis

The cell cycle analysis was conducted for sorghum accession PI329694 as having highest total phenolic content (Table 1), and PI570481 as having lowest IC\textsubscript{50} value (Table 2), as well as the sample control F10000. Cells were treated with extracted sorghum phenolics at various concentrations (0-200 μM GAE) and harvested after 72 hrs. After centrifugation, cells were fixed in 70% ethanol at 4 °C and then re-suspended in propidium iodide (PI) staining solution containing 20 μg/mL PI and 5 U/mL RNase in PBS at 37 °C for 15 min before analysis by a flow cytometry (LSR Fortessa X-20 and FACSCalibur, BD, Franklin Lakes, NJ 07417-1880 USA) with excitation at 488 nm and emission at 617 nm.

2.9. Statistics

Data were analyzed by the SAS statistical system (version 9.2). The significance of the trend for cell growth inhibition at various concentrations for three exposure times was analyzed with linear regression. The effect of extracted phenolic at various concentrations for 72 hours on cell cycle arrest and apoptosis was analyzed by one-way ANOVA with Tukey adjustment. Pearson correlation coefficients (r) were used to analyze the relationships between total phenolic contents and IC\textsubscript{50} values for all sorghum accessions, as well as cell numbers and percentages of cells arrest at G2/M or apoptosis for accession PI329694, PI570481 and control for both cell lines.
3. Result

3.1. Total phenolic content

The total phenolic contents for the 13 sorghum accessions were determined by Folin-Ciocalteu method as described previously, results were expressed as mg gallic acid equivalent per g dry weight (GAE/g DW). The total phenolic contents ranged from 31 to 63.7 mg GAE/g DW with the highest being PI329694, while the control (F10000) was as low as 2.3 mg GAE/g DW (Table 1).

3.2. Cell growth inhibition

The cell growth inhibition assay was conducted for all accessions in HepG2 cells (Figure 1), IC$_{50}$ values (table 2) were calculated for each accession by linear regression of cell growth inhibition data at 72 hours. Results showed a dose-dependent decrease in cell numbers for all the accessions in HepG2 cell. Although the cell growth inhibition patterns were similar among the 13 sorghum accessions examined in our study. However, IC$_{50}$ values (table 2) varied from 85.8 to 221.8 mg DW/mL comparing with the control that has an IC$_{50}$ value as high as 1275.6 mg DW/mL. The IC$_{50}$ value for accessions PI570481 was the lowest which suggested that PI570481 was the most efficient in suppressing HepG2 cells growth. Based on the result of total phenolic content and IC$_{50}$ values, PI329694, PI570481, and the control (F10000) were selected for Caco-2 cells and comparing with the result in HepG2 cells. Results as shown in figure 2 indicated that treatment of both cell lines with sorghum phenolic extracts at 0 to 200 μM GAE for up to 72 hours resulted in a dose- and time- dependent reduction in cell number. The IC$_{50}$ values were calculated at 72 hours for the three representative accessions in both cell line, PI329694 had a lowest value (102.4 mg DW/mL) in Caco-2 cells while PI570481 (85.8 mg DW/mL) being the lowest in HepG2 cells. The IC$_{50}$ values of F10000 in both cell lines were around 10 times higher than the other two accessions.
3.3. Cytotoxicity assay

Cytotoxicity was tested in both cell lines, and assessed by lactate dehydrogenase (LDH) leakage into the culture medium using a commercially available kit CytoSelect™ LDH Cytotoxicity Assay Kit from Cell Biolabs, Inc. The % Relative Cytotoxicity values at each concentrations of sorghum extracts for various sorghum accessions were calculated using the equations mentioned previously, all the values were lower than 15% and the treated cells did not differ significantly from the vehicle control, which suggested that sorghum phenolic extracts were non-toxic to both cell lines (data not shown).

3.4. Cell cycle arrest and cell death

Sorghum accession PI329694, PI570481 and F10000 was selected as representative for cell cycle analysis. The effect of sorghum phenolic extracts on cell cycle was assessed by using a DNA flow cytometry, results were shown in Figure 3. Treatment of HepG2 with sorghum phenolic extracts at 0-200 μM GAE for 72 hours showed an increase in the percentage of cells at G2/M phase and a decrease in the percentage of cells at G1 phase. While in Caco-2 cells, it did not show an increase in G2/M phase or decrease in G0/G1 phase until phenolic concentration reached 100 μM GAE. Proportions of cells at S-phase were not significantly altered by sorghum phenolic extracts at any concentration in the two cell lines. The treatment of HepG2 with sorghum phenolic extracts at 0-200 μM GAE for 72 hours also resulted in an increase in the percentage of dead cells in both cell lines for the three accessions (Figure 4).

3.5. Correlation coefficient

Correlation coefficient (r) was calculated to determine whether an association existed between: (1) total phenolic contents and IC₅₀ values from 13 sorghum accessions in HepG2 cells;
(2) cell number decreasing and accumulation % of cells at G2/M phase or dead cells for accessions PI329694, PI570481 and control in both cell lines. As shown in Table 3, significant inverse correlation was observed between total phenolic contents and IC\textsubscript{50} values in all accessions (r = -0.6806, p<0.05). Meanwhile significant inverse correlations (Table 3) were also observed between the decreasing of cell number and increasing accumulation of G2/M and dead cells in sorghum accessions PI 329694, PI570481 and control in both HepG2 and Caco-2 cells.

4. Discussion

Thirteen sorghum accessions were selected from previous studies as having high antioxidant activity, accession F10000 that having the least phenolic content was selected to serve as control. Phenolic compounds could be classified as antioxidants because of their phenol rings and hydroxyl groups in the structure. In the present study, total phenolic contents determined by Folin-Ciocalteu assay were 30-64 mg GAE/g DW in various accessions (Table 1) while the control only contains 2.3 ± 0.2 mg GAE/g DW total phnoelics. This result indicate that the thirteen accessions could be potential candidates of phenolic resource for cancer prevention because higher phenolic content are potentially linked to higher antioxidant activity. Antioxidants are reported having the potential ability to prevent the initiation of carcinogenesis via either direct scavenging of reactive oxygen/nitrogen species (ROS/RNS) or induce cellular defense detoxifying/antioxidant enzymes that contribute to cellular protection against ROS/RNS and reactive metabolites of carcinogens.[19]

