

NUTRACEUTICAL USES OF SORGHUM BRAN (*SORGHUM BICOLOR*)

by

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(Under the Direction of Phillip Greenspan)

ABSTRACT

Select varieties of sorghum grain (*Sorghum bicolor*) are excellent sources of antioxidants, phytochemicals and very long chain fatty aldehydes, alcohols and acids. Studies have linked the consumption of food sources enriched in these beneficial compounds to reduced incidence of inflammatory and cardiovascular diseases. Although sorghum grain is one of the top five most important grains produced worldwide, the health promoting effects of sorghum have not received significant attention. In this dissertation, the anti-inflammatory effects of ethanol extracts of sorghum bran varieties are investigated. The hypocholesterolemic action of the wax and bran fractions of sorghum grain alone and in combination with other nutraceutical ingredients is also examined. The secretion of cytokines TNF- α and IL-1 from LPS-stimulated human peripheral mononuclear cells was significantly reduced by extracts of black sorghum bran. In the phorbol myristate (TPA) inflamed mouse ear edema model, markers for acute inflammation were measured after the treatment with various varieties of sorghum bran extracts or indomethacin. Ethanolic extracts of black and sumac sorghum bran produced reductions in both ear edema and myeloperoxidase activity. No effect was observed with white or mycogen sorghum bran varieties. The anti-inflammatory activity positively correlated with the high phenolic contents and antioxidant activities of black and sumac sorghum brans. Black bran extract did not affect

COX-2 protein expression, an enzyme involved in prostaglandin biosynthesis. Black sorghum bran extract in combination with indomethacin did not produce a greater effect than each agent alone suggesting that this bran may also block an enzyme involved in prostanoid production. Additional studies undertaken demonstrated that integration of sorghum wax into hypercholesterolemic diets did not produce any lipid-altering effects. A diet composed of 20% sorghum bran significantly lowered plasma cholesterol. A formulation comprised of a blend of phytosterols, *Spirulina*, turmeric and pantethine, produced lipid-lowering effects in the hypercholesterolemic hamster. The addition of 5% or 10% sorghum bran to this formulation produced a further modest lowering of plasma cholesterol levels. In conclusion, select sorghum bran varieties may be a beneficial food/nutraceutical ingredient for the moderation of inflammatory diseases and maintaining healthy plasma cholesterol concentrations.

INDEX WORDS: Sorghum bran, cholesterol, anti-inflammatory, nutraceutical and phytochemicals

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DEDICATION

To my grandparents that I have lost along this journey...

Your love and friendship has had a large impact in the person I am today.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

The human diet has consisted of whole grains for many thousands of years. However, recently a shift from whole grain to refined grains has occurred in the human diet [1]. The grindmills that were used previously to grind the grain did not separate the bran and germ from the endosperm. With the introduction of the roller mill, a machine that more efficiently separated the bran and germ from the endosperm, refined grain entered the human diet [1, 2]. Refined grain contains much more starch and fewer phytochemicals and vitamins than whole grains [3]. However, in the 1970's consumption of whole grain increased due to the 'fiber hypothesis' which suggested that whole foods that provide fiber result in significant health benefits.

Today, whole grain consumption has been associated with a decreased incidence of cancer, cardiovascular disease (CVD), diabetes and obesity [1]. In fact, the consumption of whole grains is associated with a decrease in the incidence of cardiovascular events [4-7]. The 2006 diet and lifestyle recommendations by the American Heart Association states that a diet rich in whole grains can decrease the risk of CVD and that half of all grain consumption should consist of whole grains [8]. The cholesterol lowering and improved glucose response is associated with the effects of soluble fiber, although insoluble fiber is well known for its effects on bowel stimulation [1].

However, the fiber hypothesis has not tested well in accounting for benefits of whole grains. Anderson and colleagues [9] have shown that protective effects of whole grains on CVD are independent of fiber, suggesting that additional components in the bran and germ fraction have important biological effects. Whole grains contain many antioxidant compounds including fat and water-soluble vitamins, vitamin E, phenolic acids and phytoestrogens which may account for other mechanisms by which the consumption of grains can benefit human health [1]. Additionally, the glycemic index, a system that ranks carbohydrates on their effects on blood glucose, is lower for whole grains due to the slower rate of both digestion and absorption [3]. In response to the overwhelming evidence that the consumption of whole grains is health-promoting, the current United States Dietary guidelines are 3 servings of whole grain foods daily rather than refined grains.

The most common cereal grains include wheat, rice and maize, oats, rye, barley, millet and sorghum [2]. Grain is composed of three parts: bran, germ and endosperm (**Figure 1.1**). The endosperm is the bulk of the kernel and contains proteins and starch. The germ contains the plant embryo and is thus the part where new plants sprout. The bran consists of the seed coat or testa and the pericarp. The bran fraction is rich in phytochemicals, fiber, vitamins, minerals and nutrients located in the pericarp [1]. The pericarp can be further divided into three sections: epicarp, mesocarp and endocarp. The outermost layer or epicarp is covered with a thin layer of wax [10] that contains constituents such as very long chain fatty acids and alcohols that may be biologically active [11]. A study conducted by Jensen et al. found that there was an even stronger inverse relationship between bran consumption and a decrease risk of CVD than with whole grain consumption [5].

The research presented in this dissertation focuses on the hypocholesterolemic activity of the sorghum wax and bran fractions and the anti-inflammatory effects of sorghum bran originating from the sorghum grain (*Sorghum bicolor*).

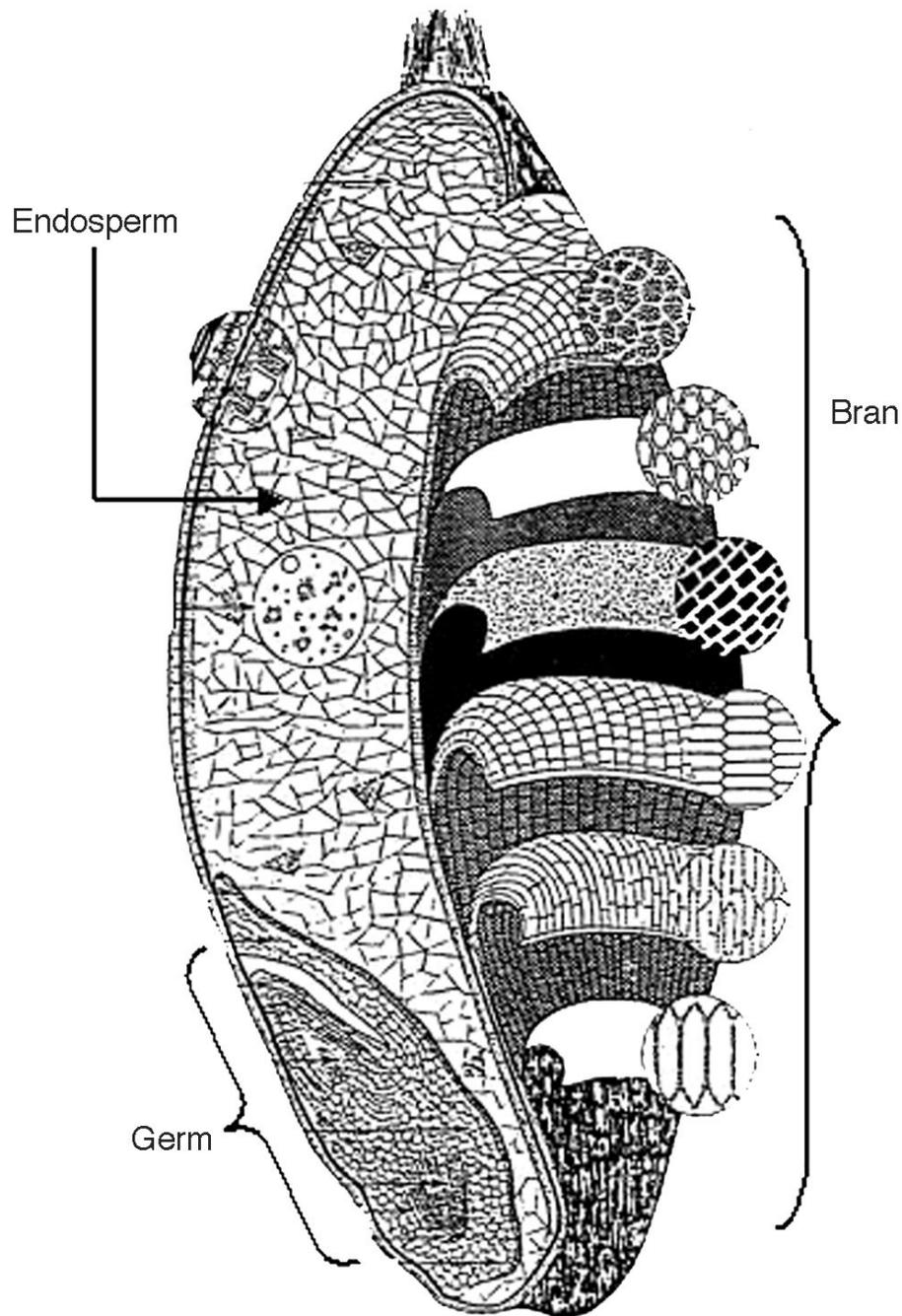


Figure 1.1: Structure of a whole grain. Adapted from Slavin et al., 2004.

SORGHUM GRAIN

Sorghum belongs to the grass family and has long been an important food staple in the semi-arid tropics of Africa. Archeological evidence dates the usage of sorghum as food back to 6500 B.C. Cultivation of sorghum began around 2000 B.C. and later spread to arid areas of China, India and Asia [12]. Sorghum arrived in America in the middle of the nineteenth century [13]. One remarkable characteristic of sorghum grain is its drought tolerance [14].

Sorghum is one of the five top cereal grains produced worldwide, along with maize, wheat, rice and barley. The United States is responsible for nearly 25% of worldwide sorghum production followed closely by India at 21%. The majority of USA sorghum is produced in Kansas, Nebraska, Texas and Arkansas. Although human cereal grain consumption has increased significantly within the last thirty years in the USA, the consumption of sorghum has remained stagnant. While sorghum is widely eaten by humans in developing countries, it remains primarily an animal feed in developed countries. In fact, the use of sorghum grain for animal feed is one of the main driving forces behind its global production [15]. Sorghum has the advantage of being gluten-free and is therefore a safe grain in breads, cookies, and snacks for celiac patients [13, 16].

The genus *Sorghum* is a genetically diverse with both wild (*Sorghum halepense* and *Sorghum propinquum*) and cultivated (*Sorghum bicolor*) species [12]. Select varieties of sorghum have considerably high concentrations of phenolic compounds and antioxidant capacities that are located primarily in the bran fraction of the grain. Flavonoids, phenolic acids and tannins are three phenolic categories found in sorghum. The flavonoids consist of anthocyanins, flavanols, flavones, and flavanones and the phenolic acids are benzoic and cinnamic derivatives [17]. One type of anthocyanin that is unique to sorghum is 3-

deoxyanthocyanin [18]. Sorghum contains only non-hydrolysable tannins as proanthocyanidins. Of the total anthocyanin content, nearly all black and brown varieties of sorghum contain 36-50% of apigeninidin & luteolinidin, two types of 3-deoxyanthocyanins [19]. The proanthocyanidins are found in highest concentrations in brown varieties of sorghum. The phenolic, flavonoid and tannin contents vary greatly among the different varieties of sorghum.

Biological Activities of Sorghum

Decreased risks of CVD are associated with the increased consumption of cereal grains. This may be due to the phytosterol, polyphenolic and fiber content of whole grains. Sorghum has relatively low amounts of β -glucans, a soluble fiber component that is responsible for the hypocholesterolemic actions in other cereal grains [20, 21]. An increased fecal bile acid secretion and increased high-density lipoprotein (HDL) cholesterol was observed when animals were fed a diet composed of 30% sorghum [22]. In rat microsomes, hexane extracts of sorghum dose-dependently inhibited 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, the rate-limiting enzymatic step in the synthesis of cholesterol [22].

Carr et al. [23] supplemented the diets of hamsters with sorghum lipids extracted from the grain with hexane. Plasma cholesterol and plasma non-HDL-cholesterol fractions, consisting mostly of low-density lipoprotein (LDL) cholesterol, were significantly lowered in a dose-dependent manner with grain sorghum lipids at 1% of the diet. A further increase of grain sorghum lipids in the diet to 5% significantly reduced plasma triglycerides [23]. Long-chain fatty alcohols, long chain fatty aldehydes, long chain fatty acids, phenolic compounds and plant sterols in sorghum are present in this hexane preparation and could participate in the hypocholesterolemic activity of the sorghum extract.

Ethanol sorghum bran was shown to inhibit the formation of advanced glycation end (AGE) products *in vitro* [24]. The formation of AGE products can lead to the generation of reactive oxygen species, activation of nuclear factor- κ B (NF- κ B) and the production of pro-inflammatory cytokines. All of these events have been linked to the pathogenesis of diabetes. The high-proanthocyanidin varieties of sorghum bran (i.e. sumac bran) were more potent inhibitors than the lower tannin varieties (black bran) and very low total phenolic varieties (white and mycogen brans). Additionally, sorghum bran was more efficacious in inhibiting AGE product formation than wheat, rice and oat bran extracts [24].

Obesity is a large problem in the western world and is related to several disease conditions such as diabetes and CVD. Tannins in sorghum are thought to bind to proteins and carbohydrates to form complexes that cannot be further broken down by digestive enzymes, thereby reducing the feed efficiency in animals without significantly affecting the absorption of nutrients [19]. Typically animals must consume more feed to produce the same weight gain. Although this is not ideal for livestock, sorghum may be used to help lower calorie absorption to help manage obesity.

Another mechanism by which tannins may slow digestion is by directly binding to sucrase, amylase, trypsin, chymotrypsin, lipase, other digestive enzymes [25] and to intestinal brush-border bound amino acid transporters [26], thereby rendering them less active. In fact, certain cultures in Africa prefer the consumption of high-tannin sorghum to other types of cereal grains due to its 'staying power' in the stomach. This may be related due to its slower digestibility.

Epidemiological studies indicate possible protective effects of sorghum consumption on the incidence of cancer. Countries that consume large quantities of sorghum (Africa, India,

China, etc.) have a low incidence of esophageal cancer, whereas, wheat and corn consumption is associated with an elevated incidence of this disease. In addition to a low glycemic index, sorghum may contain chemopreventative compounds not present in wheat and corn [25].

Very little work has been done on the anti-inflammatory actions of sorghum bran. Our laboratory, previous previously found that sorghum bran extracts are potent inhibitors of hyaluronidase activity. This enzyme is responsible for generating low molecular weight hyaluronan (HA) which is involved in intensifying the inflammatory processes by up-regulating CD44 receptors, pro-inflammatory cytokines, and matrix metalloproteinases. Therefore, the balance between the synthesis and degradation of HA is critical in the maintenance of joint function [27]. High-phenolic sorghum bran (black and sumac varieties) was more effective in inhibiting hyaluronidase activity than the low-phenolic containing sorghum varieties. In contrast, two of the most common brans consumed in the human diet, rice and wheat bran, had very little anti-hyaluronidase activity.

PHYTOCHEMICALS

Figure 1.2 illustrates the classification of the many different types of phytochemicals. Phytochemicals are defined as bioactive non-nutrient plant compounds in fruits, vegetables, grains and other plant foods that are linked to reducing the risk of many chronic diseases. More than 5000 phytochemicals have been identified but a large percentage still remains undocumented [28]. Phenolic compounds are one of the most studied phytochemicals. Sorghum brans can contain large concentrations of phenolic compounds [25].

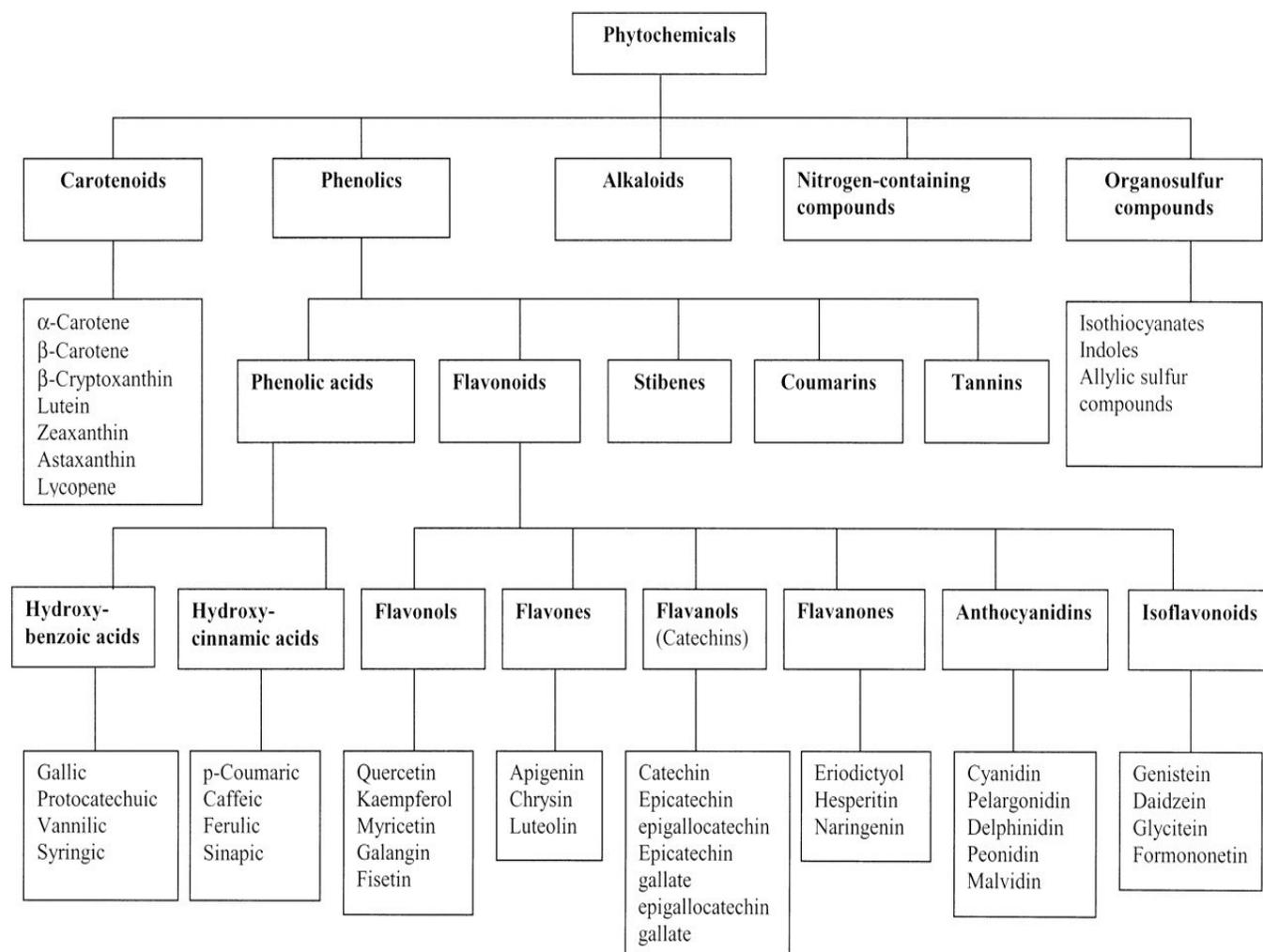


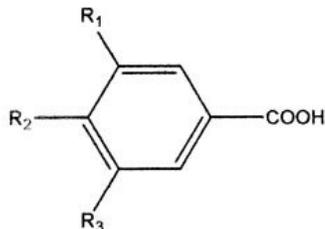
Figure 1.2: Classification of dietary phytochemicals. Adapted from Liu, R.H. J. Nutr. 2004:134:3479S-3485S.

Phenolic Acids

Phenolic acids are further divided into hydroxybenzoic acids and hydroxycinnamic acids (**Figure 1.3**) and are present in all types of cereal grains. Hydroxybenzoic acids are derived from benzoic acid and include gallic, p-hydroxybenzoic, vanillic, syringic, and protocatechuic acids. Caffeic, ferulic, and sinapic acids constitute the hydroxycinnamic acids derived from cinnamic acid [19]. Phenolic acids occur in cereal in both free and bound states. Free phenolic acids are located in the pericarp and bound phenolic acids are esterified to cell walls in the endosperm

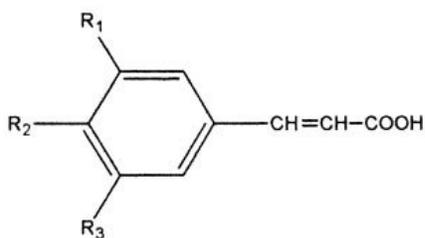
[29]. Brown sorghum (a high-tannin category) is a particularly rich source of ferulic and coumaric acids [30].

(a) Benzoic acid



Benzoic acid Derivatives	Substitutions		
	R ₁	R ₂	R ₃
p-Hydroxybenzoic	H	OH	H
Protocatechuic	H	OH	OH
Vannilic	CH ₃ O	OH	H
Syringic	CH ₃ O	OH	CH ₃ O
Gallic	OH	OH	OH

(b) Cinnamic acid



Cinnamic acid Derivatives	Substitutions		
	R ₁	R ₂	R ₃
p-Coumaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	CH ₃ O	OH	H
Sinapic	CH ₃ O	OH	CH ₃ O

Figure 1.3: Structure of a) benzoic acid and derivatives and b) cinnamic acid and derivatives. Adapted from Liu, R.H. J. Nutr. 2004:134:3479S-3485S.

In agreement with Hahn et al. [30] our laboratory identified high concentrations of protocatechuic, ferulic and caffeic acid in sumac bran (a brown sorghum) and ferulic, p-coumaric and caffeic phenolic acids in black sorghum bran (see chapter 2). Phenolic acids found in grains, fruits and vegetables and are known for their strong antioxidant capabilities [25]. For instance, ferulic acid significantly prevented DNA damage in rat peripheral blood lymphocytes and improved plasma antioxidant status of rats during nicotinic toxicity [31]. Ferulic, caffeic and p-coumaric protect LDL from oxidation and inhibit the formation of plasma thiobarbituric acid reactive substances [32]. Protocatechuic acid has been shown to effectively reduce the actions of nitrosoamines, known carcinogens in rats [33]. Nitric oxide production was significantly inhibited by gallic acid [34] and ferulic acid [35] in LPS-stimulated macrophages

Flavonoids

Flavonoids are compounds with a C6-C3-C6 skeleton that consists of two aromatic rings joined by a three carbon link. They are subdivided by their differences in the generic structure of the heterocycle C ring as flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins, and isoflavonoids (**Figure 1.4**).

Some of the common types of flavonoids found in fruits and vegetables include flavonols (quercetin, kaempferol, and myricetin), flavones (luteolin and apigenin), flavanols (catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate), flavanones (naringenin), anthocyanidins, and isoflavonoids (genistein) [28]. Flavonoids contribute the blue, purple and red colors in plants. Flavonoids possess various biological activities including anticancer, antimicrobial, antiviral, anti-inflammatory, immunomodulatory and antithrombotic effects [36].

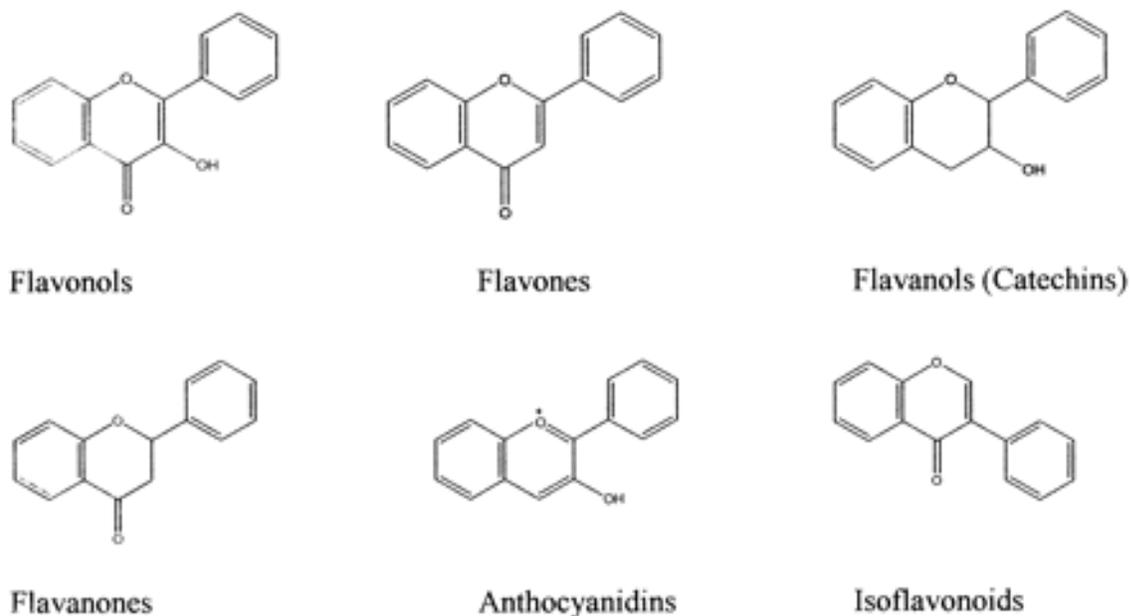


Figure 1.4: Structure of flavonoids. Adapted from Liu, R.H. *J. Nutr.* 2004;134:3479S-3485S.