Treatment of sorghum phenolics at various concentrations for up to 72 hours resulted in a significant inhibition of cell growth in a dose- and time- dependent manner (Figure 1 and 2) in both HepG2 and Caco-2 cell lines. The selected two cell lines which were derived from intestine
and liver respectively, are believed to have the opportunity of exposing to sorghum phenolic compounds after ingestion. IC_{50} values were calculated based on the data of cell number reduction at 72 hrs in cell growth inhibition assay and found to vary from 85.8 to 221.8 mg DW/mL (Table 2), with sorghum accession PI570481 being the lowest and PI559855 the highest when comparing with that (1275.6 mg DW/mL) of control in HepG2 cells. Similar results also found in Caco-2 cells treated with various concentrations of phenolic extract from sorghum accession PI329694, PI570481 and control (Table 2 and Figure 2). These results indicated that sorghum accessions rich in phenolic compounds effectively suppressed the cell growth in both HepG2 and Caco-2 cell lines. Possible inhibition mechanisms including: (1) perturb cell cycle specific proteins, affect and/or block the continuous proliferation of tumorigenic cells[20]; (2) induce apoptosis by activation of caspases-3 and -9, diminished levels Bcl-2 and Mcl-1[21][22], or cell cycle arrest, etc.

To investigate the underlying mechanisms of cancer cell growth inhibition by sorghum phenolics, cytotoxicity was tested by using a commercial available kit, this assay is based on the conversion of lactate to pyruvate in the presence of LDH (Lactate Dehydrogenase) with parallel reduction of NAD. The formation of NADH from the above reaction results in converting WST-1 (present in the reagent) to an orange formazan that can be detected under absorbance at 450 nm. The % Relative Cytotoxicity values at each concentrations of sorghum phenolic extracts for various sorghum accessions in both cell lines were lower than 15% and the treated cells did not differ significantly from the vehicle control, which suggested that sorghum phenolic extracts were non-toxic to the cells. However, we still found dead cells in cell cycle analysis by flow cytometry, the percentage of dead cells were increased in a dose-dependent manner in both cell lines (Figure 4). In addition, both cell lines undergone accumulation of G2/M phase and decrease of percentage
of cells at G1 phase (Figure 3). These findings indicated that the death of the cells appears to be cytostatic, not cytotoxicity, since the cytotoxicity assay showed the sorghum phenolics are non-toxic to the cells. To further confirm the cause of cell death, we could use tunnel assay or apoptosis marker such as annexin V. Cancer progression has been suggested to involve the loss of cell-cycle checkpoint controls that regulate the passage through the cell-cycle. These checkpoints monitor the integrity of the DNA and ensure that genes are expressed in a coordinated manner.[23] The cell cycle arrest may trigger the DNA repair machine, leading to apoptosis which causes cell death. The apoptosis in HepG2 and Caco-2 cells may also induced through changing mitochondrial membrane potential and downregulation of the anti-apoptotic protein, bcl(xl). [24]

Coefficient correlations were further calculated between (1) total phenolic contents and IC_{50} values in the 13 accessions in HepG2 cells; (2) cell number decreasing and proportion of cells arrested at G2/M phase for the three representative accessions in both cell line; (3) cell number decreasing and proportion of dead cells for the three representative accessions in both cell line. Significant inverse correlation observed between total phenolic contents and IC_{50} values of the thirteen sorghum accessions (Table 3) suggested the cell growth inhibition by sorghum extracts was associated with their phenolic contents. While, strong significant inverse correlations observed between dose-dependent cell number decreasing and accumulation of cells in G2/M phase and dead cells (Table 3) in both cell lines, further suggested that the inhibitory effect of sorghum phenolic compounds on cell growth is through cytostatic. The higher r values of percentage of cells at G2/M phase in HepG2 cells may indicate that these cells were more sensitive to sorghum phenolic extracts than Caco-2 cells which may due to intrinsic properties of the cells.

In conclusion, the present study shows that phenolic extracts in sorghum effectively inhibited HepG2 or Caco-2 cell growth in a dose- and time- dependent manner. The cell growth inhibition
by the sorghum extracts were significantly associated with their phenolic contents. Furthermore, the inhibition appeared to be mediated by cytostatic, not cytotoxicity. Taken together, this study is the first time to investigate and compare the anti-cancer effect of sorghum phenolic compounds in both HepG2 and Caco-2 cell lines, our findings suggested that sorghum is a valuable food crop to provide sustainable phenolics and furthermore promote human health.
References


[12] Lewis JB. Effects of bran from sorghum grains containing different classes and levels of bioactive compounds in colon carcinogenesis 2009.


[17] Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-


Table 2-1 Phenolic Contents in Various Sorghum Accessions

<table>
<thead>
<tr>
<th>Sorghum Accession No.</th>
<th>Total Phenolic Content (mg GAE/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F10000 (control)</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>PI 559855</td>
<td>31.0 ± 0.2</td>
</tr>
<tr>
<td>PI 152687</td>
<td>44.6 ± 2.9</td>
</tr>
<tr>
<td>PI 570819</td>
<td>48.2 ± 4.4</td>
</tr>
<tr>
<td>PI 570993</td>
<td>51.0 ± 1.9</td>
</tr>
<tr>
<td>PI 559733</td>
<td>51.5 ± 1.4</td>
</tr>
<tr>
<td>PI 570366</td>
<td>54.3 ± 1.7</td>
</tr>
<tr>
<td>PI 152653</td>
<td>54.6 ± 1.2</td>
</tr>
<tr>
<td>PI 570484</td>
<td>54.8 ± 0.8</td>
</tr>
<tr>
<td>PI 193073</td>
<td>55.1 ± 4.5</td>
</tr>
<tr>
<td>PI 570889</td>
<td>58.0 ± 2.0</td>
</tr>
<tr>
<td>PI 568282</td>
<td>58.3 ± 2.5</td>
</tr>
<tr>
<td>PI 570481</td>
<td>58.5 ± 2.5</td>
</tr>
<tr>
<td>PI 329694</td>
<td>63.7 ± 2.5</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD (n=3); mg GAE/g DW represent for milligram gallic acid equivalent per gram dry weight.
<table>
<thead>
<tr>
<th>Sorghum Accession No.</th>
<th>IC₅₀ (mg DW/mL)</th>
<th>HepG2</th>
<th>Caco-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>F10000 (control)</td>
<td>1275.6</td>
<td>1131.3</td>
<td></td>
</tr>
<tr>
<td>PI 559855</td>
<td>221.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI 152687</td>
<td>138.9</td>
<td></td>
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<tr>
<td>PI 570819</td>
<td>192.1</td>
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<tr>
<td>PI 570993</td>
<td>146.2</td>
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<td>PI 559733</td>
<td>90.8</td>
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<tr>
<td>PI 570366</td>
<td>120.9</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>PI 152653</td>
<td>127.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI 570484</td>
<td>158.0</td>
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<tr>
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<td>113.3</td>
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<tr>
<td>PI 570481</td>
<td>85.8</td>
<td>115.6</td>
<td></td>
</tr>
<tr>
<td>PI 329694</td>
<td>126.8</td>
<td>102.4</td>
<td></td>
</tr>
</tbody>
</table>
Table 2-3 Correlation coefficient (r) among total phenolic contents with IC\textsubscript{50} values from the phenolic extracts of 13 sorghum accessions in HepG2 cells, or cell number with cell cycle arrest and apoptotic cells from control, PI329694 and PI570481 in both cell lines.

<table>
<thead>
<tr>
<th>IC\textsubscript{50}</th>
<th>Total phenolic content</th>
<th>Cell Arrest at G2/M</th>
<th>Apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.6806*</td>
<td></td>
<td></td>
</tr>
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<th></th>
<th>HepG2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F10000 control</td>
<td>-0.8608*</td>
<td>-0.9377**</td>
</tr>
<tr>
<td></td>
<td>PI329694</td>
<td>-0.8281*</td>
<td>-0.9764**</td>
</tr>
<tr>
<td></td>
<td>PI570481</td>
<td>-0.9469**</td>
<td>-0.9097**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Caco-2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F10000 control</td>
<td>-0.7599*</td>
<td>-0.9719**</td>
</tr>
<tr>
<td></td>
<td>PI329694</td>
<td>-0.7655*</td>
<td>-0.8199*</td>
</tr>
<tr>
<td></td>
<td>PI570481</td>
<td>-0.6840*</td>
<td>-0.8925*</td>
</tr>
</tbody>
</table>

*\(p<0.05\), **\(p<0.01\).
Figure Legends

Figure 2-1 The effect of various sorghum phenolic extracts at 0-200 μM GAE on cell number in HepG2 cells after 72 hrs. HepG2 cells were cultured with phenolic extracts at various concentrations (0-200 μM GAE) in 6-well plates, and then detached by trypsin-EDTA solution, and the cell number was counted by a hemacytometer. Values are expressed as Mean ± SD (n=3), the significance of the trend for cell growth inhibition at various concentrations was analyzed with linear regression, p<0.05.

Figure 2-2 The effect of representative sorghum phenolic extracts at 0-200 μM GAE on cell number in both HepG2 and Caco-2 cells for up to 72 hrs. The representative sorghum accessions were: PI329694, PI570481 and control (F10000). Cells were cultured with phenolic extracts at various concentrations (0-200 μM GAE) in 6-well plates, then detached by trypsin-EDTA solution and harvested by resuspension in medium for an immediate counting of cell numbers by hemacytometer at each time point. Values are expressed as Mean ± SD (n=3), the significance of the trend for cell growth inhibition at various concentrations at each time point was analyzed with linear regression, p<0.05.

Figure 2-3 Cytostatic effect of representative sorghum phenolic extracts in HepG2 and Caco-2 cell lines. The representative sorghum accessions were: PI329694, PI570481 and control (F10000). Cells were cultured with sorghum phenolic extracts at 0-200 μM GAE for 72 hrs, and then cell cycle was monitored by a DNA flow cytometric analysis as follows: solid circles, % of cells at G1 phase; open circles, % of cells at G2/M phase; solid triangles, % of cells at S phase. Values are expressed as Mean ± SD (n=3), *p<0.05, **p<0.01 versus the vehicle controls.

Figure 2-4 Percentage of dead cells induced by representative sorghum phenolic extracts in HepG2 and Caco-2 cell lines. The representative sorghum accessions were: PI329694,
PI570481 and control (F10000). Cells were cultured with sorghum phenolic extracts at 0-200 µM GAE for 72 hrs, and then apoptosis was analyzed by a DNA flow cytometry. Values are expressed as Mean ± SD (n=3), *p<0.05, **p<0.01 versus the vehicle controls.
Figure 2-1 The effect of various sorghum phenolic extracts at 0-200 μM GAE on cell number in HepG2 cells after 72 hrs.

Values are expressed as Mean ± SD (n=3), the significance of the trend for cell growth inhibition at various concentrations was analyzed with linear regression, p<0.05
Figure 2-2 The effect of representative sorghum phenolic extracts at 0-200 μM GAE on cell number in both HepG2 and Caco-2 cells for up to 72 hrs.

Values are expressed as Mean ± SD (n=3), the significance of the trend for cell growth inhibition at various concentrations at each time point was analyzed with linear regression, p<0.05.
Figure 2-3 Cytostatic effect of representative sorghum phenolic extracts in HepG2 and Caco-2 cell lines.

Values are expressed as Mean ± SD (n=3), *p<0.05, **p<0.01 versus the vehicle controls.
Figure 2-4 Percentage of dead cells induced by representative sorghum phenolic extracts in HepG2 and Caco-2 cell lines.

Values are expressed as Mean ± SD (n=3), *p<0.05, **p<0.01 versus the vehicle controls.
Chapter 3 - Effect of Various Dietary Fats on Fatty Acid Profile in Duck Liver: Efficient Conversion of Short-chain to Long-chain Omega-3 Fatty Acids
Abstract

Omega-3 fatty acids, especially long-chain omega-3 fatty acids, have been associated with potential health benefits for chronic disease prevention. Our previous studies found that dietary omega-3 fatty acids could accumulate in the meat and eggs in a duck model. This study was to reveal the effects of various dietary fats on fatty acid profile and conversion of omega-3 fatty acids in duck liver. Female Shan Partridge Ducks were randomly assigned to five dietary treatments, each consisting of 6 replicates of 30 birds. The experimental diets substituted the basal diet by 2% of flaxseed oil, rapeseed oil, beef tallow, or fish oil, respectively. In addition, a dose response study was further conducted for flaxseed and fish oil diets at 0.5%, 1%, and 2%, respectively. At the end of the 5-week treatment, fatty acids were extracted from the liver samples and analyzed by GC-FID. As expected, the total omega-3 fatty acids and the ratio of total omega-3/omega-6 significantly increased in both flaxseed and fish oil groups when compared with the control diet. No significant change of total saturated fatty acids or omega-3 fatty acids was found in both rapeseed and beef tallow groups. The dose-response study further indicated that 59-81% of the short-chain omega-3 ALA in flaxseed oil-fed group was efficiently converted to long-chain DHA in the duck liver, whereas 1% of dietary flaxseed oil could produce an equivalent level of DHA as 0.5% of dietary fish oil. The more omega-3 fatty acids, the less omega-6 fatty acids in the duck liver. Taken together, this study showed the fatty acid profiling in the duck liver after various dietary fat consumption, provided insight into a dose response change of omega-3 fatty acids, indicated an efficient conversion of short- to long-chain omega-3 fatty acid, and suggested alternative long-chain omega-3 fatty acid-enriched duck products for human health benefits.