There is not a vast amount of literature concerning identification of flavonoids in sorghum. It is known, however, that different varieties of sorghum have significant differences in both their type and content of flavonoids. For example, black sorghum contains the highest amount of 3-deoxyanthocyanins, an anthocyanin lacking a C-3 hydroxylation in the C ring, when compared to red and brown varieties. Additionally, 3-deoxyanthocyanins apigeninidin and lutelinidin account for nearly 50% of the total anthocyanin content. The 3-deoxyanthocyanins are synthesized within the sorghum plant to help protect it from bacterial and fungal infections [37]. These compounds may prove useful as natural food colorants as they have increased stability in acidic solutions compared to other common anthocyanins found in fruits and vegetables that are easily converted to their anthocyanidin counterpart [19]. Lutelinidin, isolated from sorghum seedlings, was more cytotoxic than other anthocyanins in reducing human cancer cell viability of HL-60 and HepG2 cells [38]. Similar results were obtained with apigeninidin.

Six of the other common anthocyanins in sorghum include cyanidin, delphinidin, malvidin, pelargonidin, petunidin, and peonidin. Relative to fruits such as blueberries, strawberries and grapes, black sorghum bran was reported to have a higher anthocyanins content and antioxidant activity. Evidence that anthocyanins in black sorghum are responsible for its high antioxidant activity was confirmed by a positive correlation between total anthocyanin content and antioxidant activity [39]. Black sorghum bran was reported to contain the highest amount of flavan-4-ols compared to other varieties of sorghum with luteoforol and apiforol predominating [40]. Two common flavones isolated and identified in sorghum include apigenin and luteolin [19]. Inducible nitric oxide synthase (iNOS) expression is down-regulated by apigenin in LPS-induced macrophages [41]. Naringenin and kaempferol are two flavanones that have been identified in sorghum [29]. Kaempferol and apigenin bind to the transcription factor, peroxisome proliferator activated factor (PPAR), acting as a possible ligand in activating the transcription of numerous genes [41].

Tannins

Tannins convert animal skin to leather during the tanning process, hence their name. The two types are condensed tannins or proanthocyanidins and hydrolysable tannins. The latter type has never been found in sorghum. Proanthocyanidins were named because anthocyanidins are released when tannin polymers are treated with mineral acids [30]. The proanthocyanidins in sorghum are mainly polymerized flavan-3-ol and/or flavan-3,4-diol units, consisting largely of catechin (88%) and epicatechin (12%) subunits as seen in **Figure 1.5** [42]. Sumac bran (sorghum variety) contains nearly 4% tannins [43]; this stands in contrast to other varieties which may be relatively free of such molecules.

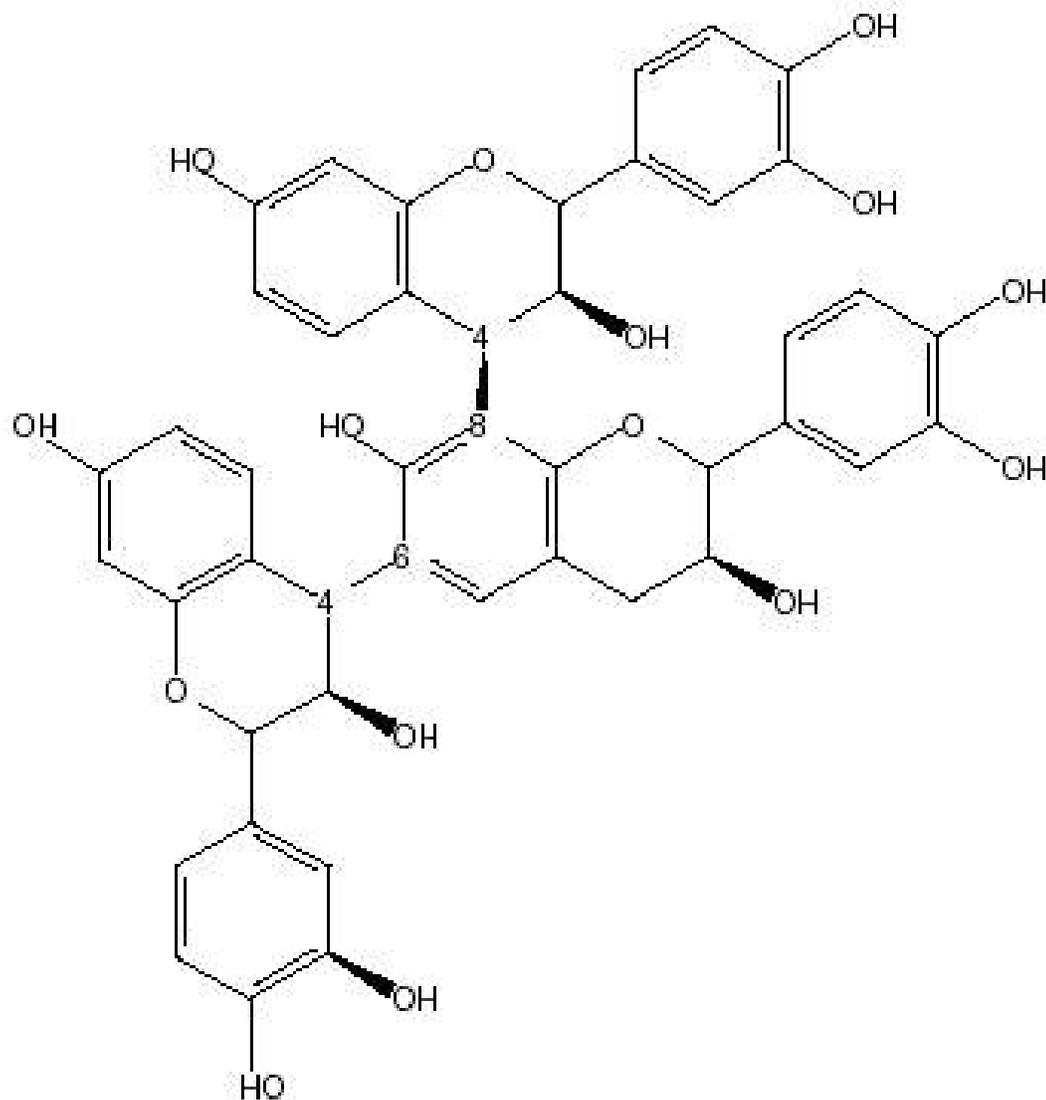


Figure 1.5: Structure of condensed tannin. Adapted from www.taninos.tripod.com/aa4.jpg

As a sorghum kernel matures, the flavonoid monomer units are condensed, forming oligomeric proanthocyanidin polymers. Due to the bitter taste of tannins in foods, many types of high-tannin sorghum may prove useful in astringent tasting foods such as dark chocolate. In addition, the bitter-tasting, high-tannin content in sorghum imparts a degree of bird resistance and protects the grain from mold growth [30].

Over 60% of the proanthocyanidins in sorghum have a degree of polymerization over 10 [43]. Since absorption of molecules either by the skin or GI tract is largely dependent upon the size of molecules, the bioavailability of these large compounds is in question. It has been reported that proanthocyanidins with degrees of polymerization measuring less than 7 were readily “absorbed” in the intestinal epithelium cell monolayer [44]. Deprez et al. [45] showed that large polymers are degraded by the colonic microflora into lower molecular weight phenolic acid compounds that can be easily absorbed. The significance of these metabolites that are bioavailability renews interest in the biological effects of high molecular weight tannins.

Condensed tannins have many beneficial pharmacological and biological effects; among these are significant anti-cancer, anti-inflammatory and antioxidant properties. Tannins have potent oxygen radical scavenging capabilities [46, 47] and can effectively inhibit oxidation of lipids contained in LDLs [48]. Tannins have also been reported to inhibit platelet aggregation *in vitro* [49] and are efficient iron and copper metal chelators, reducing oxidative damage to the myocardium [49]. Studies have shown that anthocyanidins can inhibit the formation of IL-1 β and TNF- α cytokines *in vitro* [50].

Phytosterols

Cholesterol-like compounds that are natural components of plant cell membranes are called phytosterols. Phytosterols mimic and compete with cholesterol absorption in the GI tract and can produce a cholesterol-lowering effect. These compounds are primarily found in the wax and bran fractions of cereal grains. Sitosterol, campesterol and stigmasterol have been identified in sorghum. Singh et al. [25] reported nearly 0.5 mg/g of phytosterols in sorghum while corn contains 0.9 mg/g.

INFLAMMATION

Inflammation is a response initiated by the activation of the immune system occurring when the host is threatened with infection, irritation or injury. Inflammation plays a central role in health and disease. Typically, inflammation is the first crucial step in fighting off an infection. Acute inflammation is highly protective. However, chronic inflammation participates in the development of many diseases. Cardiovascular disease, diabetes, arthritis, cancer, asthma, allergies, irritable bowel syndrome and many others are known to involve chronic inflammation.

The humoral and cellular components of the immune system interact in such a way as to result in inflammation. This event is initiated by the recognition of a foreign object by the host, followed by the reaction of the object with humoral components and inflammatory cells, resulting in the production of numerous cytokines, chemokines and other mediators [51].

Lipopolysaccharide and Inflammation

Lipopolysaccharide (LPS) is a major component in the outer cell wall of gram-negative bacteria which serves dual roles by providing great structural integrity to the bacteria and protecting the membrane from certain kinds of chemical attacks. Many of the inflammatory events caused by gram-negative bacteria can be mimicked by LPS administration, making this a well characterized model to study inflammation [52-54].

LPS is a large molecule composed of three parts: the lipid A, *O*-antigen and core oligosaccharides. Lipid A consists of fatty acids that are embedded in the cell membrane while the rest of molecule projects outward towards the surface. The core oligosaccharides containing various sugars such as heptose are attached to the lipid A. This region of the molecule is partly responsible for stimulating the immune system to fight off infection. The polysaccharide chain

that extends outward from the core polysaccharide is easily recognized by the host and is referred to as the *O*-antigen [51].

LPS induces local and systematic inflammation by forming a complex with LPS-binding protein (LPB) (**Figure 1.6**). The LPS-LPB complex then binds to CD14, a receptor located on monocytic cells. This complex then activates Toll-like receptors that function as signal-transducing components. MyD88, also a Toll-like molecule, binds to this complex. This event is followed by the recruitment and dissociation of inactive receptor-associated kinases resulting in the interaction with tumor necrosis factor receptor associated factor 6 (TRAF6) and the activation of transcription factors namely, nuclear factor- κ B (NF- κ B) [51, 55]. Numerous genes are activated by the transcription factors that encode for cytokines and enzymes leading to an inflammatory response. The macrophage expression and secretion of the cytokines TNF- α and IL-1 β are increased [53, 56-58]. Cyclooxygenase II (COX-II), an enzyme that catalyzes the synthesis of prostaglandins is also upregulated upon the activation of NF- κ B [54, 58-63]. Alternatively, in chronic inflammatory states in which external pathogenic stimuli are absent, TNF- α and IL-1 β have been shown to promote the dissociation of I κ B, rendering NF- κ B active. Therefore the production of these cytokines can further perpetuate the inflammatory responses [64].

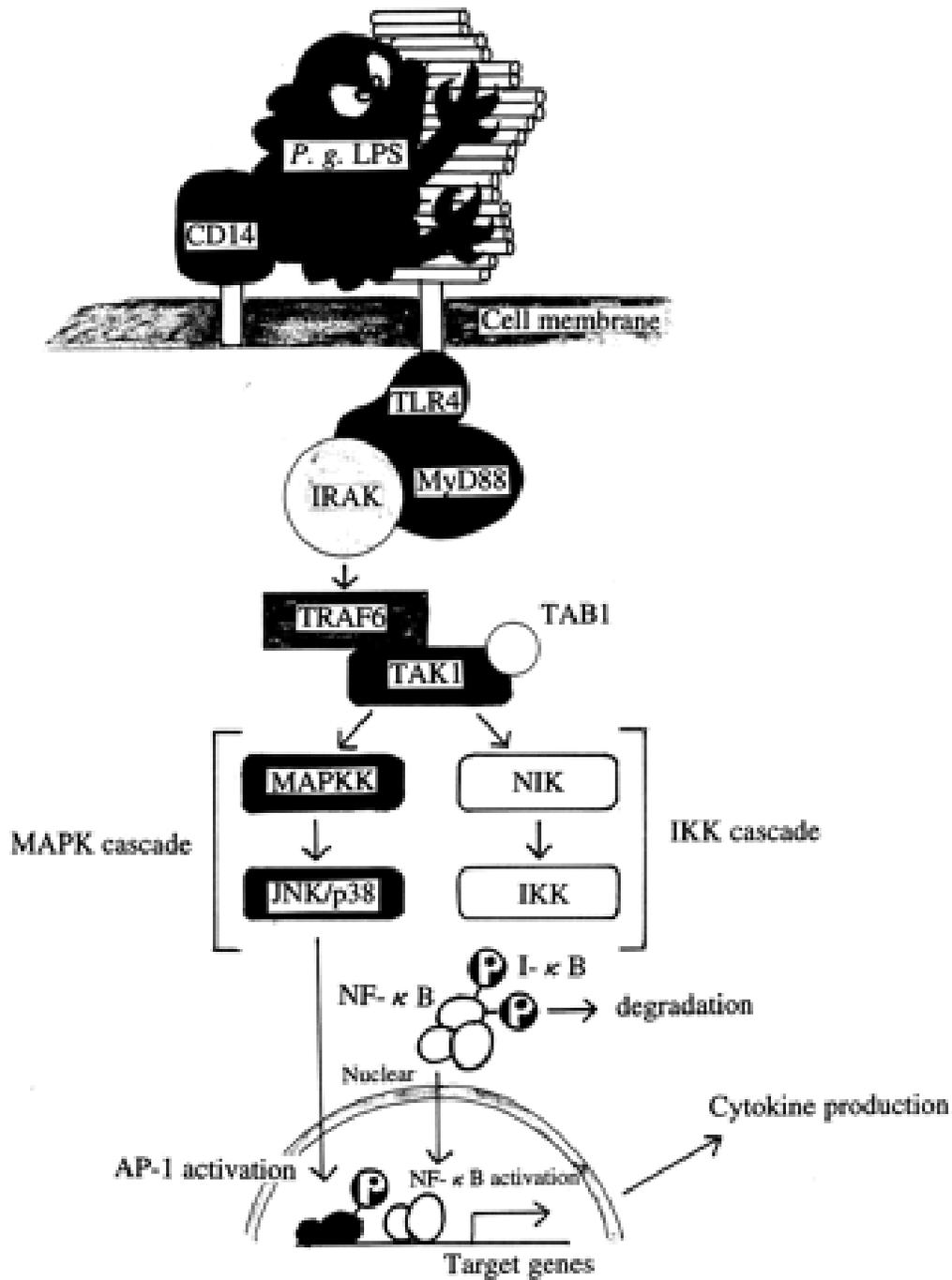


Figure 1.6: Signaling by LPS. Adapted from <http://crobm.iadrjournals.org/cgi/content-nw/full/13/2/132/F6>.

12-O-tetradecanoylphorbol-13-acetate and Inflammation

Mouse ear edema induced by phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) has been widely used as an animal model for testing anti-inflammatory activity. In this model, TPA has been associated with an increase in ear edema and hyperplasia by an increased production of many inflammatory mediators including prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄). These eicosanoids are responsible for recruiting macrophages, neutrophils and other leukocytes ultimately leading the release of histamine and bradykinin, two mediators of inflammation [65, 66]. TPA also stimulates keratinocytes to release cytokines TNF- α and IL-1 β which have been reported to exacerbate edema in the mouse ear [67, 68].

The mechanism of TPA activity is not completely elucidated but is thought to start with the activation of protein kinase C [69]. PKC has been implicated in events that lead to keratinocyte differentiation, epidermal tumor growth and cutaneous inflammation [70]. The PKC pathway is part of a signal transduction system (**Figure 1.7**) that is dependent upon the turnover of phosphatidylinositol bisphosphate (PIP₂). PKC is activated by diacylglycerol (DAG), a second messenger that is formed by the actions of phospholipase C. PKC contains binding sites for both DAG and phorbol esters. Therefore, TPA activates PKC. Activated PKC phosphorylates various cellular enzymes and receptors, such as the nuclear transcription factor NF- κ B [71, 72].

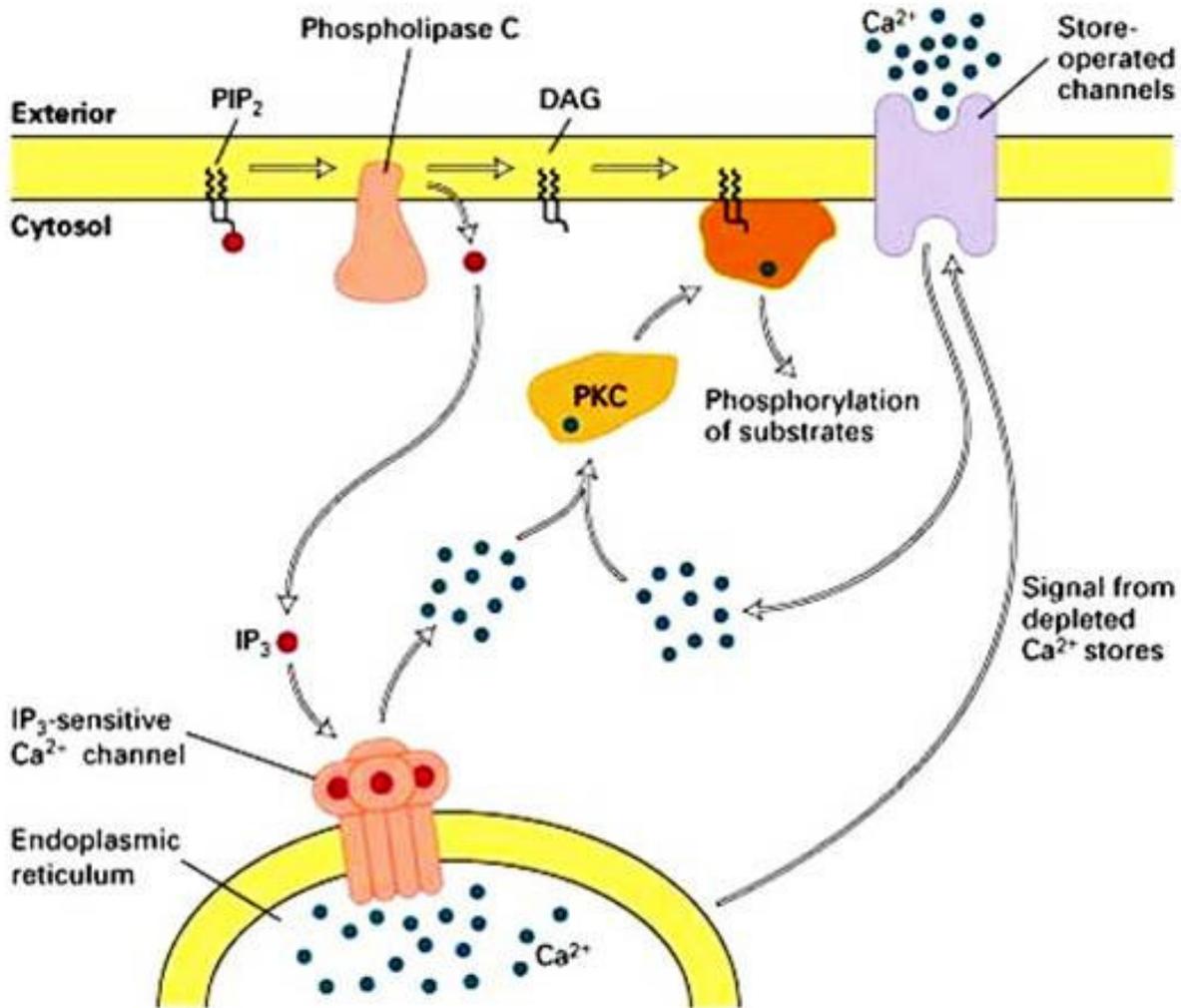


Figure 1.7: Signaling pathway of protein kinase C.

NF-κB

NF-κB, when complexed with its inhibitory protein IκB, remains in a silent state in the cytosol. Phorbol esters, bacterial toxins, free radicals, ultraviolet radiation and cytokines can phosphorylate IκB, thereby rendering NF-κB active. Following the degradation of IκB, the p50 and p65 subunits of NF-κB translocate to the nucleus and participate in initiating transcription of over 200 genes that have a broad range of functions (**Figure 1.8**) [73]. NF-κB is one of the most pivotal regulators of pro-inflammatory gene expression [73] promoting chemotaxis of

neutrophils [69], cyclooxygenase and lipoxygenase induction [74, 75] and pro-inflammatory cytokine production (IL-1, TNF- α , IL-6) [67]. The activation of NF- κ B has been associated with cancer, autoimmune diseases, atherosclerosis, diabetes and other inflammatory diseases [76-81].

Inflammatory mediators such as prostaglandins and leukotrienes are synthesized during the metabolism of arachidonic acid (AA) by cyclooxygenase (COX) and lipoxygenase (LOX) enzyme pathways. These mediators promote inflammation by recruiting macrophages, neutrophils, and other leukocytes that release histamine and bradykinins [83].

AA is a 20-carbon fatty acid that is localized in cellular membrane phospholipids. Phospholipase A₂ catalyzes the release of AA from the membrane phospholipids. This reaction can be activated by the phosphorylation of phospholipase A₂ which is regulated by PKC α [82], cytokines [83] and by nuclear transcription factors such as NF- κ B [75, 84-86]. The subsequent release of AA from the phospholipids pools allows for AA to be metabolized by either the COX pathway or the LOX pathway (**Figure 1.9**).

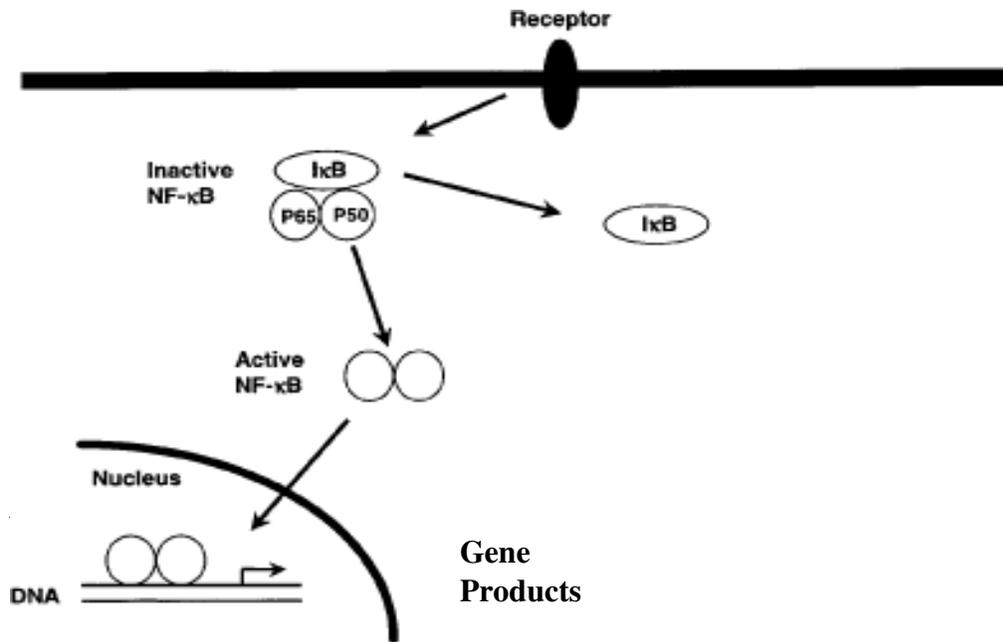


Figure1. 8: Activation of NF-κB pathway from www.biochemsoctrans.org

Prostaglandins and leukotrienes affect the initial responses to inflammation by increasing vasodilatation (redness) and vascular permeability (swelling) [87]. LTB₄ promotes neutrophil chemotaxis and PGE₂ can increase free intracellular calcium resulting in keratinocyte differentiation and vascular permeability changes produced by histamine and bradykinin [85]. Prostaglandins and leukotrienes play a large role in inflammation. The topical application of prostaglandins and leukotrienes, specifically PGE₂ and LTC₄ abolished the anti-edematous effects of indomethacin and zileuton, respectively, in AA-injured mouse ears. The loss of the protective effects of indomethacin and zileuton by PGE₂ and LTC₄ application resulted in ear edema similar to the TPA values [88].