Keywords: omega-3 fatty acid, duck, liver, dietary fat, health benefits
Introduction

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs), in addition as essential nutrients, have been associated with potential health benefits in chronic disease prevention. There are two types of ω-3 PUFAs, known by short-chain ω-3 PUFAs like ALA (alpha-linolenic acids, C18:3n-3) or long-chain ω-3 PUFAs such as EPA (eicosapentaenoic acid, C20:5n-3) and DHA (docosahexaenoic acid, C22:6n-3). Short-chain ω-3 PUFAs are presented in plant oil such as flaxseed and soybean oil, while long-chain ω-3 PUFAs are usually found in marine products such as fish oil. Although short-chain ω-3 PUFAs are more common and less expensive, the potential health benefits of ω-3 PUFAs have been related to long-chain ω-3 PUFAs only.

Compelling data from epidemiological and interventional studies have demonstrated an inverse correlation between long-chain ω-3 PUFAs and risk of some chronic diseases, including cardiovascular diseases, myocardial infarction, psoriasis, mental illnesses, cancer and bronchial asthma. Although it is not conclusive, some clinical trials also found long-chain ω-3 PUFAs contributed to a lower risk of cancers, such as colon, breast and prostate cancers. Therefore, the 2015 Dietary Guidelines for Americans recommends the consumption of 8 oz. of seafood per week to provide an average of 250 mg/day of long chain omega-3 Fatty acids for health benefits. Moreover, The American Heart Association’s Strategic Impact Goal Through 2020 and Beyond recommends at least two servings with 3.5-oz. fish every week to increase EPA and DHA intakes, while an adequate intake of ω-6 linoleic acid as 17 g/d for men and 12 g/d for women at the age of 19 to 50 years.

Humans can convert short-chain to long-chain omega-3 fatty acids, but the conversion
efficiency is limited, usually below 5% in adults\textsuperscript{18} or even less than 1\% in infants and aging people.\textsuperscript{19} When compared with humans, however, waterfowls such as geese have been reported to convert short-chain to long-chain omega-3 fatty acids more efficiently by a series of desaturase and elongase in the liver\textsuperscript{20} and subsequently excreted into blood circulation to other tissues.\textsuperscript{21} The diverse conversion rates between human and waterfowl have driven scientists to consider that waterfowls may provide an alternate and sustainable source of long-chain ω-3 PUFAs from plant-derived short-chain ALA.\textsuperscript{22-24}

Ducks are aquatic birds which have a high rate of lipogenesis to meet their energetic requirements during ancient migratory flight.\textsuperscript{25-26} It has been reported, for instance, the percent body fat could be reached as high as 37-42\% in Peking ducks and 20-30\% in Muscovy ducks.\textsuperscript{27} Although duck products are popular with its unique flavor and juicy texture, the high fat content has raised health concerns. While certain species of lean ducks, such as the Shan Partridge contains 7.5\% of body fat only have been developed, modification of the fat composition in favor of higher ω-3 PUFAs in replace of ω-6 PUFAs and/or saturated fatty acids may provide promising healthy benefits. It has been noted that supplemented diets with vegetable oil and fish oil effectively enhanced ω-3 PUFAs in the products of pork,\textsuperscript{28} broilers\textsuperscript{29,30} and broiler eggs.\textsuperscript{31,32} Dietary ALA was also reported to promote EPA and DHA contents in chicken liver.\textsuperscript{33} Furthermore, Chen and Hsu reported an increased trend of EPA, DHA, and total ω-3 PUFAs in duck egg yolks by feeding cod liver oil diet.\textsuperscript{34} In addition to storage and transportation of lipids, liver of birds has a very high capacity of lipogenesis\textsuperscript{35}. Fatty acids can be synthesis or converted in the liver, and then transported to other tissues such as adipose, cardiac and skeletal muscle. The conversion of fatty acids in the liver and incorporation of them into various tissues are well-established in response to the observed change of fatty acid composition\textsuperscript{36} in the relative tissues. Therefore, fatty acid
conversion in the liver may provide impact not only on a varied fatty acid composition but also on the meat quality including flavor and muscle color\textsuperscript{37}. From an aspect of nutrition value, efficient conversion of short- to long-chain $\omega$-3 PUFAs in the liver may boost the levels of long chain $\omega$-3 PUFAs and thus improve the meat quality. Our previous study found that fish oil and sunflower oil could significantly enhance the levels of EPA and DHA in the leg and chest muscles as well as eggs of Shan Partridge duck.\textsuperscript{38} However, the effect of various dietary fats on fatty acid profile and the conversion efficacy of $\omega$-3 PUFAs in duck liver, to our knowledge, has yet to be well studied.

The aim of this study was to assess the modification of fatty acid profiles in the liver of Shan Partridge duck after feeding various dietary fats, including ALA-enriched flaxseed oil, $\omega$-6 PUFA-enriched rapeseed oil, saturated fatty acid-enriched beef tallow, and EPA/DHA-enriched fish oil. The conversion efficacy of short-chain to long-chain $\omega$-3 PUFAs was further investigated by a dose-response study for flaxseed and fish oil treatment, respectively.

**Materials and methods**

**Animals**

Female Shan Partridge Ducks of the same genetic background and of comparable body weight at the age of 370 days were housed in the same room with incandescent lighting on 15:9 h light-dark cycle. Feed and water were provided for ad libitum consumption.

**Experimental design**

Shan Partridge Ducks were randomly assigned into 5 dietary treatment groups
including a control group (each group with 6 replicates of 30 birds). The experimental diets substituted the basal diet by 2% of flaxseed oil, rapeseed oil, beef tallow, or fish oil, respectively. Control group was feed with the basal diet. In addition, a dose response study was further conducted for flaxseed and fish oil diets only. Total 7 experimental groups fed various substituted basal diet by 0.5%, 1%, and 2% flaxseed or fish oil respectively, and the control group fed with the basal diet. Each group had 6 replicates. The ingredients and calculated nutrient level of the basal control diet was formulated to meet the nutrient requirements of the National Research Council (Table 1). The measured fatty acid values of the experimental diets in the present study is shown in Table 2. Diets were balanced to similar levels of protein, fat, total energy, and fiber. At the end of the 5-week dietary treatment, ducks were sacrificed and fresh duck livers were stored at -20°C for further lipid extraction.

**Lipid extraction**

One gram of the liver sample was grinded and mixed with 2 mL of chloroform/methanol (1:2, v/v in 0.001% Butylated hydroxytoluene), 1 mL of chloroform, and 1 mL of water. The mixture was then centrifuged at 1,000 rpm for 15 min. The lower layer was then collected. The above procedure was repeated twice. All the three lower layers were combined together and evaporated under a stream of N₂ gas. One mL of chloroform was added to the dried tube before stored at -80°C until further lipid analysis.

**Fatty acid analysis**

Fatty acid methyl esters were synthesized according to the protocols of the Kansas Lipidomics Research Center. Briefly, each lipid extracted sample was transferred to Teflon-lined
screw cap tube. An internal standard, pentadecanoic acid (C15:0), was added to each sample. Derivatization was performed with 1mL of 3 M methanolic hydrochloric acid at 78°C for 30 min. Then 2 mL of water and 2 mL of hexan:chloroform (4:1, v/v) were added to each tube. The upper phase was collected after vortex and centrifuge. After the above procedure was repeated twice, three upper phase were combined and dried under nitrogen gas, re-dissolved in 200 μL of hexane and transferred into a GC vial with insert.

Fatty acid methyl esters were analyzed using an Agilent 6890N gas chromatography equipped with a programmed temperature vaporization injector, an Agilent 7683 autosampler, and Agilent flame ionization detector (Santa Clara, CA). The GC was fitted with a HP-88 capillary column (100m × 0.25mm × 0.2μm, Agilent, Santa Clara, CA). The injector temperature was operated at 275°C with an injection volume of 1 μL. The detector temperature was set at 260°C. Helium was used as the carrier gas at a flow rate of 1.6 mL/min. The flow rate of air and hydrogen were 400 mL/min and 30 mL/min, respectively. The oven temperature ramp was programmed from an initial value of 150°C for 1 min to 175°C at 10°C /min for 10min, and then to 210°C at 5°C/min for 5 min hold, finally to 230 °C at the same speed for 11 min. The total run time per sample is 40.5 min and the sampling rate of the FID was 20 Hz. Fatty acid peaks were identified by comparison of the relative retention times with the Supelco® 37 component fatty acid methyl ester mix standards. The content of each fatty acid was calculated based upon the area of each identified peak.

**Statistical analysis**

Data are expressed as mean ± SD. All the data were analyzed by two-way analysis of variance (ANOVA) and followed by pairwise comparison with Tukey adjustment using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). A value of P < 0.05 considered to be statistically significant.
Results

Fatty acid profile in duck liver

A representative gas chromatography selected from each treatment group was showed in Figure 1. Total 23 fatty acids including the internal standard (peak 2, pentadecanoic acid, 15:0) were identified and analyzed in the duck liver samples, including saturated fatty acids (SFA 14:0, 15:0, 16:0, 17:0, 18:0, 22:0, and 24:0), monounsaturated fatty acids (MUFA 16:1n-10, 16:1n-7, 18:1n-9, 18:1n-7, and 20:1), ω-3 PUFAs (18:3, 18:4, 20:5, 22:5, and 22:6), and ω-6 PUFAs (18:2, 20:2, 20:3, 20:4, 22:4, and 22:5). As shown in Table 3, the contents of fatty acids in duck liver fed various dietary fats for 5 weeks varied. No significant difference of total SFA, total MUFA, total ω-6 PUFAs, or individual ω-6 PUFA 18:2 and 20:2 was found among the treatment groups. Both short-chain ω-3 PUFA ALA (C18:3) and long-chain ω-3 PUFA DHA (C22:6) were significantly abundant in flaxseed oil group, while long-chain ω-3 PUFA DHA only were considerably found in fish oil group. The highest content of total ω-3 fatty acids was detected in fish oil-fed group, followed by flaxseed oil-fed and rapeseed oil-fed group. The content of arachidonic acid, one of the ω-6 PUFAs (20:4), was significantly lower in both flaxseed oil and fish oil groups when compared with the control. The ratios of total PUFA/SFA and Σn3/Σn6 were significantly higher in the flaxseed oil, rapeseed oil, and fish oil groups than that in the beef tallow or the control groups.

A dose response study

In order to investigate the conversion efficacy of short-chain to long-chain ω-3 fatty acids in the liver, a dose response study using flaxseed oil diet at 0.5%, 1%, and 2% doses versus fish oil diet was conducted. As shown in Table 4, the contents of ALA in the livers of ducks fed various
doses of flaxseed oil increased from the basal line of 0.06 to 0.16, 0.36, and 0.65 mg/g fresh weight gradually. Meanwhile, DHA content in flaxseed oil-fed group also increased from 0.29 to 0.72, 1.06, and 1.01 mg/g fresh weight. In fish oil-fed groups, DHA but not EPA content increased significantly from 0.29 to 1.11, 1.76, and 2.08 mg/g fresh weight. On the contrary, the content of ω-6 arachidonic acid (AA, 20:4) decreased in 2% of flaxseed oil- and 1-2% of fish oil-fed groups significantly.

**Conversion between ω-3 fatty acids**

The effect of short-chain ω-3 ALA-enriched flaxseed oil diet and long-chain ω-3 fatty acids-rich fish oil diet on liver DHA content is shown in Figure 2. DHA in duck liver became predominant in both fish oil and flaxseed oil groups. Among of ω-3 fatty acids, 91%, 92%, and 85% were DHA in the liver of ducks fed various fish oil doses at 0.5%, 1%, and 2%, respectively. Meanwhile, 81%, 73%, and 59% of total ω-3 fatty acids were converted to DHA in the duck liver fed flaxseed oil at 0.5%, 1%, and 2%, respectively. When compared with fish oil group, 1% of flaxseed oil produced an equivalent level of DHA as 0.5% of dietary fish oil.