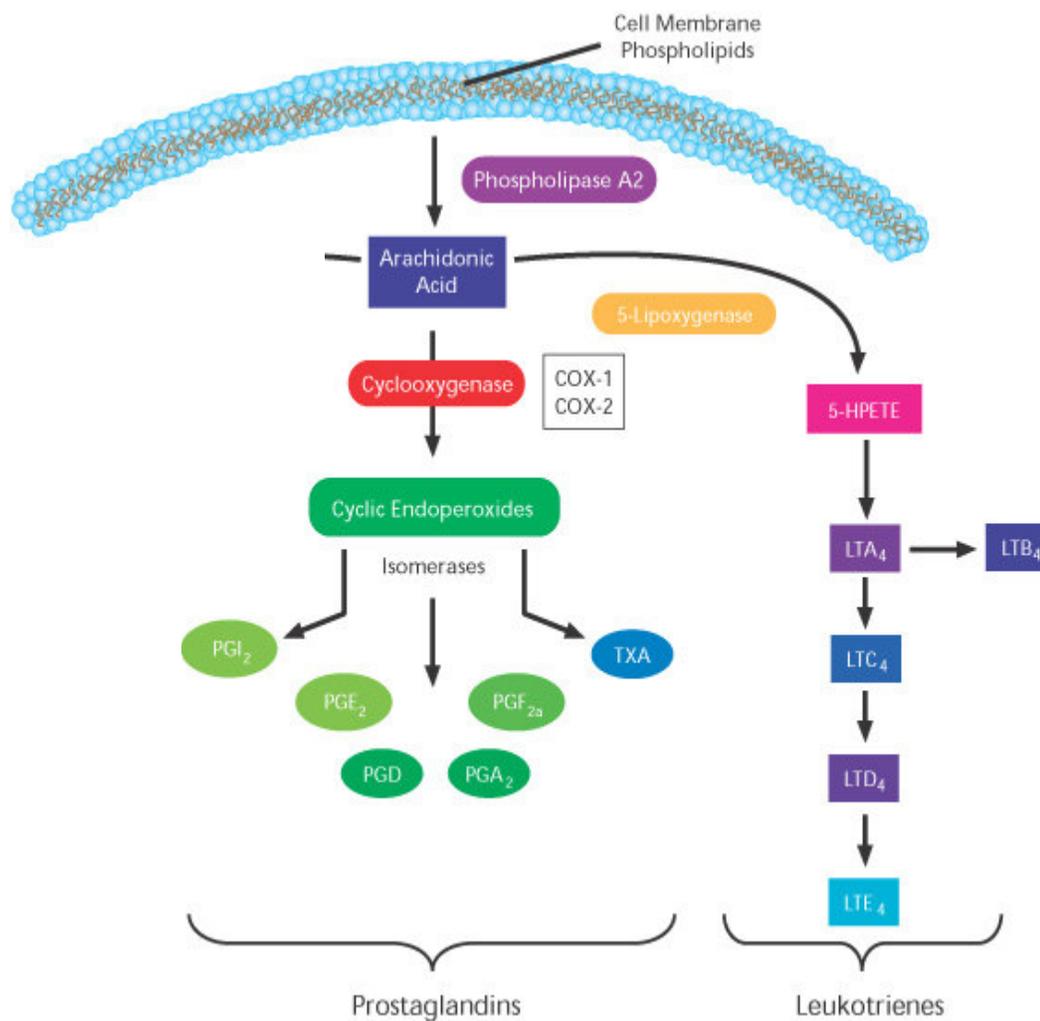


Figure 1.9: Pathway of prostaglandin and leukotriene synthesis from www.sigmaaldrich.com

Pharmaceutical Agents and Inflammation

Non-steroidal anti-inflammatory drugs (NSAIDs; e.g. aspirin, ibuprofen, and indomethacin) are pharmaceutical agents widely used in the treatment of inflammation and management of pain. NSAIDs decrease prostaglandin synthesis by inhibiting the COX enzymes. Two isoforms of COX have been identified. The constitutively expressed COX-1 enzyme is a housekeeping enzyme that plays a critical role in the protection of the gastric mucosa and platelet aggregation [85, 87]. COX-2 enzyme is induced by a number of stimuli including cytokines,

tumor promoters, carcinogens and endotoxins [89]. Aspirin acetylates and inactivates COX activity in an irreversible manner, whereas all other members of the NSAID class act by competitive inhibition at the active site. As a consequence of non-selective COX inhibition, NSAIDs cause toxic side effects including gastrointestinal effects (gastric bleeding, abdominal pain, and hemorrhages) and renal toxicities [90]. These effects are due largely to the inhibition of the housekeeping enzyme, COX-1.

A newer generation of NSAID drugs are the selective COX-2 enzyme inhibitors. These pharmaceutical agents have been called the coxibs (e.g. celecoxib and rofecoxib) and are used clinically for the management of arthritis and pain. It was thought that the select inhibition of COX-2 enzyme and the preservation of COX-1 activity would decrease many of the toxicities encountered with NSAID usage. Although coxibs decrease gastrointestinal toxicities, they also suppressed the production of prostacyclin, an inhibitor of platelet aggregation. In addition, the balance of prostanoid synthesis then favors the formation of eicosanoids produced by COX-1 such as pro-thrombotic thromboxanes. These imbalances due to selective COX-2 inhibition may explain the increase in cardiovascular complications in many patients taking coxibs. Subsequently coxibs have been pulled off the market [87, 91]. Other classes of anti-inflammatory agents such as corticosteroids and disease modifying anti-rheumatic agents, all produce adverse side effects.

Natural Agents and Inflammation

Phenolic acids have also been shown to be anti-inflammatory agents but are not associated with many of the side effects of pharmaceutical agents. Phenolics are capable of changing gene expression and inhibiting both enzyme expression and enzyme activity. Protocatechuic [70] and ferulic acids [92] inhibited PKC activity in an experimental model of

TPA-induced inflammation by gene expression. Flavones such as apigenin [41] have been shown to inhibit the activation of NF- κ B in LPS-activated macrophages, thereby suppressing the promotion of the COX-2 gene. Delphinidin, an anthocyanin, inhibited NF- κ B activation by blocking LPS-induced I κ B- α degradation and p65 translocation to the nucleus [93]. In LPS-activated macrophages, gallic acid [94], ferulic acid [95], and catechin [94] significantly inhibited the production of TNF- α . Apigenin, luteolin, kaempferol and naringenin were all found to inhibit the release of AA from rat neutrophil membranes [96]. Gallic acid [94] and derivatives of ferulic acid [97] have been shown to inhibit the activity of the COX-2 enzyme. The transcription of COX-2 was down-regulated by both kaempferol and cyanidin in LPS-activated macrophages [41]. Each of these phytochemicals are known constituents in the bran fraction of many varieties of sorghum.

CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is the number one cause of death in the United States. It is estimated that every 26 seconds an American will suffer from a coronary event. CVD also comes with a high price tag putting a major financial strain on many Americans. It is estimated that nearly \$432 billion dollars will be spent on healthcare or loss of productivity as a result of CVD in 2007 [98]. CVD is a broad term that describes many different diseases that affect the heart and/or arteries such as coronary artery disease, heart attack, heart failure, high blood pressure and stroke. The pathology of many of these diseases is believed to involve inflammation. There are many risk factors to CVD such as cigarette smoking, family history, hypercholesterolemia, diabetes, hypertension, age and obesity [99].

Pathogenesis of Cardiovascular Disease

Atherosclerosis is a disease affecting the arteries in which plaque composed of a fatty/wax-like substances is deposited on the sides of the arterial walls. The building of the plaque subsequently results in the narrowing of the arterials over time. This narrowing causes blood flow to become abnormally turbulent. A heart attack or stroke can occur when part of the plaque breaks off or a blood clot forms, at the site of a plaque and block blood flow. The formation of a plaque can start at a very young age and can progressively worsen in individuals in their 30s [100].

While the exact etiology of atherosclerosis is unknown, cholesterol in the atherosclerotic plaque is derived from circulating blood lipoproteins. There is an association between high plasma cholesterol levels and increased CVD risks [101]. Low-density lipoprotein (LDL) is often referred to as the 'bad cholesterol'. LDL contains a surface monolayer of phospholipids and free cholesterol, and TG and cholesterol esters are located in the core of the particle. Cholesterol and triglycerides are packaged into very-low density lipoproteins (VLDL) and are secreted by liver (**Figure 1.10**). Free fatty acids and intermediate-density lipoproteins (IDL) are yielded from the VLDL through lipolysis. The fatty acids are used primarily for energy production while the IDL is eventually converted to LDL by further loss of triglycerides. It is here that the cholesterol-rich LDL particle has two fates: 1) uptake by cells in the periphery or 2) uptake by hepatocytes via the LDL-receptor (LDLR) [102].

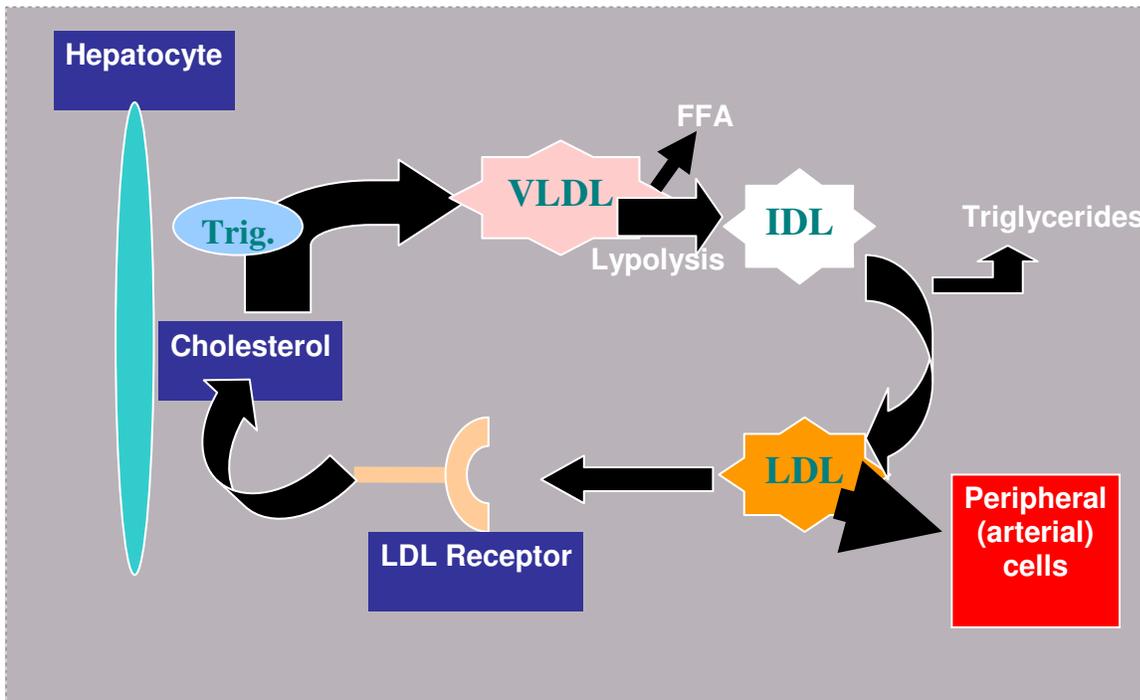


Figure 1.10: Overview of lipoprotein metabolism.

Once the LDL particles move from the plasma to the arterial walls, they can become modified and/or oxidized. The oxidized LDL is highly atherogenic [103] and subendothelium space (**Figure 1.11**). Modified LDL then promotes recruitment of monocytes from the blood to the artery walls and into the endothelium by activating the expression monocyte chemotatic protein-1 (MCP-1) on the surface of endothelial cells. Once in the subendothelial space, modified LDL stimulates monocytes to differentiate into macrophages. The macrophages also express and recruit numerous cytokines including TNF- α and IL-1 which are responsible for activating endothelial cells to express adhesion molecules (i.e. intercellular cell adhesion molecule and vascular cell adhesion molecule). This viscous cycle promotes accelerates the progression of atherosclerosis [102]. Macrophages aggressively endocytose modified LDL via scavenger receptors resulting in the formation of foam cells. These foam cells occur in growing atherosclerotic plaques. Foam cells also release growth factors and metalloproteinases, resulting

in matrix degradation and cell proliferation. As macrophages and foam cells accumulate, they develop into fibro-fatty plaques in human arteries and progresses into atherosclerotic lesions that have the potential to rupture [104] and precipitate myocardial infarctions and strokes.

High-density lipoprotein (HDL) is often referred to as the ‘good cholesterol’ due to its anti-atherogenic activities. This particle plays a role in removing excess lipids from the vascular walls and foam cells, inhibiting MCP-1 and adhesion molecule expression, promoting the efflux of cholesterol from foam cells, and protecting LDL from modifications [102].

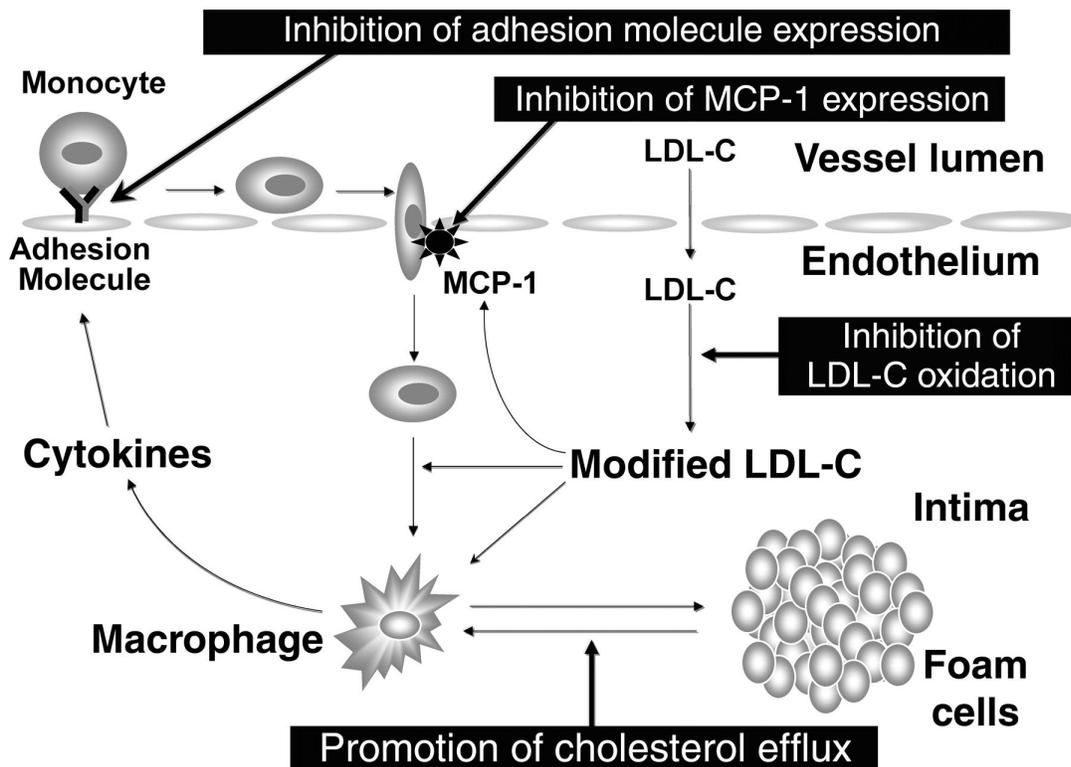


Figure 1.11: Steps in atherogenesis. Picture adapted from www.lipidsonline.gov.

For these reasons, the strategy of many lipid altering pharmaceuticals focuses on lowering plasma LDL and/or increasing plasma HDL concentrations to decrease risks of CVD.

The National Cholesterol Education Program, Adult Treatment Panel III (NCEP ATP III) has suggested an optimal target value of LDL cholesterol should be less than 100 mg/dl, total cholesterol less than 200 mg/dl, and HDL cholesterol should be higher than 40 mg/dl to lower the risk of CVD [105].

Lipoprotein levels are not the only cardiovascular disease risk markers. Several other biomarkers that serve as indicators of high cardiovascular risks include plasma levels of homocysteine, fibrinogen and C-reactive protein (CRP). High levels of homocysteine, an amino acid intermediate in methionine metabolism, are known to damage endothelial cells, promote artery damage and increase potential for abnormal blood clotting. One study found that men with high levels of homocysteine were three times more likely to have a heart attack [106]. Fibrinogen, a protein involved in blood clotting, has been associated with artery disease by restricting blood flow, accelerating plaque deposits, and promoting damage to arteries [107, 108]. CRP, a marker for systemic inflammation, is a strong predictor of heart attacks and strokes demonstrating the relation between atherosclerosis and inflammation [109]. CRP was found to be one of the best markers for future cardiac events in a study that evaluated over 28,000 women. Women with the highest CRP levels had a four-fold risk of experiencing a cardiac event in contrast to the women with lowest levels of this protein [110].

Hamster Model of Hypercholesterolemia

Hamsters are a widely used animal in experiment models of hypercholesterolemia. Bile acid excretion is not altered in response to dietary cholesterol in this animal [111]. Ultimately, hepatic LDLR activity is reduced resulting in increased plasma cholesterol. These responses in the hamster mimic that seen in some humans in which the downregulation of cholesterol synthesis and increased bile acid secretion are unable to prevent increases in plasma cholesterol.

A diet enriched in coconut oil and cholesterol will induce hypercholesterolemia in hamsters [112-117]. Coconut oil is composed of 92% saturated fats and of this nearly 44% is lauric acid, 17% myristic acid and 8% palmitic acid [118]. These fatty acids have been shown experimentally to decrease LDLR activity and mRNA expression, thereby increasing plasma LDL cholesterol concentrations in hamsters [118-121]. This model has been used to test the efficacy of many cholesterol-lowering pharmaceuticals such as statins [122].

Pharmaceutical Agents Used to Treat Hypercholesterolemia

While hypercholesterolemia is associated with CVD, there are pharmaceutical agents available that lower cholesterol levels. The statins are one class of lipid altering agents that inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), the rate limiting step of cholesterol synthesis. Less cholesterol is packaged into VLDL and secreted from the liver. Since VLDL is a precursor to LDL, blood concentrations of LDL are decreased. A homeostatic response of the liver to decreased cholesterol synthesis is to increase hepatic LDL receptors thereby clearing more LDL cholesterol from the bloodstream [123]. Statins have been shown to lower LDL cholesterol concentrations by more than 50% [124], blood triglyceride levels [125], and CVD morbidity [126]. Statin drugs include lovastatin, rosuvastatin, atorvastatin, simvastatin, and pravastatin. Although statin therapy is one of the most popular cholesterol-lowering avenues in the management of hyperlipidemia, there are many side effects. Statin therapy can result in liver toxicities which are detected by measuring transaminase levels. Therefore, monitoring of transaminase levels is recommended to those undergoing statin therapy. Rhabdomyolysis, a serious muscle/kidney toxicity has been documented with statin usage. Additionally, patients complain of muscle aches and tenderness, [127] memory loss and alterations in behavior [128].

Bile acids are metabolites of cholesterol that are secreted by the liver into the bile. Once bile acids are secreted into the intestine, nearly 95% are reabsorbed into circulation and returned to the liver for reutilization. Bile acid resins bind to bile acids in the intestine preventing their reabsorption, and thereby promoting their excretion in the feces. In response to lower concentrations of bile acids, the liver upregulates HMG-CoA reductase and 7- α -hydroxylase, the rate-limiting enzyme in bile acid synthesis. The conversion of more cholesterol into bile acids then promotes increased expression of LDLR pulling LDL out of the plasma to replenish hepatic cholesterol stores [123]. However, the upregulation of HMG-CoA reductase increases cholesterol synthesis resulting in an increase in triglyceride levels which is counter productive [129]. Bile acid resins have been shown to effectively lower plasma LDL and can slightly increase plasma HDL concentrations [130-132]. Unfortunately, the use of bile acid resins is limited by their high incidence of adverse gastrointestinal effects [133] and their ability to block the absorption of many commonly used drugs [123]. Bile acid resins are often used in combination with other lipid altering agents such as statins [130].

Ezetimibe is a pharmaceutical agent approved by the U.S. Food and Drug Association in 2002 to lower blood lipid levels by blocking the absorption of both hepatic excreted biliary cholesterol and dietary cholesterol in the intestine without inhibiting the absorption of other fats. It is thought that the site of inhibition occurs at the tips of the intestinal villi [134]. Ezetimibe has been shown to effectively decrease plasma LDL concentration by 18% when used in monotherapy [135]. Often, ezetimibe is used in combination with other cholesterol-lowering agents such as the statins. A further decrease (10-20%) in plasma LDL is observed when used with statin therapy. There are no significant adverse effects compared to placebo with ezetimibe treatment [136].

Although fibric acid derivative or fibrates do not produce favorable reduction in LDL cholesterol they have been shown to effectively reduce plasma triglycerides, increase plasma HDL levels and stimulate bile acid secretion. Fibrates appear to facilitate these effects by activating of transcription factors known as peroxisome proliferators activated receptors (PPAR), specifically PPAR α , which is expressed in the liver. PPARs modulate the transcription of many genes involved in lipoprotein metabolism through peroxisome proliferator response elements. Additionally, plasma fibrinogen levels are significantly reduced by 25% with fibrate therapy. The mechanism of this response still needs to be elucidated. Fibrates can produce adverse gastrointestinal effects such as nausea and diarrhea and liver toxicity. The formation of gallstones can occur with the long-term usage of fibrates [137]

Natural Agents Used to Treat Hypercholesterolemia

Dietary intervention is an important means of treating hypercholesterolemia. A diet low in saturated fat and high in fiber is currently emphasized in reducing the risk of CVD. However, other dietary components may have equivalent or larger effects on lipid profiles without the many disadvantages associated with pharmaceutical agents, including the expensive costs and adverse side effects. Natural compounds that decrease cholesterol when incorporated in a daily food product may contribute to a reduction in the risk of CVD.

Niacin is a B vitamin that lowers plasma LDL cholesterol up to 25%, significantly lowers plasma triglycerides and increases plasma HDL levels up to 35% and is as efficacious as prescription drugs. Cholesterol is synthesized, packaged in VLDL and secreted by the liver. Niacin produces these lipid-lowering effects by blocking the secretion of VLDL from the liver [138]. More recently, it has been shown that niacin has a high affinity for a receptor that is highly expressed in adipose tissues, GPR109A. The alterations in the plasma lipid profiles may

be mediated through GPR109A receptor but the exact mechanism remains to be elucidated [139]. The usage of niacin in the treatment of hypercholesterolemia is limited due to its low patient compliance. Many people taking niacin complain of a burning sensation and flushing [123]. Slow release forms of niacin maintenance have fewer side effects [123].

The cholesterol-lowering capabilities of plant sterols have been recognized since the late 1950s. Plant sterols and stanols are collectively referred to as phytosterols or phytostanols [140]. For dietary cholesterol to be absorbed in the GI tract, it must be incorporated into micelles which partition cholesterol from the oil phase to the aqueous micellar phase. The chemical structure of phytosterols is similar to that of cholesterol allowing for the phytosterols to compete with cholesterol in this process. The affinity of phytosterols in micelles is greater than that of cholesterol. The reduction in cholesterol absorption with the GI tract increases hepatic LDLR activity thereby decreasing plasma LDL by 10% with the consumption of 2 g per day of phytosterols [123, 140, 141].

Policosanol is a mixture of very long chain fatty alcohols isolated from sugar cane wax (*Saccharum officinarum L.*) that has been incorporated into Bayer's One-A-Day® Cholesterol Plus™ and many other products to help maintain healthy cholesterol levels. The very long chain fatty alcohols must first be metabolized to very long chain fatty acids to exert their effects. The bioavailability of the very long chain fatty alcohols is low; therefore, to increase bioavailability, these constituents must be metabolized to very long chain fatty acids [11]. For metabolism in gut to occur, chylomicrons deliver the very long chain fatty alcohols to the liver where they enter the endoplasmic reticulum and undergo oxidative metabolized to their acid counterparts [142]. The acid then undergoes β -oxidation in the peroxisome [11]. Studies show that policosanol produces beneficial changes in plasma LDL, plasma triglycerides, plasma HDL and total

cholesterol concentrations in both experimental animals [143, 144] and humans [145-147]. It is believed that policosanol mediates these changes by inhibiting cholesterol biosynthesis. Although policosanol does not directly inhibit HMG-CoA reductase, the key enzyme involved in cholesterol biosynthesis, it may occur at a step between acetate utilization and mevalonate formation [148]. Additionally, policosanol may increase the uptake of LDL by the LDL-receptor, consequently lowering the concentration of circulating LDL [149]. However, these mechanisms have never been elucidated. In contrast, many recent studies undertaken to investigate the hypocholesterolemic actions of policosanol failed to produce any significant effects on plasma levels [150-157]. The controversy surrounding policosanol as an effective lipid-lowering supplement is discussed in further detail in later chapters of this dissertation.

Turmeric (*Curcuma longa*) is a member of the ginger family. Turmeric is traditionally powdered and used as a major ingredient in curry powder. Nearly 5% of turmeric is composed of curcumin, an orange-yellowish oil that is believed to be one active component in turmeric. Turmeric has many pharmacological activities [158]. Turmeric prevents the development of atherosclerosis by: (1) producing cholesterol lowering effects [159], (2) blocking platelet aggregation, and (3) inhibiting LDL oxidation [160]. Curcumin is believed to reduce plasma LDL and triglycerides by significantly increasing the activity of cholesterol 7- α -hydroxylase, the rate-limiting enzyme involved in bile acid synthesis. Thus, the rate of cholesterol catabolism is increased [161, 162]. LDLR mRNA expression is dose-dependently increased in HepG2 cells treated with curcumin. These results suggest that curcumin may decrease plasma LDL by increasing the amount of LDL cleared from the plasma [163].