**Ratio of total ω-3/ω-6 fatty acids**

As shown in Figure 3, the ratios of Σω3/Σω6 in duck liver gradually increased as the doses of flaxseed oil or fish oil increased. Fish oil group possessed a higher Σω3/Σω6 value than flaxseed oil group at each dose, while a comparable value was observed between 1% of flaxseed oil and 0.5% of fish oil treatment.

**Discussion**

Fatty acid manipulation via dietary means may provide an effective method to obtain
healthy animal products for humans. Our previous studies investigated the effect of dietary fat on fatty acid composition showing that different dietary fats could change ω-3 fatty acid composition in the duck eggs and muscle tissues. However, little information is available about ω-3 fatty acid profile in the liver modified by various dietary fats. Therefore, this present study, to our knowledge, is the first time to examine the modulation of different dietary fats on fatty acid profile and contents in the duck liver.

After 5-week’s dietary treatment, all the dietary fats except for beef tallow showed significant modifications of the fatty acid profile and content in the duck liver. Although beef tallow provided more SFA than the control diet, no significant difference was found in 2% beef tallow-fed group, suggesting an effective transport and storage of SFA into non-hepatic tissues such as adipose tissues. Furthermore, the MUFA-enriched rapeseed oil treatment did not affect any fatty acids except for ALA that significantly increased in hepatic tissues.

The most significant modification was observed in the groups fed with either flaxseed oil or fish oil. Ducks fed with flaxseed oil and fish oil diets were found to have much higher total ω-3 PUFA and ω-3/ω-6 ratio than other groups. The ratio of ω-3/ω-6 was achieved as high as 0.28 for flaxseed oil-fed group and 0.36 for fish oil-fed group. Such ratio is much higher than the modern Western diet and is compatible with that of our ancestors about 100-150 years ago.39 The increase of both ratios in flaxseed oil and fish oil groups directly not only due to the increase of ω-3 fatty acids but also due to the decrease of ω-6 fatty acids, especially for AA. AA is a precursor of the derived eicosanoids such as PGE2, TXA2 and LTB4. The decrease of ω-6 fatty acids like AA may thus reduce risk of platelet aggregation, hemorrhage, and vasoconstriction.40-41 Some studies also suggested that a lower ratio of ω-3/ω-6 diets suppress inflammation in patients with rheumatoid arthritis,42,43 and have a beneficial effect on patients with asthma.44 The ω-3/ω-6 ratio
maybe a useful indicator to evaluate the healthy benefits of the functional food products.

It is interesting that duck liver possesses an efficient conversion of all the short-chain ω-3 fatty acids into long-chain DHA. About 60% of ALA in the flaxseed oil was converted to DHA in the duck liver, while 85% of total ω-3 fatty acids, mostly EPA and DHA, in the fish oil was converted to DHA. Such high conversion efficiency may be related to the broad substrate specificity of the duck elongase enzymes that convert the short-chain ω-3 PUFAs to final DHA exceptionally.23

The results of dose response study showed that the total ω-3 fatty acids, specifically DHA, and the ratio of ω-3/ω-6 increased as the dose increased in both flaxseed oil and fish oil treatments. It should be noted that 60-81% of the short-chain omega-3 ALA in flaxseed oil-fed group was efficiently converted to long-chain DHA in the duck liver and 1% of dietary flaxseed oil produced an equivalent level of DHA or ω-3/ω-6 ratio as 0.5% of dietary fish oil. Therefore, the ducks fed flaxseed oil could be an alternative source of fish DHA. Considering that the cost of flaxseed oil is much less expensive than fish oil, it appears commercially applicable for flaxseed oil-enriched diet to be used by waterfowl to provide healthy products.

Taken together, this study investigated the effects of various dietary fats on fatty acid profile and contents of ω-3 fatty acids in duck liver. Total ω-3 fatty acids and the ratio of total ω-3/ω-6 significantly increased in both flaxseed oil- and fish oil-fed groups. About 60-81% of the short-chain ω-3 ALA in flaxseed oil-fed group was efficiently converted to long-chain DHA in the duck liver, whereas 1% of dietary flaxseed oil could produce an equivalent level of DHA as 0.5% of dietary fish oil. It is significant that the short-chain ALA was efficiency converted to long-chain DHA in the duck liver, which may provide an alternative DHA-enriched duck products for human
health benefits.

**Reference**


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Table 3-1 Composition and main characteristics of the basal diet.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Content (g/kg)</th>
<th>Nutrient</th>
<th>Content (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize grain</td>
<td>400</td>
<td>Metabolizable energy</td>
<td>11.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wheat</td>
<td>290</td>
<td>Crude protein</td>
<td>16.5</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>120</td>
<td>Total phosphorus</td>
<td>0.70</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>90</td>
<td>Total calcium</td>
<td>3.35</td>
</tr>
<tr>
<td>Calcium hydrophosphate</td>
<td>12</td>
<td>Total lysine</td>
<td>0.79</td>
</tr>
<tr>
<td>Stone powder</td>
<td>80</td>
<td>Total methionine</td>
<td>0.40</td>
</tr>
<tr>
<td>Salt</td>
<td>3</td>
<td>Ether extract</td>
<td>29.0</td>
</tr>
<tr>
<td>Premix&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Supplied per kg of diet: vitamin A 1,500 U, cholecalciferol 200 U, vitamin E (DL-α-tocopheryl acetate) 10 U, riboflavin 3.5 mg, pantothenic acid 10 mg, niacin 30 mg, cobalamin 10 μg, choline chloride 1,000 mg, biotin 0.15 mg, folic acid 0.5 mg, thiamine 1.5 mg, pyridoxine 3.0 mg, Fe 80 mg, Zn 40 mg, Mn 60 mg, I 0.18 mg, Cu 8 mg, Se 0.3 mg; <sup>b</sup> Unit: MJ/kg
Table 3-2 Measured fatty acids in the experimental diets.