Spirulina is a dried form of the nutrient-rich blue-green alga or cyanobacterium. The U.S. Food and Drug Administration in 2003 approved *Spirulina* as a whole food in 2003.

Traditionally, *Spirulina* has been used as food dating back to the Aztec civilization [164]. *Spirulina* has many therapeutic effects such as anti-viral, immunomodulatory, anti-cancer, anti-diabetic, and hypocholesterolemic effects [165]. Consumption of *Spirulina* in rats reduces plasma LDL, plasma VLDL, and total cholesterol concentrations [164]. *Spirulina* contains a high chlorophyll content which may be responsible for its pharmacological actions. Chlorophyll is metabolized to phytanic (3,7,11,15-tetramethylhexadecanoic) acid, an isoprenoid-derived fatty acid [166]. This fatty acid has been shown to have a high affinity for the nuclear transcription factor, PPAR- α [167]. The beneficial lipoprotein effects have been shown to be mediated via the activation of PPAR- α by stimulating lipoprotein lipases; subsequently the hydrolysis of VLDL triglycerides is increased [168]. Therefore, the ligand-binding properties of phytanic acid to PPAR may be responsible for *Spirulina*'s hypocholesterolemic actions.

Pantetheine is a major component and precursor of coenzyme A and is further metabolized to pantothenic acid and cysteamine. Pantethine, the stable form of pantetheine, has produced hypolipidemic activities in both experimental animals [169, 170] and humans [171]. In humans, a significant reduction in plasma lipoproteins was observed which was accompanied by an increase in plasma HDL concentrations [171]. Pantethine when added to human skin fibroblast cells decreased cholesterol synthesis by 80% [172]. This inhibition occurred at the steps between the conversions of lanosterol to cholesterol [172]. Additionally, pantethine decreased fatty acid synthesis by 50% and decreased oxidation of plasma LDL [173].

HYPOTHESES TESTED

In Chapter Two the following hypothesis was tested:

Sorghum bran of several varieties will inhibit acute inflammation associated with the topical application of phorbol myristate, a potent inflammatory tumor promoter, to mouse ears. It is

anticipated that highest phenolic acid and antioxidant capacity brans will have the greatest anti-inflammatory activity and certain sorghum brans will be more efficacious inhibitors than wheat, rice and oat brans.

In Chapter Three the following hypothesis was tested:

The very long chain fatty acids, alcohols and aldehydes in the wax fraction of sorghum will produce cholesterol-lowering effects when integrated into the diets of hypercholesterolemic hamsters. In addition, the consumption of sorghum bran will decrease plasma cholesterol concentrations in hamsters consuming a high fat diet.

In Chapter Four the following hypothesis was tested:

The combination of four natural components (a blend of phytosterols, turmeric, *Spirulina*, and pantethine) into the diets of hypercholesterolemic hamsters will reduce plasma cholesterol to a greater extent than previously reported for each agent alone. The formulation will produce additive pharmacological effects allowing for the administration of significantly smaller doses than previously documented. The integration of sorghum bran into the cholesterol-lowering formulation will produce a further decrease in plasma cholesterol concentrations in animals fed a high fat diet.

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CHAPTER TWO

ANTI-INFLAMMATORY EFFECTS OF SELECT VARIETIES OF SORGHUM BRAN EXTRACTS¹

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ABSTRACT

The bran fractions of certain varieties of sorghum (*Sorghum bicolor*) grain are rich sources of phytochemicals and antioxidants. In this paper, the anti-inflammatory actions of extracts of select sorghum brans were evaluated in two experimental inflammatory systems: 1) the release of cytokines by lipopolysaccharide (LPS)-activated peripheral blood mononuclear cells, and 2) 12-O-tetradecanoylphorbol acetate (TPA) ear edema in mice. A 1:100 dilution of an ethanol extract of black sorghum bran significantly inhibited the secretion of pro-inflammatory cytokines IL-1 β and TNF- α . Black and sumac varieties of sorghum bran significantly reduced edema in inflamed ears as measured by ear thickness and ear punch weight 6 h following TPA application. The degree of inhibition was similar to that observed with indomethacin. Black sorghum bran produced a significant inhibition of myeloperoxidase (MPO) activity 24 h following the application of TPA. No anti-inflammatory activity was observed with white and mycogen sorghum bran varieties or with oat, wheat or rice brans. The anti-inflammatory activity correlated with the phenolic content and antioxidant activity in the ear model. Furthermore, the combination of black sorghum bran extract and a low dose of indomethacin produced an additive effect in reducing the edema in TPA-treated ears, suggesting that the mechanism of action of the bran extract may be similar to indomethacin. Gene expression of the COX-2 enzyme was not altered in the TPA-injured ears by sumac and black sorghum bran varieties. These results demonstrate that select sorghum bran varieties possess potent anti-inflammatory actions in both LPS-activated cells and in the topical TPA-ear model.

INTRODUCTION

Sorghum grain, one of the world's five most important grains along with maize, wheat, rice and barley, has been a dietary staple for millenia in parts of India, Africa, and China [1]. Some sorghum varieties have extremely high contents of phenolic compounds that aid in the natural defense of plants against pests and diseases. These phytochemicals, primarily located in the bran fraction, result in the plant having significant antioxidant properties [2, 3]. These antioxidant compounds fall into three major categories: phenolic acids, flavonoids and tannins. The phenolic acids are divided among two classes: benzoic and cinnamic acid derivatives, whereas the tannins are largely condensed tannins or proanthocyanidins. The major flavonoids in sorghum grain are 3-deoxyanthocyanins, including apigenidin and luteolinidin. The phytochemical content of specific varieties of sorghum bran is much higher than most widely cultivated grains [4-6]. Flavonoids have been shown to reduce inflammation in many experimental models [7-9]. However, the effect of sorghum on inflammation has not been examined.

Tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) are two pro-inflammatory cytokines that have been implicated in the pathogenesis of many inflammatory diseases [10-12]. The progression of rheumatoid arthritis, inflammatory bowel disease and sepsis syndrome have been linked to the dysfunction of cytokine regulation, resulting in the alteration in the balance of pro- and anti-inflammatory cytokines predominately favoring a pro-inflammatory state [10]. In addition, pro-inflammatory cytokines play a major role in mediating other inflammatory events such as the induction of cyclooxygenase-2 (COX-2), an enzyme that is responsible for the edema and vasodilatation associated with inflammation [13]. The dermal administration of TPA, a

phorbol ester, to mouse ears is a well characterized model that has been shown to elicit an inflammatory response mediated via the upregulation of COX-2 expression, resulting in increased vascular permeability and edema [14, 15]. The results presented demonstrate that certain varieties of sorghum brans possess significant anti-inflammatory activity.

The present study tested anti-inflammatory effects of ethanolic extracts of sorghum bran varieties using an *in vitro* cell culture model and an *in vivo* animal model. The purposes of this study were: (i) to examine the inhibition of the release of pro-inflammatory cytokines using lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells and (ii) to test the ability of sorghum bran extracts to inhibit inflammation, edema, and polymorphonuclear (PMN) leukocyte infiltration in the mouse ear following topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA).

METHODS

Materials

12-O-tetradecanoylphorbol-13-acetate (TPA), indomethacin, hexadecyltrimethylammonium bromide, 3,3',5,5'-tetramethylbenzidine dihydrochloride, gallic acid, and 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) and vanillin, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 culture media was purchased from GIBCO (Grand Island, NY), Ficoll-Hyaque was obtained from ICN Biomedicals, Inc. (Aurora, OH), and enzyme-linked immunosorbent assay (ELISA) kits for interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) were products of Biosource International (Camarillo, CA). Laemmli buffer and non-fat dried milk were purchased from BioRad (Hercules, CA).

Animals

Male Swiss Webster mice (Charles River Laboratory; Wilmington, MA) weighing 20-24 g were housed in polycarbonate cages with Tek-Fresh bedding. The hamsters were kept in an environmentally controlled room maintained at 21°C with a 12:12-hr light:dark cycle. The animals were allowed free access to rodent chow (Purina Lab Diet 5001, Ralston Purina, St Louis, MO, USA) and water throughout the duration of the experiment. All experimental procedures were approved by the Animal Care and Use Committee at the University of Georgia.

Preparation of Sorghum Extracts

To extract phytochemicals, the brans were diluted with 50% ethanol (1:4, wt:vol) for 1 hour at room temperature with constant vortexing. The extract was then centrifuged at 2500 rpm for 20 min to remove the precipitate and filter sterilized to obtain the final extract.

HPLC Analysis of Sorghum Bran Flavonoids

For the HPLC fingerprint analysis of phenolic compounds [16] present in 50% ethanolic extracts, a Agilent Technologies 1200 system and a prepacked LUNA C₁₈ column (4 x 150 mm, 5 µm, Phenomenex) was employed. The column was eluted in a gradient of acetonitrile-water-acetic acid (5:93:2, v/v/v) [solvent A] in 30 min and acetonitrile-water-acetic acid (40:58:2, v/v/v) [solvent B] running from 0-30 min with solvent B going from 0 to 100% at 1 mL/min. The extracts were initially dissolved in methanol at 5 mg/mL and the injection vol was 10 µL. Detection of the sorghum bran flavonoids was performed at 360 nm.

Total Phenolic Content

Total phenolic content of each sample was assayed in triplicate using the Folin-Ciocalteu method as described by Singleton [17]. In a polystyrene cuvette, 20 µL of each diluted sample, 1.58 mL of distilled water, and 100 µL of Folin-Ciocalteu reagent were added and mixed well.

After 10 min, 300 μ L of sodium carbonate solution was added. After the 2 h incubation at room temperature, the absorbance was measured at 765 nm in a Beckman DU 600 series spectrophotometer. Gallic acid was employed as the standard. Results are expressed as mg of gallic acid equivalent (GAE) per g of bran.

Ferric Reducing Antioxidant Power (FRAP)

FRAP values were determined in triplicate using a modified Benzie and Strain method [18]. 10 μ L of each sample, 30 μ L distilled water, and 300 μ L of FRAP reagent were added to a cuvette. The FRAP reagent was prepared with 25 mL of 300 mM acetate buffer pH 3.6, 2.5 mL of 10 mM 2,4,6-tri[2-pyridyl]-s-triazine solution (TPTZ) dissolved in 40mM HCl, and 2.5 mL of 20 mM ferric chloride solution. After 6 min at 37°C, 340 μ L of distilled water was added to the cuvette and absorbance was measured at 593 nm in a Beckman DU 600 series spectrophotometer. The results are expressed as mmoles of ferrous sulfate formed per 100 g of dry bran.

Vanillin Assay for Tannins

The tannin concentration was determined for each sorghum bran extract variety in triplicate following a modified version of the vanillin-HCL method [19]. 200 μ L of each sample was added to 2 mL of the vanillin reagent. The vanillin reagent was prepared by mixing 1% vanillin in methanol to 8% concentrated HCL diluted in methanol (1:1 vol:vol) and warmed to 30°C prior to use. The absorbance of the samples was measured at 500 nm in a Beckman DU 600 series spectrophotometer following a 20 min incubation at 30°C. Catechin was employed as the standard and the results are expressed as mg of catechin per g of bran.

Isolation of Human Blood Monocytes

Human blood was collected in heparinized tubes, diluted 2-fold with RPMI-1640 culture media and layered over Ficoll-Hypaque (2.5:7 vol:vol). The mononuclear cells were collected by centrifugation for 20 min at room temperature at 400 g. The cells were washed and cultured with RPMI-1640 culture medium containing 10% fetal calf serum, 50 units/mL of penicillin, streptomycin (50 µg/mL), and 2 mM *L*-glutamine.

Cellular Toxicity of Sorghum Bran Extracts

Human peripheral blood mononuclear cells were cultured for 24 h with various concentrations of black sorghum bran extract in RPMI medium containing 10% fetal calf serum. At the end of the incubation period, propidium iodide was added to a final concentration of 25 µg/mL. A FACScan flow cytometer (Becton Dickinson [BD], San Jose, CA) and was used to determine the percentage of dead cells using WinMDI 2.8 software (<http://facs.scripps.edu/software.html>) [20].

Cellular Release of Pro-inflammatory Cytokines

1×10^5 human peripheral blood mononuclear cells were cultured in round bottom microtiter plates in 0.2 mL of RPMI culture medium containing 1 µg/mL *Escherichia coli* O111:B4 lipopolysaccharide and various concentrations of black sorghum bran extract. After 24 h, the culture supernatants were collected by centrifugation and stored at -70°C. The culture supernatants were thawed and assayed for IL-1β and TNF-α using ELISA commercial kits. A standard curve using recombinant cytokines was included in each assay.

TPA Ear Edema Model

TPA (2 µg/20 µL) was applied to the inner and outer surfaces of the mouse ear for inducing acute-type skin inflammation. 20 min following the TPA-induced injury, a series of

test compounds in 50% ethanol were applied (20 μ L/ear) to the same site. The anti-inflammatory effects of sorghum and other bran extracts (1:4 wt:vol 50% ethanol) were compared to those of indomethacin (0.5 mg/20 μ L acetone), a non-steroidal anti-inflammatory drug. Each experiment included a vehicle control and a treatment group that received TPA and 50% ethanol.

Mice were euthanized 6 h later after TPA treatment. 7-mm diameter sections of the right and left ear were weighed and the thickness was recorded. Ear edema was expressed as the increase in ear thickness due to the inflammatory challenge. Ear thickness was measured before (0 h) and after (6 h) the inflammatory response by using a micrometer (Mitutoya Series IP65, Aurora, IL). To minimize variation due to technique, a single investigator performed the measurements throughout any one experiment.

Myeloperoxidase Activity

MPO activity was used to assess PMN infiltration. Mice were euthanized 24 h after TPA treatment and 7-mm diameter sections of the right and left ear were removed for determination of MPO [21, 22]. The tissue punch was homogenized with a small sample laboratory Tissue Tearor Homogenizer Model 985-370 (Biospec Products, Bartlesville, OK) in 0.75 mL of 80 mM phosphate-buffered saline (PBS) pH 5.4, containing 0.5% hexadecyltrimethylammonium bromide (HTAB) for 45 s on ice. The homogenate was transferred to a microfuge tube and the vessel was washed with 0.75 mL of hexadecyltrimethylammonium bromide in PBS buffer and added to the microfuge tube. The sample was centrifuged at 12,000 g at 4°C for 15 min. 30 μ L of sample was added to a 96-well microtitre plates in triplicate. 200 μ L of a mixture containing 100 μ L of 80 mM PBS pH 5.4, 85 μ L of 0.22 M PBS pH 5.4, and 15 μ L of 0.017% hydrogen peroxide was added to each sample well. The reaction was started with the addition of 200 μ L of

18.4 mM tetramethylbenzidine HCL in 8% aqueous dimethylformamide followed by the incubation of the plates at 37°C. After 3 min, the plates were placed on ice and 30 µL of 1.46 M sodium acetate, pH 3.0 was added to stop the reaction. MPO enzyme activity was assessed colorimetrically using Bio-Tek Microplate Reader (ELx 808) at an absorbance of 630 nm, and expressed as OD/biopsy.

Western Blot Analysis

Ear punch protein extracts were prepared in 10 mM Tris-HCL buffer, pH 7.4 containing 1mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride and homogenized for 45 s. After quantification of protein concentration, lysates containing 40 µg protein were boiled in Laemmli buffer containing β-mercaptoethanol for 10 min before electrophoresis on a 7.5% SDS-polyacrylamide gel (BioRad Laboratories, Hercules, CA). The proteins from the gel were then transferred to a PVDF membrane (BioRad Laboratories) and the blots were blocked in 3% nonfat dry milk PBST buffer (phosphate buffered saline containing 0.1% Tween-20) for 2 h at room temperature. The membrane was incubated for 2 h at room temperature in 3% nonfat dry milk PBST buffer with a 1:1000 dilution of COX-2 antibody (Cayman Chemicals, Ann Arbor, MI). The blots were washed in PBST buffer 4 times each. Washed blots were incubated for 1 hr at room temperature with a 1:7500 dilution of the horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (BioSource, Carlsbad, CA) in 3% nonfat dried milk PBST buffer and washed 4 times for 10 min each with PBST buffer. Levels of expression for COX-2 protein were visualized by treating the blots with an ECL detection kit (Amersham Pharmacia Biotech, Inc.) and exposing them to X-ray film. The image was captured using a BioImager (BioRad Laboratories, model #107-8010) and digitized using Quantity One 1-D Analysis Software (BioRad Laboratories).

Statistical Analysis

All results are reported as the mean \pm SEM. Student's t-test or one-way ANOVA was used to statistically analyze data subsequently followed by Tukey's test to detect significant differences among treatment groups ($P < 0.05$). SigmaStat (SPSS Science, Chicago, IL) was used to perform all statistical analyses.

RESULTS

Polyphenolic content of sorghum brans

The relative polyphenolic acid concentration and FRAP values of sumac, black, white, and mycogen sorghum bran varieties were determined using 1:4 (wt:vol) extraction with 50% ethanol. The phenolic acid concentrations of sumac and black sorghum bran varieties were 20 and 7.5 fold higher than white sorghum bran extract and 8.9 and 3.3 fold higher than mycogen sorghum bran extract, respectively (**Table 2.1**). The FRAP values of sumac and black sorghum bran extracts were higher than white and mycogen sorghum bran extracts at 48.5 ± 0.6 mmol/100g and 15.6 ± 0.4 mmol/ 100 g, respectively, which directly correlated with the high total phenolic compounds present in these fractions. The phenolic acid concentration and FRAP values were also measured for oat, rice and wheat brans (**Table 2.1**) extracted with 50% ethanol at 1:8 (wt:vol) dilutions. These brans were found to be low in phenolic content and antioxidant capacity when compared to sumac and black sorghum bran varieties. The tannin content was determined for each ethanolic extract of sorghum bran. Sumac sorghum bran had a significantly higher concentration of tannins (113 mg/ g) compared to black, white and mycogen sorghum bran extract varieties contained no detectable tannins.

In **Figure 2.1**, the flavonoid profile of the four sorghum bran varieties was analyzed using reverse-phase HPLC. The profiles of the four sorghum bran varieties illustrate the high

flavonoid content in the black bran when compared to the other varieties. The elution profile of black and sumac sorghum brans was compared to 7 phenolic standards. Of the phenolic acids analyzed, esterified ferulic acid was the major non-tannin phenolic compounds in black sorghum followed by esterified caffeic and p-coumaric. Esterified protocatechuic, caffeic and ferulic acids were found to be the predominant compounds in sumac sorghum bran. These results indicate that sumac and black sorghum bran have a higher phenolic acid content and antioxidant power when compared to other types of sorghum or non-sorghum brans.

Cytokine release from mononuclear cells

The anti-inflammatory effects of high phenolic black sorghum bran extract were evaluated using LPS-stimulated peripheral blood mononuclear cells. As illustrated in **Figure 2.2a**, black sorghum bran extract inhibited in a dose-dependent manner the release of pro-inflammatory cytokine, TNF- α . A 1:200 dilution significantly inhibited TNF- α release by 52% and a 1:100 dilution by 84%. A 1:400 dilution did not produce a decrease in cytokine release. Furthermore, black sorghum bran extract had a more profound effect on the release of cytokine IL-1 β at dilutions ranging from 1:400 to 1:100 (**Figure 2.2b**). At a 1:400 dilution, IL-1 β release was significantly inhibited by 30% while a 1:100 dilution produced a near total inhibition of release.

The effects of black sorghum bran extracts on cell viability were also measured to determine if the effects of black sorghum bran on mononuclear cell cytokine secretion were related to cell death. The proportion of dead cells in the untreated and ethanol treated cells was similar to the cells treated with black sorghum bran extract at a dilution of 1:400 (1.7%) and a 1:200 (2.4%). A dose of 1:100 of sorghum bran extract was associated with a 3.3% of cell death.

These results indicate that the reduction of TNF- α and IL-1 β was not a result of toxicity of black sorghum bran.

Effects on TPA-induced mouse ear inflammation

The change in ear thickness from 0 to 6 h mediated by TPA was from 0.005 ± 0.002 mm to 0.24 ± 0.01 mm and ear punch weight increased from 10.5 ± 1.6 mg to 18.6 ± 0.6 mg. As illustrated in **Figure 2.3a**, sumac and black bran extracts applied 20 min following the induction of inflammation using TPA, significantly decreased ear thickness by 63% and 66%.

Furthermore, the weights of the ear punches were reduced by 30% and 32% with black and sumac bran extracts, respectively (**Figure 2.3b**). Similar results are observed when ears were pretreated with black and sumac sorghum bran extracts 20 min prior to TPA application (data not shown). The anti-inflammatory effects of sorghum extracts were then compared with indomethacin, a non-steroidal anti-inflammatory drug. Ear edema and the weight of the ear punches decreased by 45% and 35%, respectively, by treatment with indomethacin following TPA administration (**Figure 2.3a and 2.3b**). This decrease was not significantly different from the anti-inflammatory effect produced by black and sumac sorghum bran extracts.

The effects of various sorghum bran varieties on ear inflammation were examined. White and mycogen sorghum bran extracts applied 20 min following TPA treatment did not effectively decrease ear edema, as measured by ear thickness (**Figure 2.4**). In the same experiment, black and sumac sorghum bran extracts significantly reduced ear thickness by 60% and 57%, respectively. These results demonstrate that anti-inflammatory activity of sorghum bran varieties was observed in only the high phenolic containing brans.

To determine the dose-response effect of black sorghum bran extract, extracts were applied to mouse ears in dilutions ranging from 1:4 to 1:12 (wt:vol 50% ethanol) following TPA

induced inflammation. At a dilution of 1:12, black bran extract did not alter ear thickness (**Figure 2.5**). Black sorghum bran extract at 1:8 significantly reduced ear edema by 31%. A 1:4 dilution further lowered ear edema to 48% of that observed in the TPA treated animals.

The anti-inflammatory activity of non-sorghum brans was examined in the topical TPA-induced inflammatory model. In this experiment all bran dilutions were prepared at 1:8 (wt:vol 50% ethanol). Rice, oat and wheat bran extracts were ineffective in reducing mouse ear edema (**Figure 2.6**). Black sorghum bran significantly reduced the change in ear edema by 32% in this experiment. The results of these experiments demonstrate that the anti-inflammatory activity of grains is rather unique to select sorghum brans.

The effect of black sorghum bran in combination with indomethacin was also examined in the TPA ear mouse model. As illustrated in **Figure 2.7**, a 0.08 mg dose of indomethacin did not significantly decrease the weight of the ear punches (12% decrease) after TPA treatment. In this experiment, a black bran extract (1:4 wt:vol) significantly reduced the weight of the ear punches by 15%. The combination of black sorghum bran extract and 0.08 mg of indomethacin produced an additive effect, significantly decreasing the weights to a greater extent than either treatment alone (**Figure 2.7a**). Since the anti-inflammatory effect of the combination lowered the weight of the ear punches to control levels, the effect of a higher dose combination was examined by measuring ear thickness. In this experiment, the change in ear thickness was also significantly lowered by the application of 0.5 mg of indomethacin (**Figure 2.7b**). However, there was not a further inhibition observed when black bran extract (1:4 wt:vol) was combined with 0.5 mg of indomethacin. The maximal effect was a 50% lowering in inflammation. This appears to represent the maximum inhibition that can occur by these agents in this model.

An additional *in vivo* experiment was undertaken to determine the MPO activity in mouse ear homogenates biopsied 24 h after the application of TPA. A 1:4 dilution of black bran extract applied 20 min after TPA treatment, significantly reduced MPO activity by 70% (**Figure 2.8**); this reduction was similar to the inhibition observed with indomethacin treatment (85%).

Western blotting of COX-2 protein expression was performed on ear biopsies to elucidate the mechanism of action of sorghum treatment. Mouse ear punches were collected 6 h following application of treatment groups, homogenized and probed for COX-2 protein expression. TPA treatment considerably induced the expression COX-2 protein (**Figure 2.9**). COX-2 protein expression, analyzed by digitometry, was not altered by the treatment of black sorghum bran extract and was slightly, but not significantly increased by the application of sumac sorghum bran extract.

DISCUSSION

Sorghum bran extract inhibited the release of TNF- α and IL-1 β in LPS-stimulated peripheral blood mononuclear cells at dilutions ranging from 1:200 to 1:100 and 1:400 to 1:100, respectively. Important processes related to TPA-induced skin inflammatory responses were significantly reduced with the topical application of sumac and black bran extract varieties. Decreases were observed in migration of polymorphonuclear leukocytes to the dermis (24 h) and in acute edema (6h) when black and sumac bran extract were applied following TPA-induced injury. The ear thickness and ear punch weight in the groups treated with black and sumac bran extracts were statistically similar to the ear thickness and ear punch weights of the mouse ears receiving indomethacin treatment. The expression of COX-2, an enzyme that plays a key role in prostanoid biosynthesis, was not affected by black or sumac sorghum bran extracts. Non-

significant reductions in ear edema was observed in the treatment groups receiving bran extracts of white and mycogen sorghum varieties and oat, wheat and rice.

LPS is a major cell wall component of Gram-negative bacteria that orchestrates macrophage pro-inflammatory gene expression [13]. This response begins with LPS forming a complex with LPS-binding protein and binding to the molecule CD14 on the surface of macrophages [23]. This results in the activation of toll-like receptors. The nuclear factor- κ B (NF- κ B) inflammatory cascade [24] is initiated following the activation of the toll-like receptors [25, 26]. NF- κ B appears to be responsible for the transcription, production and secretion of immunoregulators such as TNF- α and IL-1 β ; nitric oxide (NO) a pro-inflammatory radical produced by inducible nitric oxide synthase (iNOS) and prostaglandins, mediators involved in acute inflammation [27, 28]. Pro-inflammatory cytokines TNF- α and IL- β contribute to the pathogenesis of many chronic inflammatory diseases such as arthritis, colitis, and heart disease [10]. Flavonol [29], luteolin [30], vanillin [31] and ferulic acid [32] have been shown to decrease inflammation by either inhibiting the expression of numerous genes or inhibiting the activity of mediators involved in the inflammatory process including adhesion molecules, chemokines, cytokines, and enzymes that control prostaglandin synthesis (COX) [12, 24, 33-35]. The quenching of reactive oxygen species is another mechanism in which inflammation is alleviated in the LPS model. Antioxidants such as quercetin [36] and resveratrol [37] blocked the release of NO by inhibiting NF- κ B activation and expression of iNOS. Black sorghum bran extract inhibited the release of TNF- α and IL-1 β in LPS-stimulated peripheral blood mononuclear cells. These results are similar to that observed with muscadine extracts [33] and garlic powder extracts [38].

The application of TPA to the ear also initiates a series of inflammatory cascades. It is well established that TPA exerts its effects through the activation of protein kinase C with the subsequent activation of NF- κ B. This event is then followed by the stimulation of cytosolic phospholipase A₂ (cPLA₂) resulting in arachidonic acid mobilization via cyclooxygenase (COX) and lipoxygenase (LOX) enzymes and the synthesis of prostaglandins and leukotrienes [39]. These mediators are responsible for the recruitment of macrophages, neutrophils, and other leukocytes that release histamine and bradykinins, thereby promoting inflammation [40]. For example, PGE₂ release from the keratinocytes amplifies vascular permeability changes produced by histamine and bradykinin [40], increases the infiltration of activated neutrophils into the dermis and increases the secretion of cytokines IL-1 and TNF- α from keratinocytes [34]. It is tempting to hypothesize that select sorghum bran extracts are modulating inflammation by decreasing the release of TNF- α and IL-1 from keratinocytes in the inflamed ear. However, the role of TNF- α in this model of inflammation has not received much attention [41]. At the present time, there is insufficient evidence supporting the hypothesis that antioxidants inhibit the release of TNF- α from keratinocytes in the acute phase of TPA-induced inflammation. However, using a four day treatment schedule of TPA, Huang et al [42] demonstrated that black tea theaflavin derivatives did inhibit both edema and the increase in cytokine IL-1 β and IL-6 levels in mouse ears, supporting the possibility that select sorghum brans may inhibit the release of cytokines in ear inflammation, similar to that observed in LPS-stimulated monocytes. It is interesting to note that IL-1 can stimulate prostaglandin synthesis via upregulation of COX-2 and PLA₂ [43].

The inhibition of prostaglandin synthesis can lead to the attenuation of the TPA-induced ear inflammation [8, 44-48]. These biochemical events correlate with the peripheral changes in

the ear. Natural products have been shown to inhibit inflammation associated enzymes and the expression of regulators of inflammation-associated genes [8, 33, 40] in the TPA model of ear inflammation; this model has become an excellent anti-inflammatory screening test for many compounds. Chi et al. [49] reported that wogonin, a flavone significantly alleviated inflammation in a subchronic TPA ear model by reducing inflammatory mRNA expression for COX-2 and TNF- α . The eukaryotic transcription factor, NF- κ B has been known to regulate COX-2 expression and is a critical target for anti-inflammatory agents. Several natural products block the phosphorylation and subsequent degradation of I κ B α , thereby inhibiting NF- κ B translocation to the nucleus and activation of gene expression [50-52]. In addition to gene expression, COX or LOX activity has been shown to be inhibited by certain phenolic acids, resulting in reduction in ear edema [53-56]. However, the efficacy of the phenolics depends directly on its structure [54]. For example, quercetin significantly reduced LOX activity whereas caffeic acid had little effect on this enzyme and inhibited COX instead [54]. Some phytochemicals have reduced ear edema via the inhibition of prostaglandin synthesis but failed to have an effect on COX and LOX activity and gene expression. Instead these natural products are believed to inhibit PLA₂ activity resulting in decreased concentrations of inflammatory mediators [47, 57].

It has previously been reported that COX-2 protein expression is significantly increased 6 h after TPA treatment [45, 58]. In the experiments reported in this communication, the expression levels of COX-2 were also found to be induced. The application of black and sumac sorghum bran varieties, however, had no effect on protein expression. In fact, sumac sorghum bran extracts slightly upregulated COX-2 protein. This could be a result of the tannin activity that has been shown to have pro-inflammatory activity in certain experimental conditions [59,

60]. In a similar manner to sorghum brans, ginkgetin, a bioflavonoid, reduced edema but did not alter COX-2 expression in this animal model [47].

In these studies, black sorghum bran extract in combination with a low dose of indomethacin reduced ear edema in an apparent additive manner. However, a combination of black sorghum extract and a high dose of indomethacin did not produce a greater effect than either treatment alone. Apigenin, luteolin, kaempferol and naringenin, constituents found in sorghum bran, were all found to inhibit the release of AA from rat neutrophil membranes [61] and gallic acid and derivatives of ferulic acid [62, 63] have been shown to inhibit the activity of the COX-2 enzyme. While indomethacin and sorghum bran only produced a 50% reduction in ear edema, curcumin and derivatives of black tea theaflavins effectively reduced ear edema by 99 and 97%, respectively [42, 64] and a topical application of hydrocortisone produced a 92% reduction in ear edema [65]. Since the combination of black sorghum bran and indomethacin only produced a 50% reduction in ear edema, less than achieved by other agents, one likely possibility is that both agents are inhibiting inflammation via the same pathway. This study did not attempt to investigate this intriguing possibility.

These biochemical inflammatory processes result in producing an animal model having specific temporal events. Ear thickness reaches its maximum at 6 h after TPA treatment and moderately decreases at 24 h [45]. The increased ear thickness is characterized by edema, increased vascular permeability and increased swelling in the dermis [45]. 24 h following TPA-induced injury, a maximum influx of neutrophils is observed in the dermis [34] and is quantified by the MPO assay. Neutrophil infiltration is associated with the generation of oxidative stress in the epidermis by releasing hydrogen peroxide and reactive oxygen species. In time glutathione and superoxide dismutase, two enzymes involved in destroying free radicals and reactive oxygen

species are depleted thereby propagating the oxidative state [66]. Flavonoids with high antioxidant capacity have been reported to potently reduce inflammation and the production of hydrogen peroxide in this animal model [67]. Select sorghum bran varieties, such as sumac and black, contain high amounts of phytochemicals and antioxidants [4]. This unusual property is not observed in white and mycogen sorghum brans and in other common brans consumed in the diet such as wheat, oat and rice brans.

Sumac sorghum bran contains a rich source of condensed tannins also known as proanthocyanidins, specifically containing epicatechin and catechin as constituent units [3, 68]. Our laboratory has determined that there are nearly 113 mg of tannins per g of sumac sorghum bran. In contrast, black sorghum bran is not rich in flavonoids (**Figure 2.1**). Black sorghum, low in tannin content, contains anthocyanins, a class of flavonoids [69, 70]. The anthocyanins present in black sorghum are unique to other types of anthocyanins as they do not contain a hydroxyl group on the C-ring and are referred to as 3-deoxyanthocyanins specifically apigeninidin and luteolinidin [3, 4]. Awika et al. [69] has identified and quantified these two anthocyanins using HPLC. Apigeninidin and luteolinidin represented 36-50% of the total anthocyanin content in black sorghum bran. These anthocyanins would appear to constitute the major peaks in the HPLC flavonoid profile (**Figure 2.1**). The antioxidant activity of black sorghum bran is higher than some fruits such as strawberries, plums and blueberries [3]. Although sumac sorghum bran had a much greater phenolic content and antioxidant capacity as black sorghum bran, their effect on inflammation was very similar. Because of the differences in phenolic composition, further investigations of the pharmacological activities of both black and sumac sorghum bran should be pursued.

Low penetration of drugs through the skin can be a major drawback for the development of percutaneous drug delivery. However, in vivo skin penetration studies have shown that topically administered proanthocyanidins can be absorbed through the skin surface and can penetrate into the epidermis and dermis layers of the skin [71, 72]. Though the majority of the proanthocyanidins were distributed on the surface and in the stratum corneum a small percentage were absorbed in the epidermal and dermal layers. The absorption of proanthocyanidins is dependent upon the structure of each tannin specifically the location of carboxyl groups [71]. Phenolic acids that are present in select varieties of sorghum bran such as gallic acid, p-hydroxybenzoic acid, protocatechuic acid, catechin, and vanillin all are reported to rapidly penetrate human skin. Absorption occurred as quickly as a half an hour [73]. These results indicate that phytochemicals such as flavonoids and proanthocyanidins can inhibit TPA-induced inflammation by percutaneous absorption.

Many non-steroidal anti-inflammatory drugs on the market suffer from many drawbacks, mainly the side effects that can occur from the usage of these pharmaceuticals. Therefore, it is desirable to develop agents that possess anti-inflammatory properties devoid of the negative aspects of conventional drug therapy. In this regard, select varieties of sorghum bran may be found to be of value in the treatment of many inflammatory diseases.

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TABLES AND FIGURES

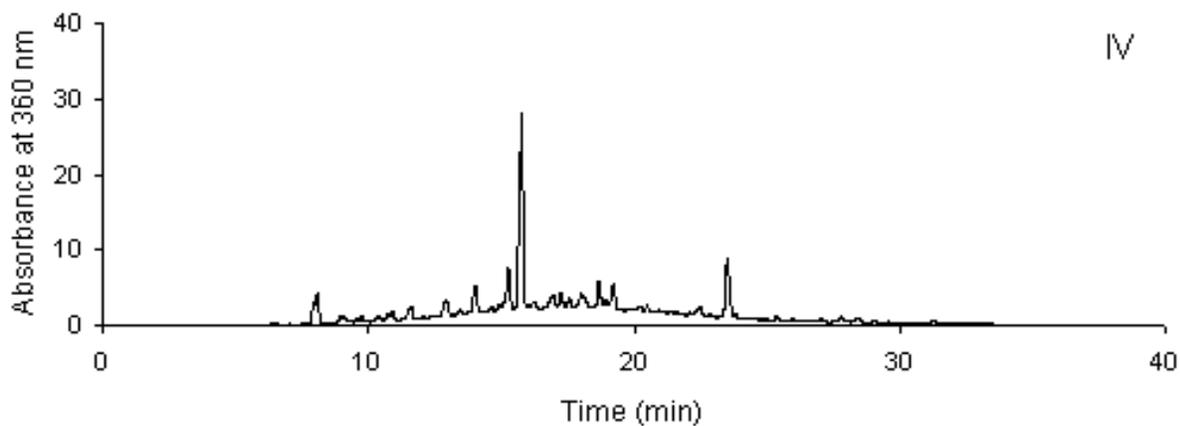
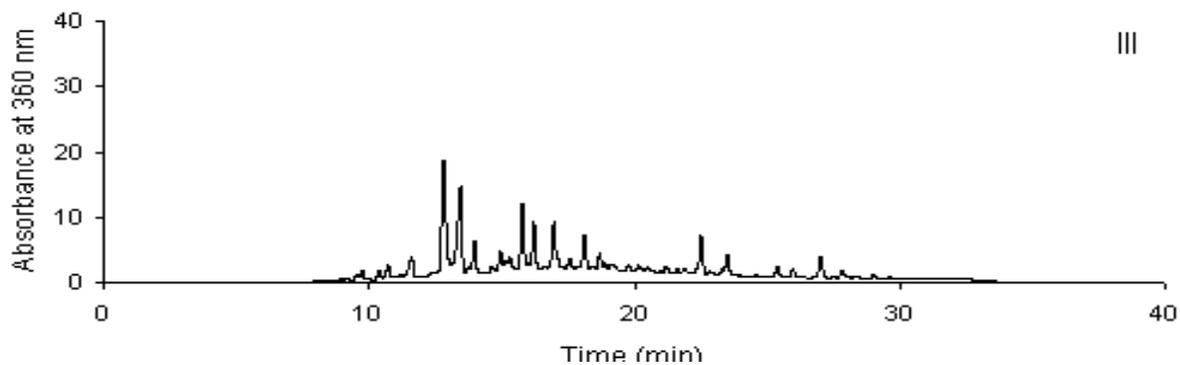
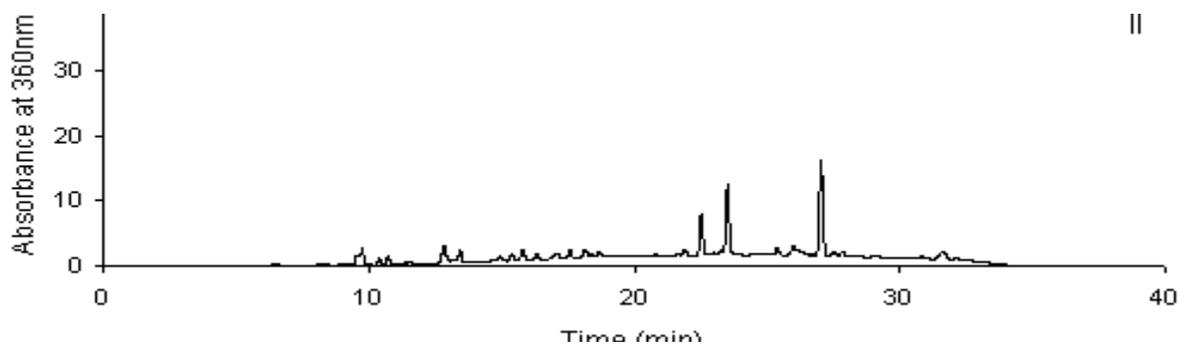
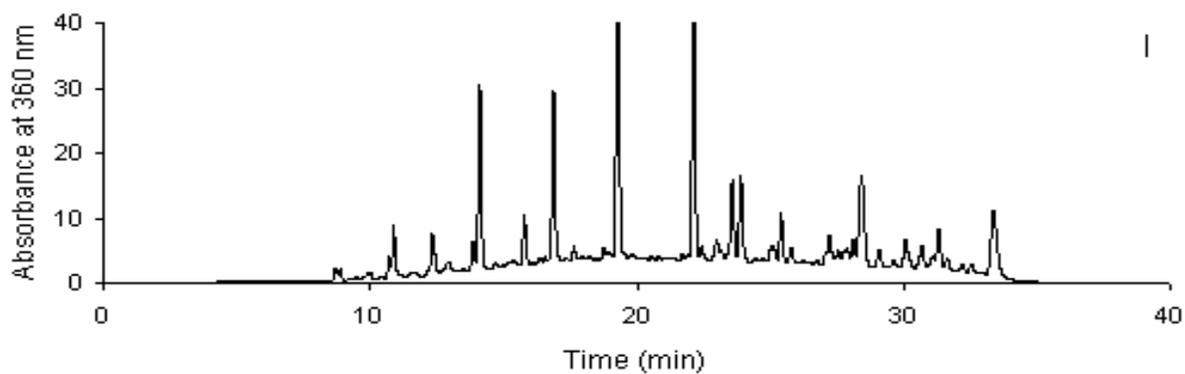


Figure 2.1: Reverse-phase HPLC fingerprint analysis of sorghum bran extract varieties at 360 nm. Gradient reversed-phase HPLC analysis of flavonoids in ethanol extracts of sorghum bran varieties. Samples: 50% ethanolic extracts of: **I.** black sorghum bran, **II.** white sorghum bran **III.** mycogen sorghum bran, and **IV.** sumac sorghum bran. Absorbance was measured at 360 nm.

Table 2.1: Total phenolic content and antioxidant (FRAP) values of various brans

Bran	Phenolic Acid (GAE mg/g)	FRAP Value (mmol/100g)
Sumac Sorghum	62.5 ± 0.6	48.4 ± 0.6
Black Sorghum	23.4 ± 0.9	15.6 ± 0.4
White Sorghum	3.11 ± 0.16	1.75 ± 0.60
Mycogen Sorghum	6.98 ± 0.41	4.37 ± 0.20
Oat	0.84 ± 0.12	0.64 ± 0.04
Rice	6.0 ± 0.3	5.35 ± 0.03
Wheat	3.88 ± 0.28	1.22 ± 0.06

Data represent mean ± of triplicate determinations.

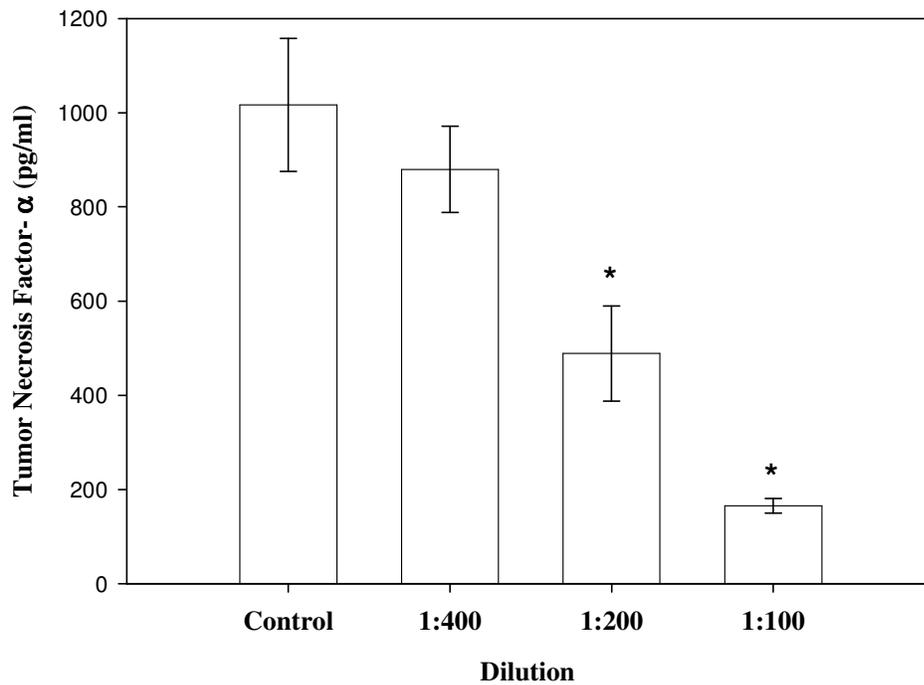


Figure 2.2a: Inhibition of TNF- α and IL-1 β release from human mononuclear cells by black sorghum. Black bran extract inhibition of cytokine release from human mononuclear cells. Cells were incubated with 1 $\mu\text{g/mL}$ *Escherichia coli* O111:B4 lipopolysaccharide and various dilutions of the black sorghum bran extract. After 24 h, the content of TNF- α (a) and IL-1 β (b) were determined by ELISA. Results represent means \pm SEM of two separate determinations. * $p < 0.05$ when compared to control cultures.

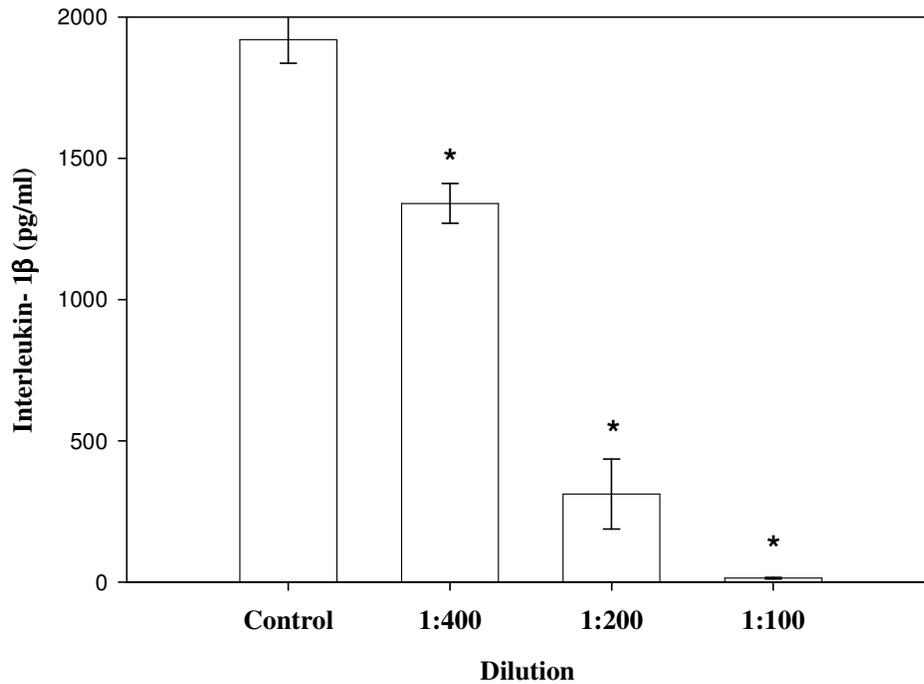


Figure 2.2b: Inhibition of TNF- α and IL-1 β release from human mononuclear cells by black sorghum. Black bran extract inhibition of cytokine release from human mononuclear cells. Cells were incubated with 1 μ g/mL *Escherichia coli* O111:B4 lipopolysaccharide and various dilutions of the black sorghum bran extract. After 24 h, the content of TNF- α (a) and IL-1 β (b) were determined by ELISA. Results represent means \pm SEM of two separate determinations. * p <0.05 when compared to control cultures.

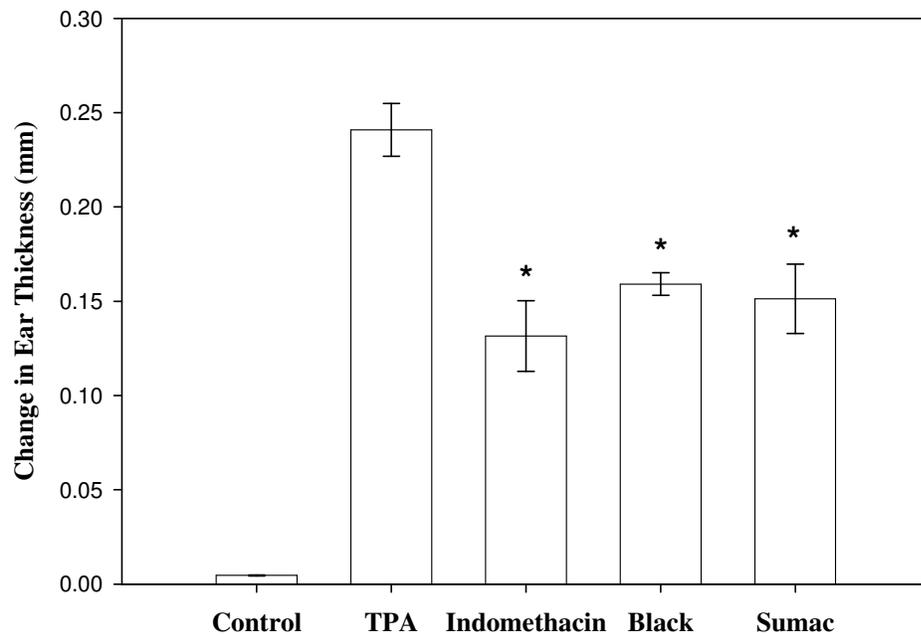


Figure 2.3a: Anti-inflammatory effects of sorghum bran extracts and indomethacin applied following TPA-induced injury. Sumac and black sorghum bran extracts or 0.5 mg/ear indomethacin were applied to mouse ears following the application of 2 μ g of TPA. The change in ear thickness (**a**) and the weight of ear punches (**b**) were determined after 6 h. Results represent means \pm SEM. * $p < 0.05$ compared to vehicle treated TPA.