<table>
<thead>
<tr>
<th>Fatty acid*</th>
<th>Control</th>
<th>Flaxseed oil</th>
<th>Rapeseed oil</th>
<th>Beef tallow</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>32.6</td>
<td>25.5</td>
<td>26.2</td>
<td>41.9</td>
<td>30.4</td>
</tr>
<tr>
<td>MUFA</td>
<td>37.8</td>
<td>33.0</td>
<td>48.5</td>
<td>36.9</td>
<td>45.7</td>
</tr>
<tr>
<td>PUFA</td>
<td>29.8</td>
<td>41.6</td>
<td>25.4</td>
<td>20.3</td>
<td>35.1</td>
</tr>
<tr>
<td>Total ω3</td>
<td>7.6</td>
<td>20.5</td>
<td>5.7</td>
<td>4.7</td>
<td>10.9</td>
</tr>
<tr>
<td>18:3ω3</td>
<td>6.2</td>
<td>19.7</td>
<td>4.8</td>
<td>4.2</td>
<td>4.4</td>
</tr>
<tr>
<td>20:5ω3</td>
<td>0.9</td>
<td>0.6</td>
<td>0.6</td>
<td>0.4</td>
<td>6.1</td>
</tr>
<tr>
<td>22:6ω3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Total ω6</td>
<td>19.3</td>
<td>19.1</td>
<td>17.8</td>
<td>13.3</td>
<td>14.2</td>
</tr>
<tr>
<td>18:2ω6</td>
<td>18.8</td>
<td>18.7</td>
<td>17.4</td>
<td>12.9</td>
<td>12.7</td>
</tr>
<tr>
<td>20:2ω6</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>20:4ω6</td>
<td>0.5</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>PUFA/SFA</td>
<td>0.9</td>
<td>1.6</td>
<td>1.0</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Σω3/Σω6</td>
<td>0.4</td>
<td>1.1</td>
<td>0.3</td>
<td>0.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids. (This table is modified from our published paper45).
Table 3-3 Fatty acid contents in the duck liver fed various dietary fats for 5 weeks.*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control</th>
<th>Flaxseed oil</th>
<th>Rapeseed oil</th>
<th>Beef tallow</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>8.5±1.9</td>
<td>8.9±0.7</td>
<td>10.0±2.5</td>
<td>9.8±1.0</td>
<td>11.1±3.7</td>
</tr>
<tr>
<td>MUFA</td>
<td>7.0±2.3</td>
<td>7.9±1.4</td>
<td>12.2±4.9</td>
<td>9.4±1.3</td>
<td>11.3±6.0</td>
</tr>
<tr>
<td>PUFA</td>
<td>5.7±0.9</td>
<td>7.8±0.5</td>
<td>8.0±2.2</td>
<td>6.3±0.6</td>
<td>9.6±3.3</td>
</tr>
<tr>
<td>Total ω3</td>
<td>0.4±0.0</td>
<td>1.7±0.1</td>
<td>1.0±0.3</td>
<td>0.3±0.0</td>
<td>2.5±0.7</td>
</tr>
<tr>
<td>18:3ω3</td>
<td>0.1±0.0</td>
<td>0.7±0.2</td>
<td>0.4±0.3</td>
<td>0.1±0.0</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>20:5ω3</td>
<td>UD</td>
<td>0.0±0.0</td>
<td>UD</td>
<td>0.2±0.0</td>
<td></td>
</tr>
<tr>
<td>22:6ω3</td>
<td>0.3±0.0</td>
<td>1.0±0.1</td>
<td>0.6±0.1</td>
<td>0.3±0.0</td>
<td>2.1±0.5</td>
</tr>
<tr>
<td>Total ω6</td>
<td>5.3±0.9</td>
<td>6.1±0.4</td>
<td>7.0±2.0</td>
<td>6.0±0.6</td>
<td>7.2±2.8</td>
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<td>18:2ω6</td>
<td>2.5±0.7</td>
<td>3.9±0.6</td>
<td>4.6±2.1</td>
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<td>5.4±2.7</td>
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<td>0.1±0.0</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>20:4ω6</td>
<td>2.8±0.3</td>
<td>2.1±0.3</td>
<td>2.4±0.3</td>
<td>2.6±0.2</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>PUFA/SFA</td>
<td>0.7±0.1</td>
<td>0.9±0.8</td>
<td>0.8±0.1</td>
<td>0.7±0.0</td>
<td>0.9±0.0</td>
</tr>
<tr>
<td>Σω3/Σω6</td>
<td>0.1±0.0</td>
<td>0.3±0.0</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
<td>0.4±0.1</td>
</tr>
</tbody>
</table>

*Values are expressed as mean ± SD (n=3-6). Means in a row without a common letter differ, p<0.05.

**SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids. UD: undetectable.

(This table is modified from our published paper45).
<table>
<thead>
<tr>
<th>Fatty acid**</th>
<th>Control</th>
<th>Flaxseed oil</th>
<th>Flaxseed oil</th>
<th>Fish oil</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5%</td>
<td>1%</td>
<td>2%</td>
<td>0.5%</td>
<td>1%</td>
</tr>
<tr>
<td>SFA</td>
<td>8.5±1.9</td>
<td>8.4±1.3</td>
<td>9.2±0.7</td>
<td>8.9±0.7</td>
<td>10.3±2.0</td>
</tr>
<tr>
<td>MUFA</td>
<td>7.0±2.3</td>
<td>6.4±2.8</td>
<td>6.9±1.2</td>
<td>7.9±1.4</td>
<td>11.2±3.6</td>
</tr>
<tr>
<td>PUFA</td>
<td>5.7±0.9</td>
<td>6.7±0.6b</td>
<td>7.8±0.7a</td>
<td>7.8±0.5a</td>
<td>7.4±1.5</td>
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<tr>
<td>Total o3</td>
<td>0.4±0.0</td>
<td>0.9±0.1c</td>
<td>1.5±0.2b</td>
<td>1.7±0.1a</td>
<td>1.2±0.2b</td>
</tr>
<tr>
<td>18:3o3</td>
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<td>0.2±0.1b</td>
<td>0.4±0.2a</td>
<td>0.7±0.2a</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>20:5o3</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>22:6o3</td>
<td>0.3±0.0</td>
<td>0.7±0.1b</td>
<td>1.1±0.1a</td>
<td>1.0±0.1a</td>
<td>1.1±0.2b</td>
</tr>
<tr>
<td>Total o6</td>
<td>5.3±0.9</td>
<td>5.8±0.7</td>
<td>6.4±0.4</td>
<td>6.1±0.4</td>
<td>6.2±1.3</td>
</tr>
<tr>
<td>18:2o6</td>
<td>2.5±0.7</td>
<td>2.6±1.1</td>
<td>3.4±0.5</td>
<td>3.9±0.6</td>
<td>3.7±1.2</td>
</tr>
<tr>
<td>20:2o6</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>20:4o6</td>
<td>2.7±0.3</td>
<td>3.1±0.4a</td>
<td>2.8±0.2a</td>
<td>2.1±0.3b</td>
<td>2.4±0.2a</td>
</tr>
<tr>
<td>PUFA/SFA</td>
<td>0.7±0.1</td>
<td>0.8±0.1</td>
<td>0.9±0.0</td>
<td>0.9±0.1</td>
<td>0.7±0.1b</td>
</tr>
<tr>
<td>Σo3/Σo6</td>
<td>0.1±0.0</td>
<td>0.2±0.0b</td>
<td>0.2±0.0b</td>
<td>0.3±0.0b</td>
<td>0.2±0.0b</td>
</tr>
</tbody>
</table>

* Values are expressed as mean ± SD (n=6). Means in a raw without a common letter differ, p < 0.05. ** SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids. Values are expressed as mean ± SD (n=6). (This table is modified from our published paper45).
Figure legends

Figure 3-1 Representative Gas Chromatography of fatty acid profile in the liver of ducks fed with various dietary fats for 5 weeks. Shan Partridge Ducks were randomly assigned into 5 dietary treatments: either the basal diet or 2% of flaxseed oil, rapeseed oil, beef tallow, or fish oil, respectively. At the end of the 5-week treatment, fatty acids in duck liver were analyzed by GC-FID.

Figure 3-2 Dose response of ω-3 fatty acids in the liver of ducks fed with various doses of flaxseed oil or fish oil for 5 weeks. Shan Partridge Ducks were randomly assigned into a dose response study by feeding either flaxseed oil or fish oil diets at 0.5%, 1%, and 2%, respectively. At the end of the 5-week treatment, fatty acids in duck liver were analyzed by GC-FID. About 59-81% and 85-92% of total ω-3 fatty acids were converted to DHA in the duck liver fed various doses of flaxseed oil and fish oil, respectively. The dose of 1% flaxseed oil produced an equivalent level of DHA as 0.5% fish oil. Values are expressed as mean ± SD (n=6). Means in a group without a common letter differ, p < 0.05.

Figure 3-3 Dose response of total ω-3/ω-6 ratio in the liver of ducks fed with various disease of flaxseed oil or fish oil for 5 weeks. Shan Partridge Ducks were randomly assigned into a dose response study by feeding either flaxseed oil or fish oil diets at 0.5%, 1%, and 2%, respectively. At the end of the 5-week treatment, fatty acids in duck liver were analyzed by GC-FID. The ratios of ω3/ω6 in duck liver gradually increased as the doses of flaxseed oil or fish oil increased. Fish oil group possessed a higher ω3/ω6 value than flaxseed oil group, but a comparable value was observed between 1% of flaxseed oil and 0.5% of fish oil treatment. Values are expressed as mean ± SD (n=6). Means in a group without a common letter differ, p < 0.05.
Figure 3-1 Representative Gas Chromatography of fatty acid profile in the liver of ducks fed with various dietary fats for 5 weeks.

Note: This figure is modified from our published paper$^{45}$. 
Figure 3-2 Dose response of ω-3 fatty acids in the liver of ducks fed with various doses of flaxseed oil or fish oil for 5 weeks.

Note: This figure is modified from our published paper45.
Figure 3-3 Dose response of total ω-3/ω-6 ratio in the liver of ducks fed with various disease of flaxseed oil or fish oil for 5 weeks.

Note: This figure is modified from our published paper⁴⁵.
Chapter 4 - Conclusions

This dissertation focused on functional food related bioactive compounds with special interests in sorghum phenolic compounds and omega-3 polyunsaturated fatty acids.

Study 1: Thirteen sorghum accessions were selected to test the effects of their phenolic extracts on cancer cell growth in vitro. In conclusion, this study shows that phenolic extracts in sorghum effectively inhibited HepG2 or Caco-2 cell growth in a dose- and time-dependent manner. The cell growth inhibition by the sorghum extracts were significantly associated with their phenolic contents. Furthermore, the inhibition appeared to be mediated by cytostatic, not cytotoxicity. Taken together, this study is the first time to investigate and compare the anti-cancer effect of sorghum phenolic compounds in both HepG2 and Caco-2 cell lines, our findings suggested that sorghum is a valuable food crop to provide sustainable phenolics and furthermore promote human health.

Study 2: This study investigated the effects of various dietary fats on fatty acid profile and contents of ω-3 fatty acids in duck liver. Total ω-3 fatty acids and the ratio of total ω-3/ω-6 significantly increased in both flaxseed oil- and fish oil-fed groups. About 60-81% of the short-chain ω-3 ALA in flaxseed oil-fed group was efficiently converted to long-chain DHA in the duck liver, whereas 1% of dietary flaxseed oil could produce an equivalent level of DHA as 0.5% of dietary fish oil. It is significant that the short-chain ALA was efficiency converted to long-chain DHA in the duck liver, which may provide an alternative DHA-enriched duck products for human health benefits.
Chapter 5 - Future plan

Study 1: This study was to investigate the effect of sorghum phenolic extracts on inhibiting of cancer cell growth and the underlying mechanisms regarding cytotoxicity, cell cycle interruption and apoptosis induction. First, this study was conducted in vitro which did not consider the possible metabolism of phenolics in vivo. Future study could be done in vivo to strengthen the conclusion that sorghum phenolic extracts could efficiently suppress cancer cell growth. Second, cell cycle analysis and apoptosis were tested using PI staining solution by flow cytometry, the results of which may influenced by factors such as loss of cells during the process of experiment. To further confirm the results, future work could focus on the level of cell cycle regulator proteins such as p34\textsuperscript{cdk2} and cyclin B1 which can be tested by western blot. In addition, apoptosis marker such as annexin V and PARP could be tested as well to confirm the result of apoptosis induction.

Study 2: This study was to assess the modification of fatty acid profiles as well as investigate the conversion of short- to long-chain omega-3 polyunsaturated fatty acids in the liver of Shan Partridge duck after feeding various dietary fats. Future study could focus on the fatty acid profile in the liver of other poultry animals such as chicken and turkey since they are more popular than duck in the USA and some other countries in the western world.