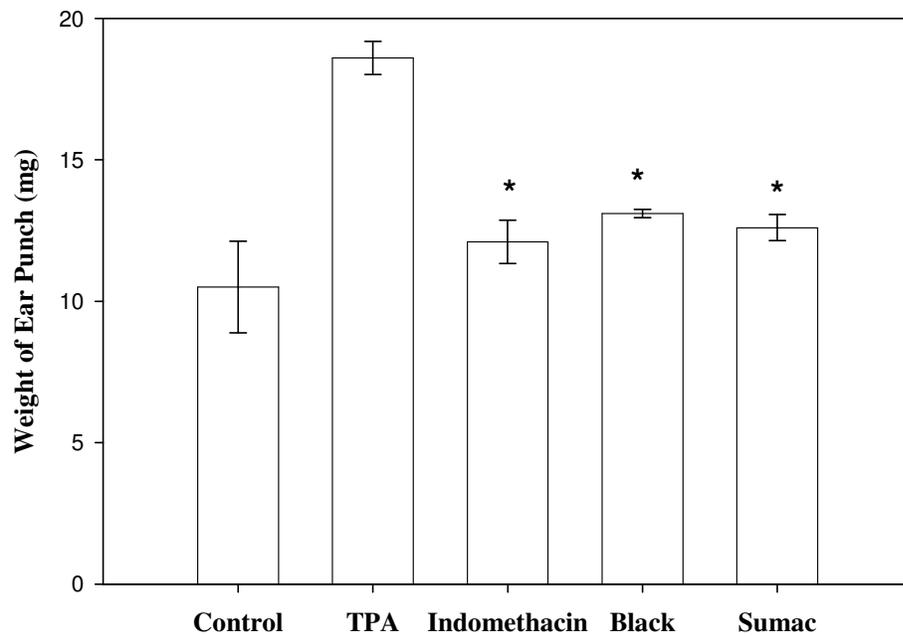


Figure 2.3b: Anti-inflammatory effects of sorghum bran extracts and indomethacin applied following TPA-induced injury. Sumac and black sorghum bran extracts or 0.5 mg/ear indomethacin were applied to mouse ears following the application of 2 μ g of TPA. The change in ear thickness (**a**) and the weight of ear punches (**b**) were determined after 6 h. Results represent means \pm SEM. * $p < 0.05$ compared to vehicle treated TPA.

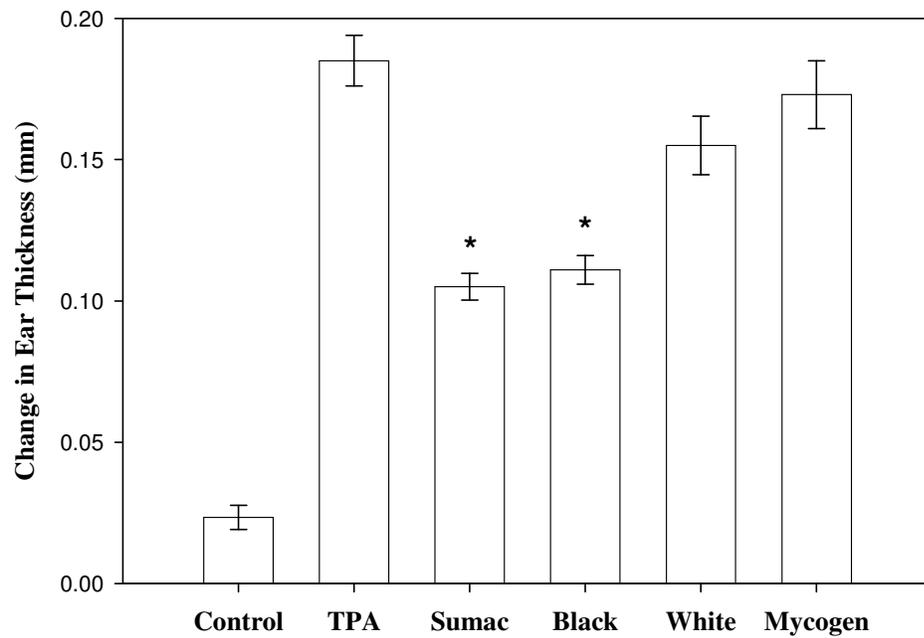


Figure 2.4: Anti-inflammatory effects of various sorghum bran extracts applied following TPA-induced injury. Sorghum bran extract varieties were applied to mouse ears following the application of 2 μ g of TPA. The change in ear thickness was determined after 6 h. Results represent means \pm SEM for 8 animals. * $p < 0.05$ compared to vehicle treated TPA.

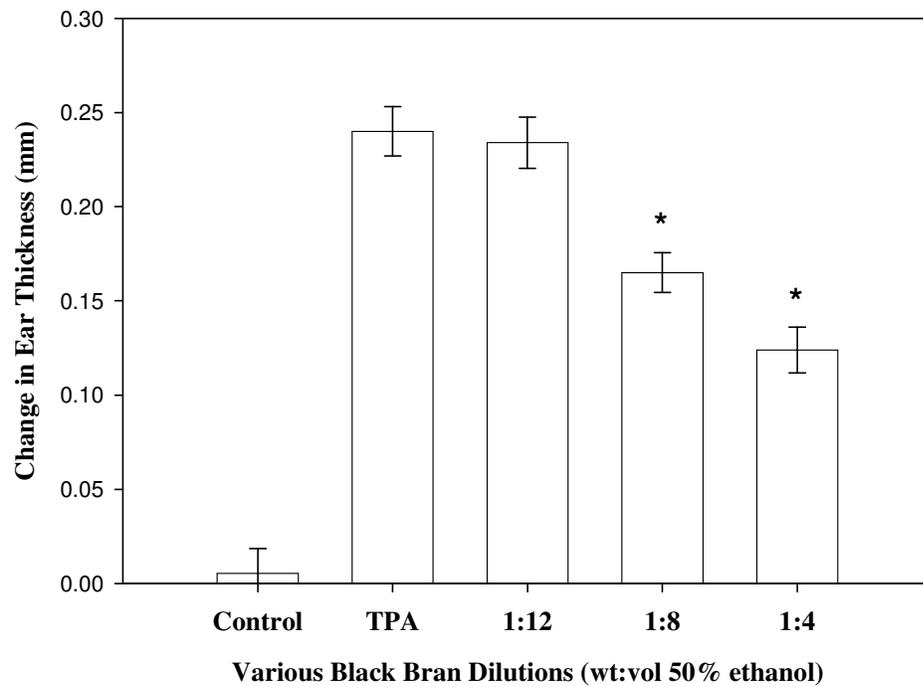


Figure 2.5: Anti-inflammatory effects of various concentrations of black bran extracts applied following TPA-induced injury. Various concentrations of black sorghum bran extract were applied to the mouse ears following the application of 2 μ g TPA. The change in ear thickness was measured after 6 h. Results represent means \pm SEM for 8 animals. * $p < 0.05$ compared to vehicle treated TPA.

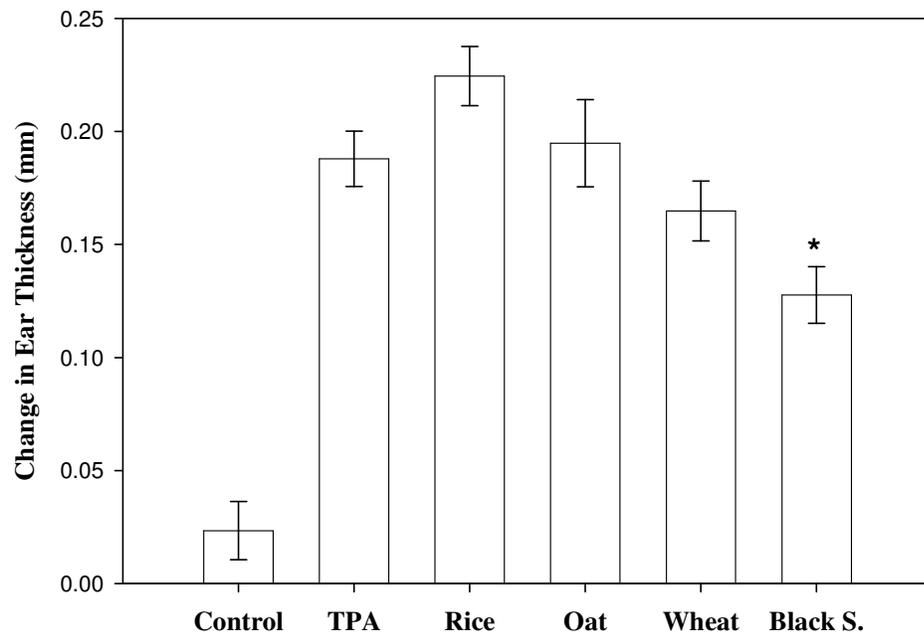


Figure 2.6: Anti-inflammatory effects of various non-sorghum bran extracts applied following TPA-induced injury. Various bran extracts were applied to the mouse ears following the application of 2 μ g TPA. The change in ear thickness was measured after 6 h. Results represent means \pm SEM for 8 animals. * $p < 0.05$ compared to vehicle treated TPA.

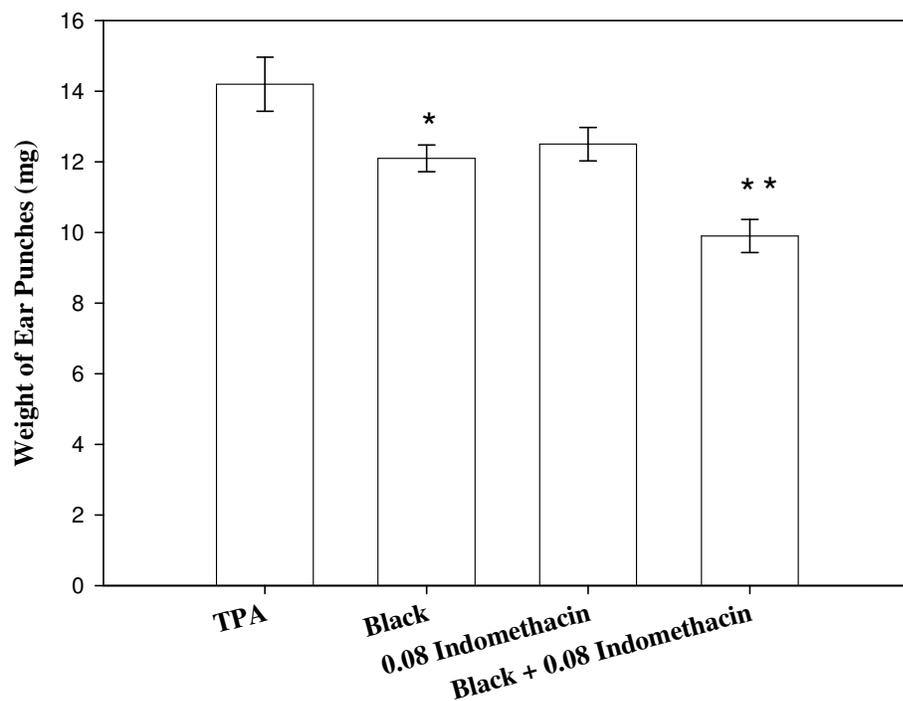


Figure 2.7a: Anti-inflammatory effects of black sorghum bran extract and indomethacin applied following TPA-induced injury. TPA-injured mouse ears received various doses of indomethacin (0.08 mg/ ear or 0.5 mg/ ear) alone or in combination with black sorghum bran extract (1:4 wt:vol) following the application of 2 μ g of TPA. The weight of ear punches (**a**) and change in ear thickness (**b**) was determined after 6 h. Results represent means \pm SEM for 8 animals. * p <0.05 compared to vehicle treated TPA, ** p <0.05 compared to black sorghum bran extract treated group.

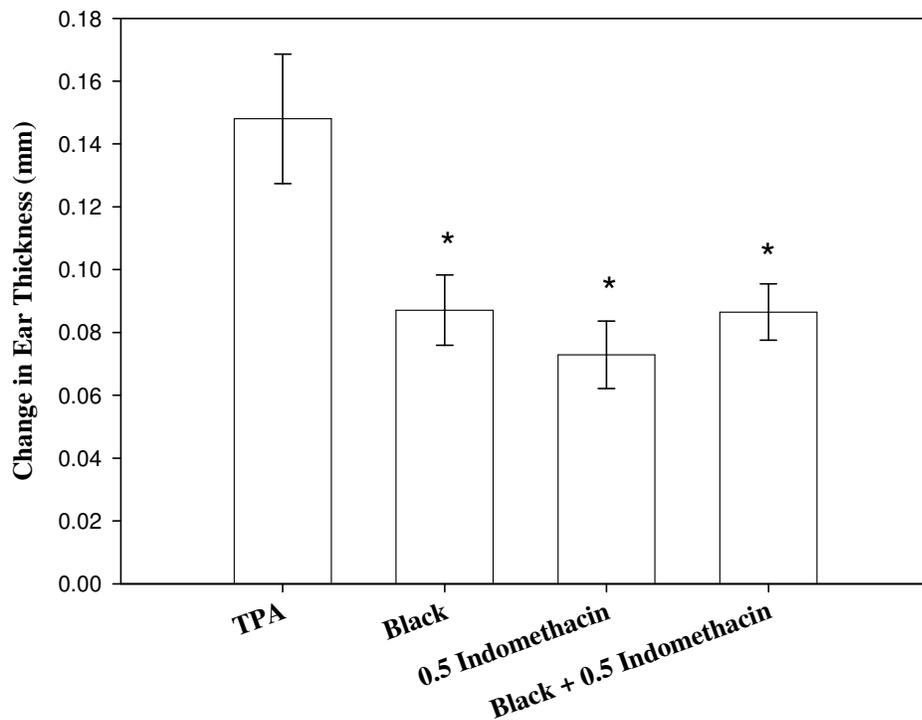


Figure 2.7b: Anti-inflammatory effects of black sorghum bran extract and indomethacin applied following TPA-induced injury. TPA-injured mouse ears received various doses of indomethacin (0.08 mg/ ear or 0.5 mg/ ear) alone or in combination with black sorghum bran extract (1:4 wt:vol) following the application of 2 μ g of TPA. The weight of ear punches (**a**) and change in ear thickness (**b**) was determined after 6 h. Results represent means \pm SEM for 8 animals. * $p < 0.05$ compared to vehicle treated TPA.

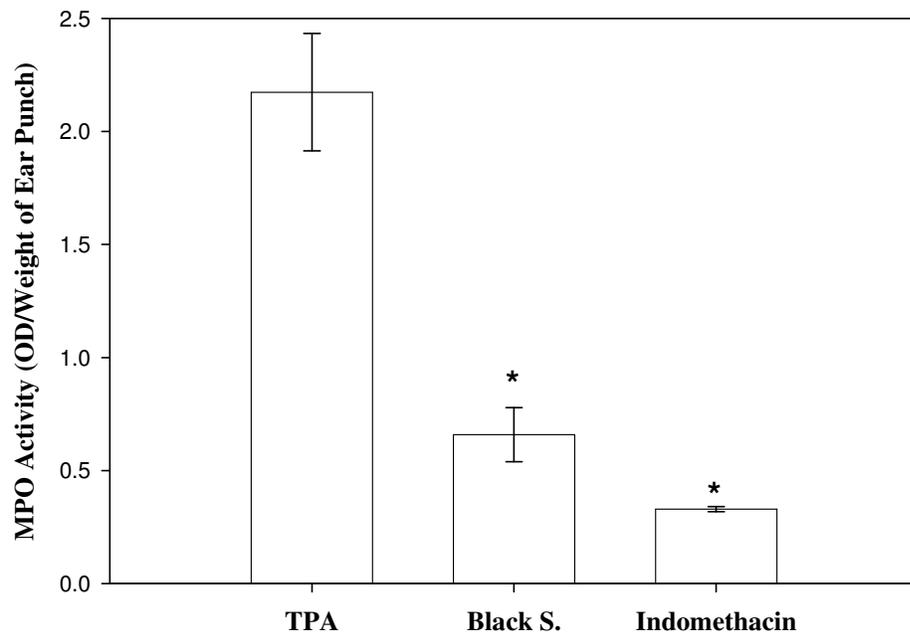


Figure 2.8: Effect of black sorghum bran extract and indomethacin on myeloperoxidase activity. Myeloperoxidase activity was measured in ear punches 24 h after TPA administration. Results represent means \pm SEM for 8 animals. * $p < 0.05$ compared to vehicle treated TPA.

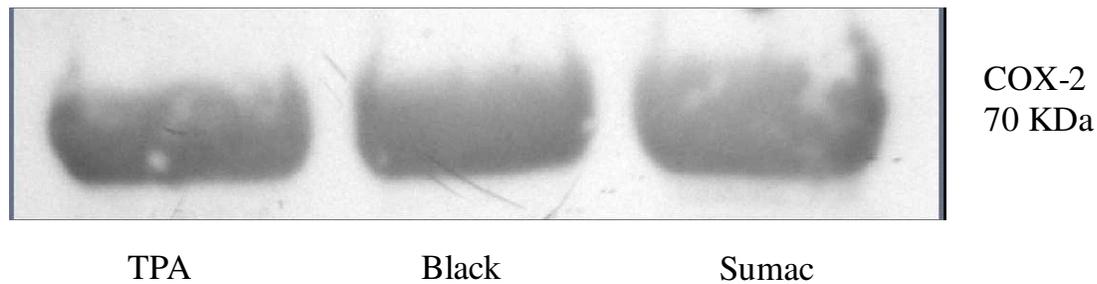


Figure 2.9a: Effect of black and sumac sorghum brans on COX-2 expression. Mouse ears were treated with black or sumac sorghum bran extracts following the application of 2 μ g TPA. Ear punches (6mm diameter) were taken 6 h after TPA application, homogenized and analyzed for COX-2 expression by western blotting (**a**) mean \pm SEM from 3 individual experiments from 8 animals (**b**).

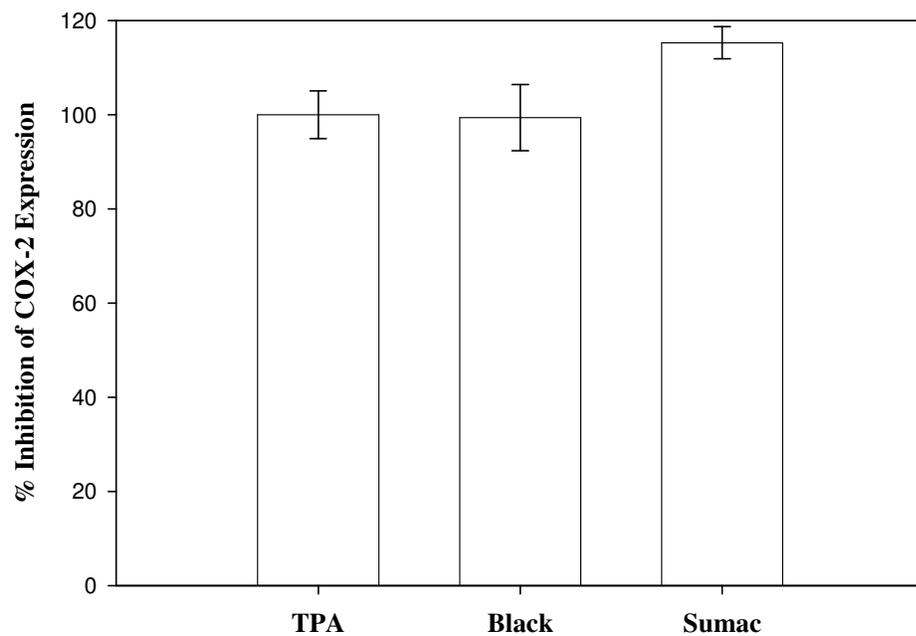


Figure 2.9b Effect of black and sumac sorghum brans on COX-2 expression. Mouse ears were treated with black or sumac sorghum bran extracts following the application of 2 μ g TPA. Ear punches (6mm diameter) were taken 6 h after TPA application, homogenized and analyzed for COX-2 expression by western blotting (a) mean \pm SEM from 3 individual experiments from 8 animals (b).

CHAPTER THREE

EFFECT OF SORGHUM BRAN AND WAX FRACTIONS ON PLASMA CHOLESTEROL CONCENTRATIONS IN HAMSTERS¹

¹ Amy Burdette, James L. Hargrove, Diane K. Hartle, Phillip Greenspan
Submitted to *Journal of Cereal Science*.

ABSTRACT

A mixture of very long chain fatty alcohols from sugar cane wax may have cholesterol-lowering capabilities. Sorghum (*Sorghum bicolor*) is a source of very long chain fatty aldehydes, alcohols and acids and other phytochemicals. In the present study, we investigated the hypothesis that feeding sumac sorghum bran or sorghum wax would lower lipoprotein cholesterol in a hyperlipidemic hamster model. Treatment groups received either a standard rodent chow diet or a high cholesterol diet consisting of 5% coconut oil and 0.12% cholesterol. In the first experiment, plasma total cholesterol was not affected by sorghum wax supplementation at concentrations ranging from 0-200 mg/kg of body wt. In a second experiment, the high cholesterol diets were supplemented with 0%, 5%, 10% or 20% of sumac sorghum bran. The consumption of sorghum bran at 5% and 10% of the diet was not effective in lowering plasma total cholesterol, plasma triglycerides or liver total cholesterol concentrations. However, hamsters receiving 20% of sorghum bran had a significant lowering of plasma total cholesterol and plasma triglyceride concentrations. Sorghum wax fractions were not effective lipid-lowering agents in the hyperlipidemic hamster model while sorghum bran produced a hypolipidemic effect when incorporated at 20% of the diet.

INTRODUCTION

Policosanols is a term that refers to a variety of commercial extracts that are enriched with very long chain fatty alcohols, but also contain very long chain acids and aldehydes. Many of the policosanols are derived from the wax of sugar cane (*Saccharum officinarum L.*) [1]. One defined sugar cane policosanols mixture consists of 63% octacosanol, a 28-carbon length aliphatic alcohol followed by triacontanol (12.6%) and hexacosanol (6.2%) [2].

The majority of the reports on the cholesterol-lowering efficacy of sugar cane policosanols have emanated from one research group in Cuba over the past sixteen years. The efficacy of policosanols in humans was reported to rival statin drugs in the magnitude of the cholesterol-lowering effects at equivalent dosages [3]. The effects included desirable reductions of serum low density lipoprotein (LDL) cholesterol, serum total cholesterol and serum triglyceride (TG) concentrations and increased serum high density lipoprotein (HDL) cholesterol concentration in experimental animals and humans. Lipid-lowering effects were observed in both normocholesterolemic and hypercholesterolemic rabbits when policosanols was orally administered at daily doses ranging from 5 to 50 mg/kg [4-6]. Additional studies in dogs, monkeys, rats and humans demonstrated a greater than 13% reduction in serum total cholesterol levels with daily consumption of policosanols [7-12].

Although sorghum (*Sorghum bicolor*) is one of the five major cereal crops produced in the USA, it is not commonly part of the American diet while it remains a food staple in many countries [13]. In the USA, this gluten-free flour is largely consumed by individuals who suffer from celiac disease [14, 15]. Sorghum bran contains anthocyanins, phytosterols and tocotrienols [13, 16-18] and is an excellent source of very long chain fatty acids, aldehydes and alcohols. These wax fractions comprise 1% of the bran. Nearly 24% and 34% of the wax fraction is

composed of long chain fatty acids and alcohols, respectively, with octacosanoic acid and octacosanol as the most abundant wax constituents [19-23]. The composition of sorghum wax is similar to that of sugar cane wax [2, 19, 24]. We hypothesized that whole sorghum bran and sorghum wax would share the same cholesterol-lowering activity as policosanol in the hypercholesterolemic hamster model.

METHODS

Materials

Cellulose, cholesterol, methanol and chloroform were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

Animals and Diets

Male golden Syrian hamsters (Charles River Laboratory; Wilmington, MA) weighing 100-120 g were housed in polycarbonate cages with Tek-Fresh bedding. The hamsters were kept in an environmentally controlled room maintained at 25°C with a 12:12-hr light:dark cycle. The animals were allowed free access to rodent chow (Purina Lab Diet 5001, Ralston Purina, St Louis, MO, USA) and water throughout the duration of the experiment. All experimental procedures were approved by the Animal Care and Use Committee at the University of Georgia.

Hamsters (7 per group) were weighed and assigned groups so that the starting body weights were equivalent in each group. One group received standard laboratory chow throughout the duration of the experiment. Every other group received a basal hypercholesterolemic diet containing 5% (wt/wt) coconut oil and 0.12% (wt/wt) cholesterol added to the standard chow diet (LabDiet 5001). Sorghum wax fraction (a gift of Dr. Curt Weller, University of Nebraska) supplemented the diet at 0, 25, 100 or 200 mg/kg body wt. Hamsters were weighed twice a week and food intake was measured weekly for two weeks.

In a second experiment, hamsters were randomly divided into groups (7 per group). Each group received a basal hypercholesterolemic diet (same as above) for three weeks. In this study sumac bran from sorghum grain (a generous gift of L.W. Rooney, Texas A&M) supplemented the diet at either 0%, 5%, 10% or 20% (wt/wt) of the diet. Cellulose was integrated into the basal diet to control for the insoluble fiber present in the bran fraction. For three weeks, the hamsters were weighed bi-weekly and food intake was measured weekly.

Hamsters were fasted 24 h prior to being euthanized by CO₂ and blood was collected via cardiac puncture. The liver was excised, weighed and immediately frozen at -80°C. The epididymal fat pads were removed and weighed.

Plasma Lipoproteins

Blood was collected in tubes containing EDTA and centrifuged at 2500 rpm for 20 min at 4°C. Plasma total cholesterol concentration and plasma triglyceride concentrations were immediately determined with fresh plasma using lipoprotein commercial kits (Wako Chemicals, Richmond, VA and Biotron Diagnostics, Hemet, CA).

Liver Lipoproteins

The liver total cholesterol content was determined by the method of Folch et al. [25]. Approximately 1 g of liver was thawed and then homogenized in 20 mL chloroform/ methanol (2:1, v/v), to extract total lipids. The liver total cholesterol content was measured using a commercial kit from Wako Chemicals (Richmond, VA).

Statistical Analysis

All results are reported as the mean of percent control \pm SEM. One-way ANOVA was used to analyze the data. Tukey's test was used to detect significant differences among treatment

groups. Differences at $P < 0.05$ were considered significant. SigmaStat (SPSS Science, Chicago, IL) was used to perform all statistical analyses.

RESULTS

Animals were fed a hypercholesterolemic diet consisting of 5% coconut oil and 0.12% cholesterol supplemented with either doses of sorghum wax ranging from 0-200 mg/kg body wt or a diet enriched with 0, 5, 10, or 20% sorghum bran. There were no statistically significant differences among diet groups in body weights (measured twice weekly), liver weights or epididymal fat pad weights.

Hamsters fed a standard rodent diet had plasma total cholesterol and plasma TG concentrations of 130 ± 1 and 217 ± 6 mg/dL respectively. The addition of 5% coconut oil and 0.12% cholesterol to the diets of the hamsters significantly raised plasma total cholesterol and plasma TG from 130 ± 1 to 303 ± 6 mg/dL and from 217 ± 4 mg/dL to 333 ± 8 mg/dL, respectively. The content of total cholesterol in the liver was increased from 17 ± 3.2 to 36 ± 2.6 mg/g liver in the 5% coconut oil and 0.12% cholesterol diet group.

Dietary supplementation of sorghum wax for three weeks was ineffective in lowering plasma total cholesterol at any dose tested (25, 100 and 200 mg/kg body weight, **Figure 3.1**). Similarly, plasma TG concentrations were not affected by sorghum wax (data not shown).

When 5% and 10% sorghum bran was fed for three weeks, plasma total cholesterol was lowered by 11% and 9%, respectively, although these findings were not statistically significant (**Figure 3.2**). Supplementation with 20% sorghum bran did significantly lower total cholesterol by 22% (**Figure 3.2**). Similarly, plasma TG values were not significantly affected with 5% or 10% sorghum bran in the hypercholesterolemic diet, but the 20% sorghum bran group had 29% lower TG than the hypercholesterolemic diet group without sorghum bran (**Figure 3.3**).

Changes in liver total cholesterol were not significantly altered by any of the sorghum bran doses (Figure 3.4).

DISCUSSION

The present study tested the abilities of either the wax or whole bran fraction of sorghum to lower lipid profiles in the hypercholesterolemic hamster model. This model has been used to successfully demonstrate the efficacy of other lipid-lowering substances such as phytosterols [26, 27] and statin drugs [28]. Research protocols used to achieve hypercholesterolemia in hamsters may vary somewhat in the literature, but in general, each utilizes some combination of saturated fat and added dietary cholesterol to achieve elevated blood total cholesterol levels [27, 29-31].

Sorghum wax was studied because its known profile of lipids overlaps that of the policosanols derived from sugar cane wax, but with a higher proportion of very long chain fatty aldehydes and acids [13, 16-19, 32]. Sugar cane very long chain fatty aldehydes and acids have been reported to lower cholesterol by the same Cuban group who has popularized the policosanols fraction [33-35]. Octacosanoic acid was a major metabolite detected after the incubation of ^3H -octacosanol with human fibroblasts and following the oral administration of policosanols to rats [36]. Upon digestion, chylomicrons presumably carry the very long chain fatty alcohols to the liver where they enter the endoplasmic reticulum and undergo oxidative metabolism to their acid counterpart [32]. Subsequently, ATP-binding cassette protein D1 transports the acid to the peroxisome where it undergoes β -oxidation. It is thought that octacosanol must be converted via oxidation to its corresponding acid, octacosanoic acid, to produce its desirable effects [32, 36]. Support for this hypothesis has come from experiments where D-003, a highly purified mixture that is a rich source of sugar cane wax very long chain fatty acids, specifically octacosanoic acid (C28), was compared directly with policosanols [37]. In a head-to-head, randomized double-

blind controlled study conducted by the Cuban groups, D-003 produced greater plasma lipid lowering in both humans and rabbits than policosanol [5, 33].

The concentration of sorghum wax administered in the present study was analogous to the doses of policosanol that lowered cholesterol in experiments performed by Cubans [4, 9-12, 33, 34]. It should be noted that the doses we used in these experiments are much higher than doses used in human clinical trials by the Cuban group (5 – 50 mg/ adult/day) [7, 8, 33, 38]. We found no cholesterol-lowering or triglyceride-lowering effects in doses as high as 200 mg/kg/day in the hamster model.

The amount of sorghum wax delivered in diets containing 5%, 10% and 20% bran is approximately equivalent to 45, 90 and 180 mg/kg/day, respectively. While no significant effects were found with the sorghum wax alone, even at the 200 mg/kg/day dose, the 20% whole bran diet did significantly lower both plasma total cholesterol and plasma TG. The fact that 20% sorghum bran effectively lowered plasma lipid profile, but not the wax fraction alone, suggests that ingredients other than the wax fraction may be responsible for the lipid lowering actions of sorghum bran. One other possibility which cannot be ignored is that the non-wax fraction may enhance the bioactivity or bioavailability of the sorghum wax fraction.

In a previous experiment, policosanol from Lipowax (Lipo Chemical), at a dose of 25 mg/kg/day, did not alter plasma lipids in a hamster model [39]. We designed our study to start at this level and tested higher doses of sorghum wax. We found no cholesterol-lowering effect, even at 200mg/kg/day. In another study, a head-to-head comparison was performed between a sugar cane policosanol originating from Cuba and a sugar cane policosanol obtained from SE Asia. Both failed to lower plasma total cholesterol levels when administered at 275 mg/kg to hypercholesterolemic hamsters [40], in agreement with our findings that 200 mg/kg of sorghum

wax was an ineffective hypocholesterolemic agent. Marinangeli et al. [41] studied the absorption of policosanol derived from Cuban-sourced (Dalmer Lab) sugar cane policosanol and policosanol from an alternative source (Deguss Bioactives). They found no detectable very long chain fatty alcohols in the plasma, liver, small intestine or adipose tissues of hamsters fed either 0.275 mg/g diet (18mg/kg/day) or 2.75 mg/g diet. Policosanol was found in the feces at both dietary doses indicating very poor bioavailability of the very long chain alcohols [41]. This is in agreement with the argument that policosanols are only marginally bioavailable unless metabolized to the corresponding fatty acids [32]. Therefore, our findings are in agreement with other investigators that have found that policosanol has no effect in the hypercholesterolemic hamster, a well established test model for studying cholesterol-lowering substances relevant to human drug/supplement development [42].

The effect of policosanol has been tested in human clinical trials in independent laboratories in Europe, Canada and the United States. In a double-blind crossover study, humans consumed 10 mg of policosanol per day for 29 days and total cholesterol, LDL-cholesterol, HDL-cholesterol or plasma TG concentrations were not found to be altered [43]. Several other human double-blind, placebo-controlled studies have found policosanol to be ineffective in lowering plasma cholesterol when administered at higher doses, ranging from 20-80 mg/day for 12 weeks [43-47]. The doses tested in humans are analogous to the doses suggested by the Cuban groups to lower cholesterol in humans. For about 16 years, the Cuban group has observed very large lipid profile changes when hypercholesterolemic humans are treated with policosanol, similar in magnitude to that of statins. Since many of the independent studies utilized Cuban policosanol or a policosanols that have similar chemical profiles, there is no easy explanation for

these disparate results. While a pharmacogenomic effect can be hypothesized, there is no current evidence in support of this argument.

In one study in hamsters, a 36% reduction of plasma non-HDL cholesterol was reported in animals consuming a 1% hexane lipid extract of whole grain sorghum [48]. The same report indicated that a 0.5% dietary sorghum lipid extract was ineffective in lowering plasma non-HDL cholesterol. The diet we used in this study containing 200 mg/kg sorghum wax represents a lower dose than the 1% dose of Carr et al. [48] and is even less than the 0.5% dose.

The cholesterol-lowering effects of cereal brans may be partially dependent upon the amount of β -glucan, a soluble fiber [49, 50]. Sorghum contains β -glucan, but its content is substantially less than other cereal sources such as oat bran, barley grains and rye bran which effectively lower plasma cholesterol [51, 52]. Other cholesterol-lowering constituents of cereal brans are the phytosterols. A diet high in plant sterols is well documented to lower plasma cholesterol by suppressing intestinal cholesterol absorption [53]. Hamster diets supplemented with 10% (wt/wt) sorghum bran are equivalent to consuming 0.6% of phytosterols per day [13]. The minimum amount of phytosterol content needed to supplement the diet in order to produce a consistent cholesterol lowering effect in the hyperlipidemic hamster is 1% [38]. Therefore, the phytosterol content in the 5% and 10% sorghum bran groups may have been subthreshold for this sterol effect, but the highest sorghum bran group would clearly have consumed enough sterols to account for the cholesterol-lowering effect.

Currently there is no international consensus supporting a dramatic lowering of plasma cholesterol concentrations by policosanol in humans at the low doses suggested in clinical trials by the Cuban group. The efficacy of current policosanol-containing nutraceutical products has not been independently verified by the scores of companies marketing products containing

policosanol. While the efficacy of policosanol containing commercial products remains to be documented, this study demonstrates that sorghum bran does possess cholesterol lowering properties in the hypercholesterolemic hamster model.

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TABLES AND FIGURES

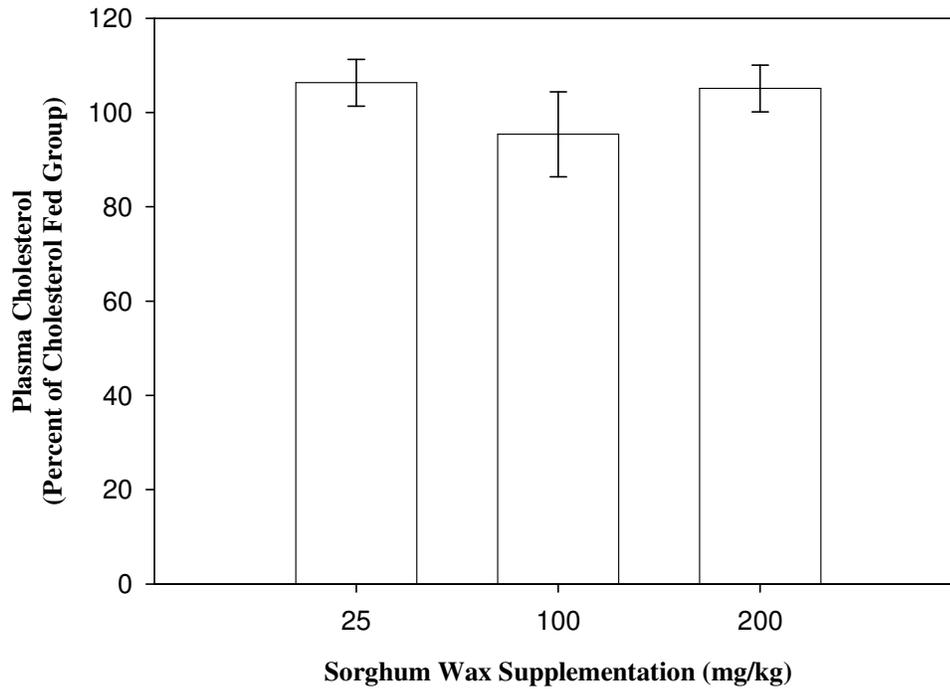


Figure 3.1: Effect of Sorghum Wax Supplementation on Plasma Total Cholesterol

Concentration in the Hypercholesterolemic Hamster. Plasma total cholesterol concentration in hamsters fed 0, 25, 100 or 200 mg/kg body weight of sorghum wax and a hypercholesterolemic diet. Values are expressed as mean \pm SEM.

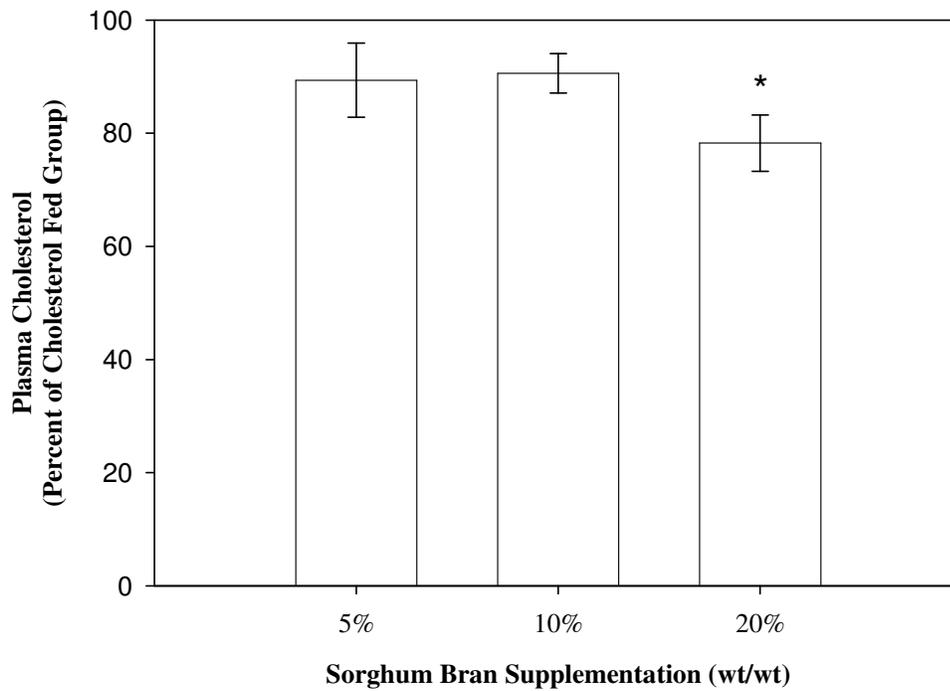


Figure 3.2: Effect of Sumac Sorghum Bran Supplementation on Plasma Total Cholesterol Concentration in the Hypercholesterolemic Hamster. Plasma total cholesterol concentration in hamsters fed 0, 5, 10 or 20% (wt/wt) sumac sorghum and a hypercholesterolemic diet. Values are expressed as mean \pm SEM. *Significantly different from treatment group receiving 5% (wt/wt) coconut oil and 0.12% (wt/wt) cholesterol, $P < 0.05$.

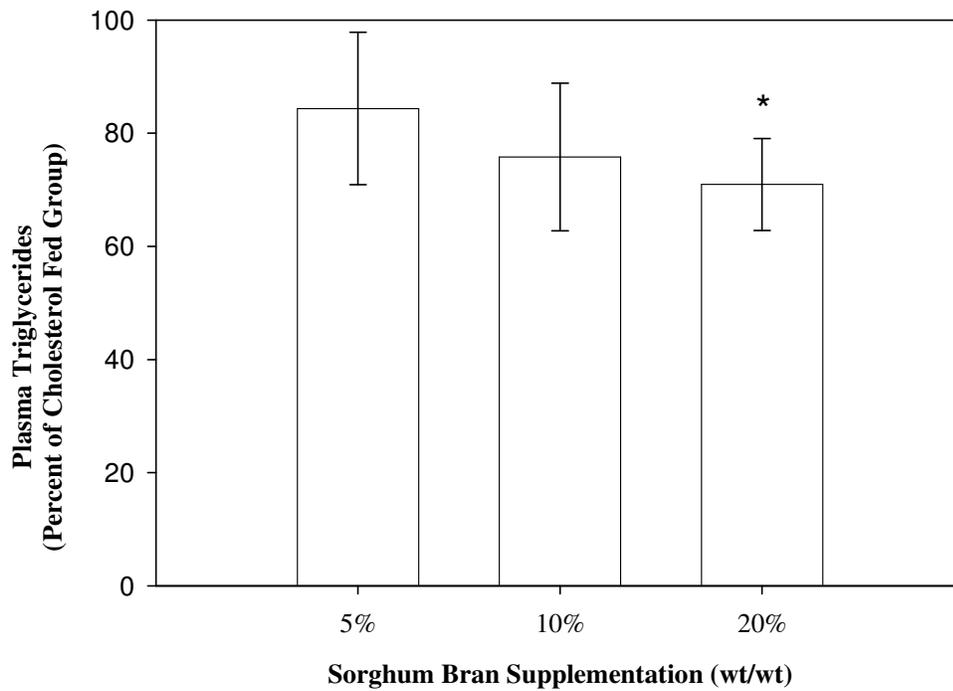


Figure 3.3: Effect of Sumac Sorghum Bran Supplementation on Plasma Triglyceride Concentration in the Hypercholesterolemic Hamster. Plasma triglyceride concentration in hamsters fed 0, 5, 10 or 20% (wt/wt) sumac sorghum bran and a hypercholesterolemic diet. Values are expressed as mean \pm SEM. *Significantly different from the group receiving 5% (wt/wt) coconut oil and 0.12% (wt/wt) cholesterol, $P < 0.05$.

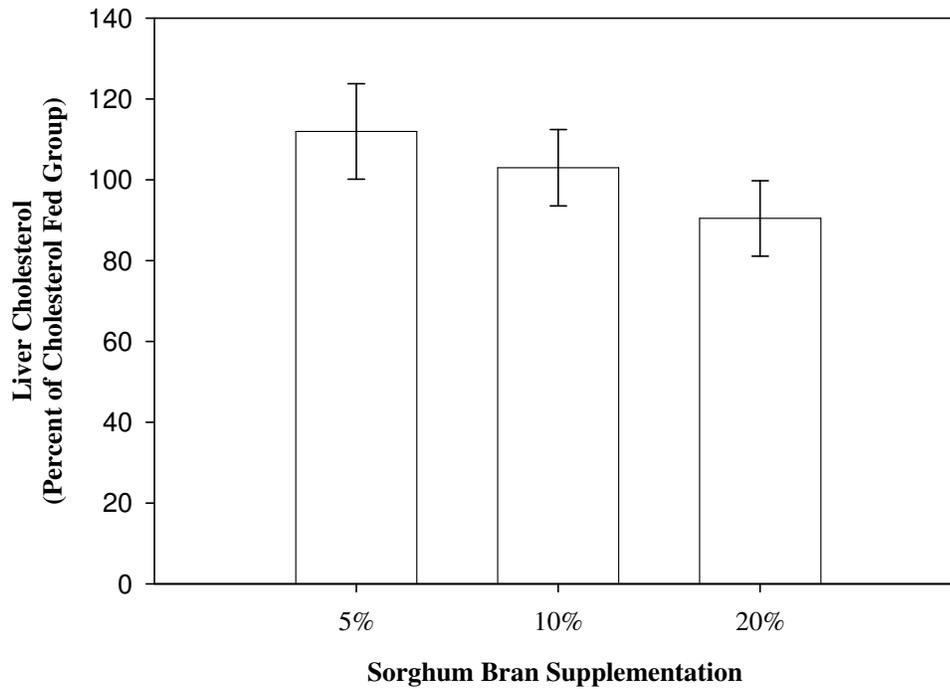


Figure 3.4: Effect of Sumac Sorghum Bran Supplementation on Liver Total Cholesterol Concentration in the Hypercholesterolemic Hamster. Liver total cholesterol concentration in hamsters fed 0, 5, 10 or 20% (wt/wt) sumac sorghum bran and a hypercholesterolemic diet.

Values are expressed as mean \pm SEM.

CHAPTER 4

A NUTRACEUTICAL FORMULATION LOWERS PLASMA LIPIDS
IN THE HYPERCHOLESTEROLEMIC HAMSTER²

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To be submitted to *Journal of Medicinal Food*

ABSTRACT

The purpose of this study was to test whether a formulation of cholesterol-lowering natural products could augment the cholesterol-lowering effects of sorghum bran. The formulation consisted of *Spirulina* (chlorophyll and phytanic acid), phytosterols, pantethine and turmeric. Sorghum bran was added to the diets at concentrations ranging from 0 to 10% (wt/wt). Hamsters were fed 5% coconut oil and 0.12% cholesterol for three weeks to produce hypercholesterolemia. When the nutraceutical formulation, without sorghum bran, was administered at a high dose (9% of the diet), the hamsters consumed approximately 6 mg chlorophyll, 24 mg curcumin, 60 mg phytosterols and 83 mg pantethine per day. This dosage of the formulation lowered plasma cholesterol concentrations by 39% and plasma triglyceride concentrations by 30%. A significant lowering of plasma cholesterol concentrations was observed at one-ninth of this dose while an effect on plasma triglycerides was seen at approximately 5% of the dose. Sorghum bran, when integrated into the formulation at 5 and 10% of the diets, produced only modest, non-significant decreases in plasma cholesterol concentrations. However, a diet of 5% sorghum bran and 9% formulation produced the greatest lowering in plasma cholesterol (44%) These results demonstrate that a combination of natural products, each with its own distinct mechanism of action and each administered at a modest dose, can lower plasma cholesterol and triglyceride concentrations in a well-studied animal model of hypercholesterolemia.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death among both men and women in the United States. Atherosclerosis is a pathological process contributing to CVD in which an excessive amount of cholesterol accumulates in the artery wall leading eventually to plaque formation. The reduction of elevated plasma total cholesterol levels, specifically low-density lipoprotein (LDL) cholesterol, is a major strategy to reduce the incidence of CVD [1]. Statins are a class of lipid altering drugs that have been shown to reduce CVD primarily by lowering LDL cholesterol [2]. However, statins have side effects including liver and muscle toxicity. They are expensive and there are problems with non-compliance [3].

A potential alternative to lipid-lowering pharmaceutical agents is incorporating beneficial foods, food ingredients, or nutraceuticals into the diet to lower plasma LDL cholesterol concentrations [4]. By combining metabolically active, lipid-lowering agents, that have distinct mechanisms of action, the potential exists for an effective nutraceutical formulation to beneficially alter blood lipid profiles. The resulting product may also allow for an increased hypocholesterolemic effect when compared to single agents because of additive or synergistic actions of the ingredients. Curcumin, phytosterols, phytanic acid and pantethine have all been shown to lower plasma cholesterol concentrations via different mechanisms [4-10]. We chose to test whether a formulation of turmeric (*Curcuma longa*), *Spirulina*, pantethine and phytosterols would collectively produce lipid-lowering effects at lower doses than previously reported for each single ingredient [11-14].

The bran layer of grain sorghum is an excellent source of very long chain fatty acids, aldehydes and alcohols, phytosterols and phenolic compounds [15-19]. Grain sorghum lipid fractions have been reported to lower plasma cholesterol in experimental animals [20]. Previous

studies conducted by this laboratory showed that the addition of sorghum bran into the diets of hypercholesterolemic hamsters slightly decreased plasma total cholesterol concentrations [21]. In addition, we observed that ethanolic extracts of sorghum bran possess anti-inflammatory effects in several *in vitro* models. The addition of sorghum bran extracts to mouse ears stimulated by phorbol myristate acetate significantly reduced topical inflammation. The secretion of pro-inflammatory cytokines, TNF- α and IL-1 was inhibited with sorghum bran extract in lipopolysaccharide-activated peripheral blood mononuclear cells [22].

Hamsters are a widely used animal model to study the lipid lowering properties of pharmaceutical or nutraceutical agents [23]. In the present study, we investigated the cholesterol-lowering actions of the *Spirulina*/turmeric/phytosterol/pantethine nutraceutical formulation with diets enriched with sorghum bran.

METHODS

Materials

Cellulose, cholesterol, methanol and chloroform were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Pantestin® was purchased from Daiichi Fine Chemicals (New York, NY, USA). Basikol® was obtained from Health from the Sun (Arkopharma, Newport, NH, USA). *Spirulina* was purchased from Earthrise (Irvine, CA, USA) and turmeric was obtained from a local grocery store.

Animals and Diets

Male golden Syrian hamsters (Charles River Laboratory; Wilmington, MA) weighing 100-120 g were individually housed in polycarbonate cages with Tek-Fresh bedding. The hamsters were kept in an environmentally controlled room maintained at 25°C with a 12:12-hr light:dark cycle. The animals were allowed free access to chow (Purina Lab Diet 5001, Ralston

Purina, St. Louis, MO, USA) and water throughout the duration of the experiment. All experimental procedures were approved by the Animal Care and Use Committee at the University of Georgia.

Hamsters were weighed and assigned to groups (n= 6 per group) so that the starting body weights were statistically equivalent in each group. One group received standard laboratory chow throughout the duration of the experiment. Every other group received a basal hypercholesterolemic diet containing 5% (wt/wt) coconut oil and 0.12% (wt/wt) cholesterol added to the standard chow diet for 21 days. Sumac sorghum bran (a generous gift of L.W. Rooney, Texas A&M) supplemented the diet at 5 or 10% (wt/wt) alone or in combination with the total formulation. The formulation contained pantethine (Pantesin®, Daiichi Fine Chemicals, New York, NY, USA), phytosterols derived from soy, corn and canola oils (Basikol®, Health from the Sun, Arkopharma, Newport, NH, USA), turmeric (obtained from local grocery store) and *Spirulina* (Earthise, Irvine, CA, USA) in a ratio of 1:1:8:8, respectively. When the formulation comprised 9% of the diet, the hamsters consumed 6 mg chlorophyll, 24 mg curcumin, 60 mg phytosterols and 83 mg pantethine per day. Diets with 0.5%, 1%, 3% and 9% by weight of the formulation (F) will be referred to as 0.5F, 1F, 3F and 9F, respectively. Cellulose was incorporated into the hypercholesterolemic diets of animals not receiving the nutraceutical supplements to control for the fiber present in the bran fraction.

A second set of experiments was undertaken to determine if the formulation was effective at lowering plasma lipid levels with a reduced number of nutraceutical ingredients. All diets contained the hypercholesterolemic diet (0.12% cholesterol and 5% coconut oil) and either (i) 0.5% pantethine, 4% turmeric and 5% sorghum bran, or (ii) 0.5% Basikol®, 4% *Spirulina* and 5% sorghum bran.

In all experiments, the hamsters were weighed bi-weekly and food intake was measured weekly. Hamsters were fasted by removing the food 24 h prior to euthanizing the animal by CO₂. Blood was collected via cardiac puncture. The liver was excised, weighed and immediately frozen at -80°C. The epididymal fat pads were removed and the weights were recorded.

Plasma Lipid Concentrations

Blood was collected at sacrifice into tubes containing EDTA and centrifuged at 2500 rpm for 20 min at 4°C to remove blood cells. Plasma total cholesterol and triglyceride (TG) concentrations were immediately determined with fresh plasma using commercial kits (Wako Chemicals, Richmond, VA and Biotron Diagnostics, Hemet, CA).

Liver Lipid Content

The liver total cholesterol content was determined according to Folch et al. [24]. Approximately 1 g of liver was allowed to thaw and was homogenized in 20 mL chloroform:methanol (2:1, v/v) solvent which extracted the total lipid. The liver total cholesterol and triglyceride contents were measured using commercial kits from Wako Chemicals (Richmond, VA) and Biotron Diagnostics (Hemet, CA), respectively.

Statistical Analysis

All results are reported as the mean \pm SEM. One-way ANOVA was used to analyze the data. Tukey's test was used to detect significant differences among treatment groups. Differences with $P < 0.05$ were considered significant. SigmaStat (SPSS Science, Chicago, IL) was used to perform all statistical analyses.

RESULTS

Animals were fed the hypercholesterolemic diet consisting of 5% coconut oil and 0.12% cholesterol for 21 days, or the same diet supplemented with various concentrations of sorghum bran and specific nutraceutical formulations. The addition of 5% sorghum bran (5SB), 10% sorghum bran (10SB), and/or concentrations of the formulation at 9% (9F), 3% (3F), 1% (1F) or 0.5% (0.5F) was made to the diet. There were no differences among treatment groups in the body weights of hamsters measured on a biweekly basis. The liver weights and epididymal fat pad weights were also similar among the groups.

Hamsters fed a standard rodent diet had plasma total cholesterol and TG concentrations of 130 ± 1 and 217 ± 6 mg/dL, respectively. The addition of 5% coconut oil and 0.12% cholesterol to the diets significantly raised plasma total cholesterol and TG to 303 ± 5 and 333 ± 7 mg/dL, respectively. The content of cholesterol in the liver was raised by 48% from 17 ± 3 to 36 ± 2 mg/g liver.

Effects of sorghum bran \pm nutraceutical formulation

After three weeks on the hypercholesterolemic diet, the effect of sorghum bran alone or in combination with the nutraceutical formulation on plasma total cholesterol values was determined (**Figure 4.1**). The addition of 5% and 10% (wt/wt) sorghum bran to the high fat diet slightly lowered plasma total cholesterol by 11 and 9%, respectively, although this reduction was not statistically significant. When the nutraceutical formulation represented 9% of the diet, plasma total cholesterol concentration was significantly reduced by 39%. The addition of 5 and 10% (wt/wt) sorghum bran to the formulation produced an additional modest decrease in plasma cholesterol concentrations (**Figure 4.1**).

The effect of sorghum bran and the nutraceutical formulation on plasma TG concentrations was examined (**Figure 4.2**). Supplementation of the diet with the formulation alone and in combination with 5 and 10% sorghum bran significantly lowered plasma TG concentrations by 30, 34, and 32%, respectively. Sorghum bran at both 5% and 10% of the diet, in the absence of the nutraceutical formulation, did not significantly decrease plasma TG concentrations.

Dose-response effect of sorghum bran and nutraceutical formulation

To determine the dose-response effect of the nutraceutical formulation, animals were fed a high fat diet and formulation doses ranging from 0.5 to 9% (wt/wt) in combination with 5% (wt/wt) sorghum bran. Sorghum bran alone and supplemented with the formulation at 0.5% of the diet slightly decreased plasma cholesterol concentrations, although these reduction were not statistically significant. Doses of the formulation as low as 1% in combination with sorghum bran significantly lowered plasma total cholesterol concentrations (**Figure 4.3**). The plasma cholesterol concentrations of animals fed the 1% formulation and 5% sorghum bran was similar to that observed when the diet was supplemented with 9% of the diet in the absence or presence of sorghum bran..

The addition of 5% sorghum bran and 0.5% formulation to the high fat diet significantly lowered plasma TG concentrations by 38%; however, a significant effect was not observed by sorghum bran alone (**Figure 4.4**). Further increases of the formulation into the diet did not result in a further reduction in plasma TG concentrations.

The liver cholesterol content was decreased in animals fed diets containing 1 to 3% of the nutraceutical formulation in the presence of 5% sorghum bran. Diets supplemented with the sorghum bran alone and sorghum bran and 0.5% formulation slightly, but not significantly,

increased liver cholesterol content (**Figure 4.5**). There were no statistically significant differences observed in liver TG content among the groups (data not shown).

Effects of different nutraceutical combinations

In another experiment, the four component nutraceutical formulation was split into two new formulations and their effect on plasma cholesterol concentrations in the hypercholesterolemic hamster examined. One formulation contained 0.5% pantethine and 4% turmeric while the other contained 0.5% phytosterols and 4% *Spirulina*. The combination of sorghum bran, pantethine and turmeric significantly lowered plasma and plasma total cholesterol by 22% while sorghum bran, phytosterols and *Spirulina* produced a 32% reduction (**Figure 4.6**). While there was no significant difference between these two formulations in reducing plasma cholesterol concentrations, the extent of inhibition by each was significantly less than the 39% reduction observed with the four-ingredient nutraceutical formulation (**Figure 4.1**).

DISCUSSION

The purpose of the present study was two-fold. First, to develop a combination of natural ingredients that produces a significant lipid-lowering effect in hypercholesterolemic hamsters. Second, to investigate whether sorghum bran can further lower plasma lipid concentrations when combined with this nutraceutical formulation. The combination of four natural ingredients: *Spirulina*, turmeric, pantethine and phytosterols significantly lowered cholesterol plasma cholesterol and TG concentrations in the hypercholesterolemic hamster

Sorghum bran was included in this study for two reasons. Previous studies in our laboratory revealed a consistent, though non-significant, 10% lowering of total plasma cholesterol concentrations with the consumption of sorghum bran at 5% of the diet [21]. In agreement with this past observation, sorghum bran also lowered plasma cholesterol

approximately 10% in these studies (**Figure 4.1**). When added to the formulation, sorghum bran produced a further, though non-significant lowering of plasma cholesterol concentrations (**Figure 4.1**). Thus, the inclusion of sorghum bran into the diet produced, at best, only a modest incremental decrease in plasma cholesterol concentrations. More importantly than its putative hypocholesterolemic effect, sorghum bran is an excellent source of phytochemicals [18, 25-27]. The antioxidants present in sorghum bran are composed of condensed tannins (epicatechin and catechin) and phenolics acids (ferulic, caffeic and coumaric). The total amount of phenolic compounds and its antioxidant capacity is higher than any other major grain bran [22]. While these phytochemicals present in sorghum appear to lack significant hypocholesterolemic effects in this animal model, sorghum bran is an inexpensive source of phytochemicals and is emerging as important dietary constituent for health conscious individuals

Natural products are becoming increasingly popular in the treatment of various disease states [28-32]. The ingredients in the formulation employed in these studies were chosen because they have unique mechanisms of actions when compared to the other constituents. These effects range from inhibition of cholesterol absorption and synthesis to increasing cholesterol and lipoprotein catabolism. While turmeric (*Curcuma longa*) has been widely used as a spice and in curry powder, recent evidence has shown that turmeric has many beneficial pharmacological effects [10, 33]. Curcumin, the active component of turmeric, can effectively lower plasma cholesterol concentrations in experimental animal models [6, 8]. The hypocholesterolemic effect may be attributed to the upregulation of hepatic cholesterol-7 α -hydroxylase, thereby increasing the rate of cholesterol catabolism [6]. Peschel et al. [34] reported a concentration-dependent increase in LDL-receptor mRNA expression in HepG2 cells, thereby, increasing the uptake of LDL-cholesterol from plasma. Additionally, curcumin has

been reported to increase HDL-cholesterol concentrations in hypercholesterolemic rats [35]. This may be attributed to the significant induction of LXR α mRNA and subsequently its direct target, the ATP-binding cassette transporter, ABCG1 [36].

The cholesterol lowering property of phytosterols has been well documented since 1953 [37, 38]. It is believed that structural similarity to cholesterol plays a major role in its cholesterol lowering activity. Phytosterols compete with dietary cholesterol for intestinal incorporation into micelles, resulting in a reduction of cholesterol absorption [9]. The phytosterols in this study were from Basikol[®], a mixture of phytosterols from soy, corn and canola oils.

Spirulina is well known for its high levels of chlorophyll [39]. Chlorophyll is metabolized to phytanic (3,7,11,15-tetramethylhexadecanoic) acid, an isoprenoid-derived fatty acid. Phytanic acid binds and activates peroxisome proliferator-activated receptor (PPAR)- α , a nuclear transcription factor [40, 41]. The stimulation of PPAR- α up-regulates lipoprotein lipase, an enzyme that plays a key role in the hydrolysis of VLDL triglycerides [42]. Thus, it is hypothesized that phytanic acid may lower blood lipid levels via its ligand-binding interactions with PPAR- α [43].

Pantethine is a stable disulfide form of pantetheine; a metabolite, cystamine appears to be responsible for its ability to inhibit acetyl-CoA carboxylase [44]. Pantethine has been shown to beneficially alter plasma cholesterol concentrations in experimental animals and humans [12, 45, 46] by inhibiting cholesterol and fatty acid synthesis; interestingly, protein, DNA and phospholipid synthesis are also inhibited in dose-dependent manners [47, 48].

At the highest dose employed in these studies, the formulation represented 9% of the diet. This dosage was chosen because it approximates the minimum dose that has been reported to be efficacious in lowering plasma cholesterol concentrations in a variety of experimental animals

[10-13]. Thus, the significant lowering of plasma cholesterol with the formulation set at 1% of the diet, in the presence of sorghum bran, demonstrates that significant effects can be seen when each ingredient is administered at a much lower dosage than previously reported. This was also observed in the capability of the formulation to lower plasma TG concentrations. In this case, the formulation at approximately 0.5% of the produced a significantly lowering of plasma TG.

The formulation appears to be effective based on incremental, additive effects of these constitutive agents. While we hypothesized that sorghum bran would significantly augment the plasma cholesterol-lowering actions of the formulation, we observed only a modest lowering of cholesterol. When an experiment was performed splitting the four ingredient formulation into two different, two-ingredient formulations, both significantly lowered plasma cholesterol concentrations. A significant difference in their hypocholesterolemic effects between these combination products was not observed. Based on the data presented in Figures 1 and 6, the addition of both formulations together to form the four ingredient nutraceutical formulation yielded a further incremental lowering in plasma cholesterol concentrations. These nutraceutical agents are apparently not acting in a synergistic manner, but each one is contributing to the overall hypocholesterolemic effect of the four ingredient formulation.

This is not the first study to demonstrate that a combination of natural products can be an effective strategy to lower plasma cholesterol concentrations; such a paradigm has been found to be successful in both experimental animals [49] and humans [29]. This report demonstrates that the dosages of the individual natural products present in a formulation can be sharply lowered without a loss of its hypolipidemic actions. This finding should eventually allow for optimization of an efficacious nutraceutical formulation for use in humans.

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TABLES AND FIGURES

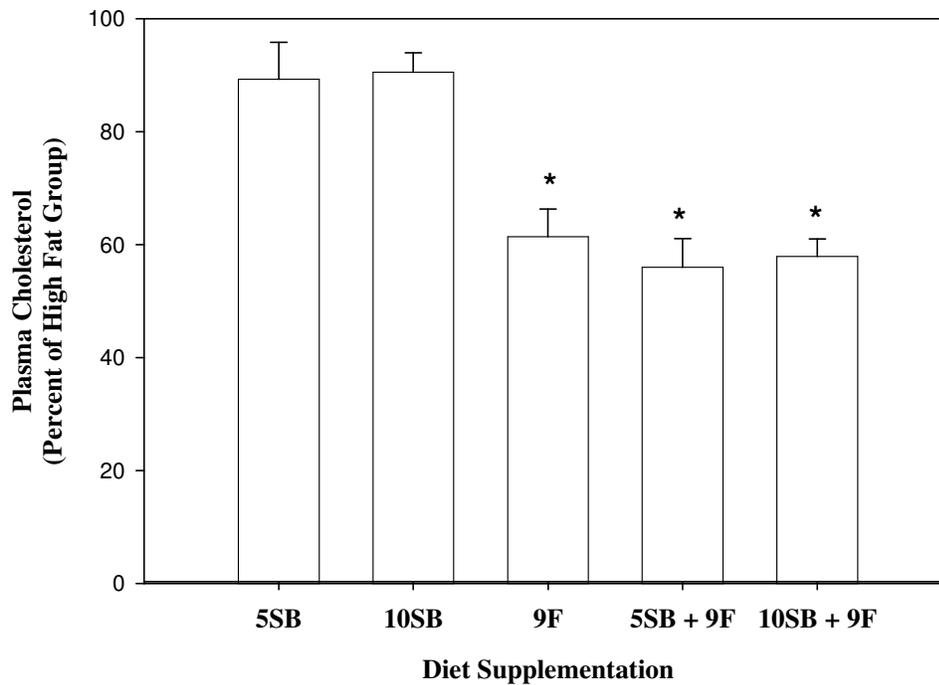


Figure 4.1. Effect of sorghum bran and the nutraceutical formulation on plasma total cholesterol concentration in the hypercholesterolemic hamster. Plasma total cholesterol concentrations in hypercholesterolemic hamsters fed 5 (5SB) or 10% sumac sorghum bran (10SB) alone or in combination with 9% (wt/wt) nutraceutical formulation (9F). Values are expressed as percentage (mean \pm SEM) of plasma cholesterol concentrations of animals fed 5% coconut oil and 0.12% cholesterol. *Significantly different from the group receiving 5% coconut oil and 0.12% cholesterol, $P < 0.05$.

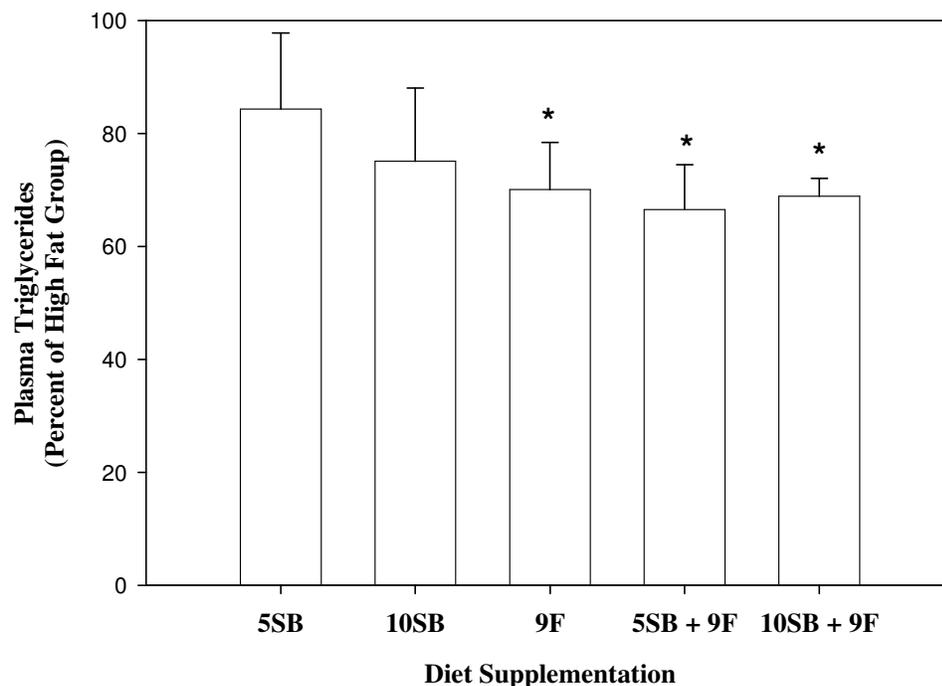


Figure 4.2. Effect of sorghum bran and the nutraceutical formulation on plasma triglyceride concentration in the hypercholesterolemic hamster. Plasma triglyceride concentrations in hypercholesterolemic hamsters fed 5 (5SB) or 10% sumac sorghum bran (10SB) alone or in combination with the 9% (wt/wt) nutraceutical formulation (9F). Values are expressed as percentage (mean \pm SEM) of plasma triglyceride concentrations of animals fed the high fat diet. *Significantly different from the group receiving 5% coconut oil and 0.12% cholesterol, $P < 0.05$.

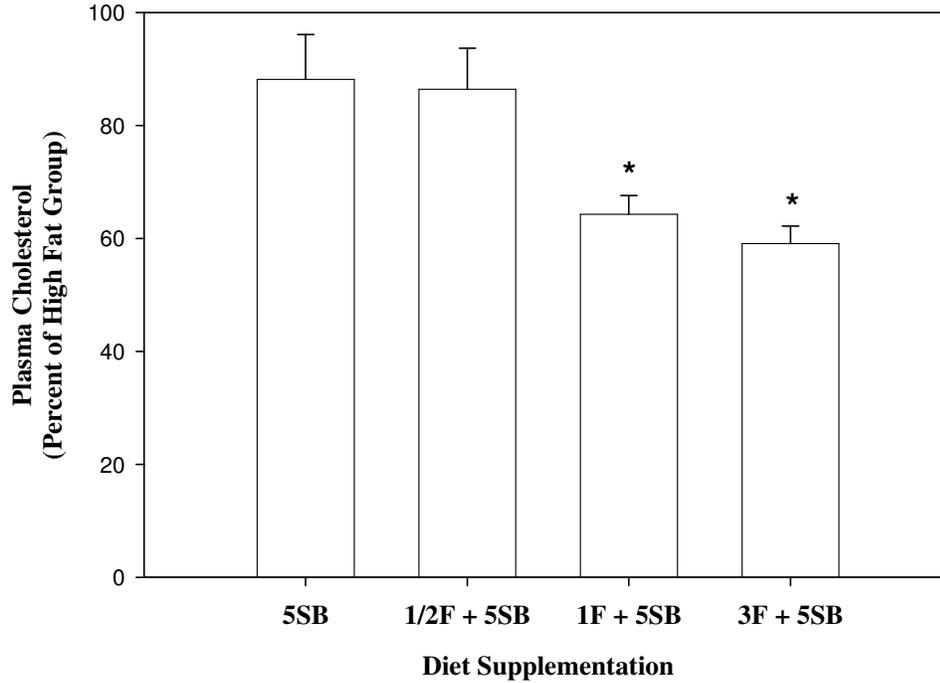


Figure 4.3. Effect of sorghum bran and the nutraceutical formulation on plasma total cholesterol concentration in the hypercholesterolemic hamster. Plasma cholesterol concentrations in hypercholesterolemic hamsters fed 5% sumac sorghum bran (5SB) alone or in combination with 0.5, 1 or 3% of the nutraceutical formulation (0.5F, 1F or 3F, respectively). Values are expressed as percentage (mean \pm SEM) of plasma cholesterol concentrations of animals fed the high fat diet. *Significantly different from the group receiving 5% coconut oil and 0.12% cholesterol, $P < 0.05$.

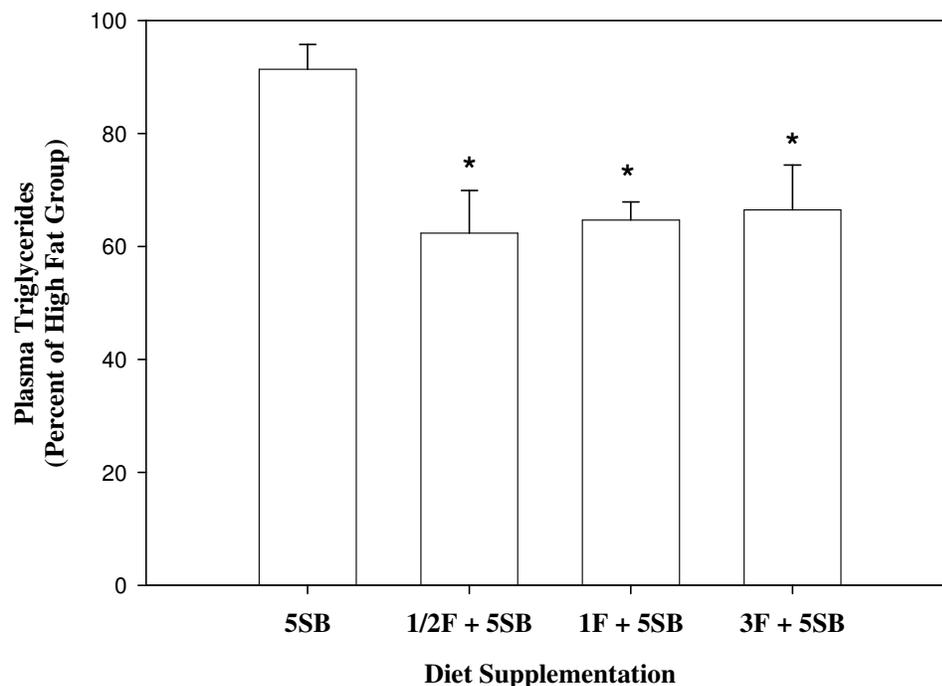


Figure 4.4. Effect of sorghum bran and the nutraceutical formulation on plasma triglyceride concentration in the hypercholesterolemic hamster. Plasma triglyceride concentrations in hypercholesterolemic hamsters fed 5% sumac sorghum bran (5SB) alone or in combination with 0.5, 1 or 3% of the nutraceutical formulation (0.5F, 1F or 3F, respectively). Values are expressed as percentage (mean \pm SEM) of plasma triglyceride concentrations of animals fed the high fat diet. *Significantly different from the group receiving 5% coconut oil and 0.12% cholesterol, $P < 0.05$.

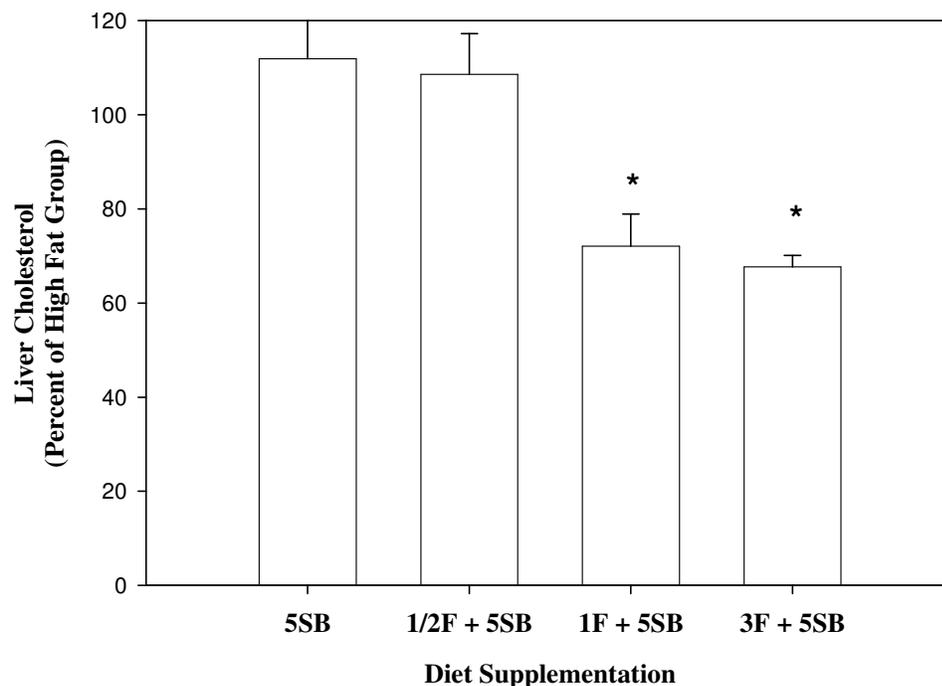


Figure 4.5. Effect of sorghum bran and the nutraceutical formulation on liver total cholesterol content in the hypercholesterolemic hamster. Liver total cholesterol content in hypercholesterolemic hamsters fed 5% sumac sorghum bran (5SB) alone or in combination with 0.5, 1 or 3% of the nutraceutical formulation (0.5F, 1F or 3F, respectively). Values are expressed as percentage (mean \pm SEM) of liver cholesterol content of animals fed a high fat diet. *Significantly different from the group receiving 5% coconut oil and 0.12% cholesterol, $P < 0.05$.

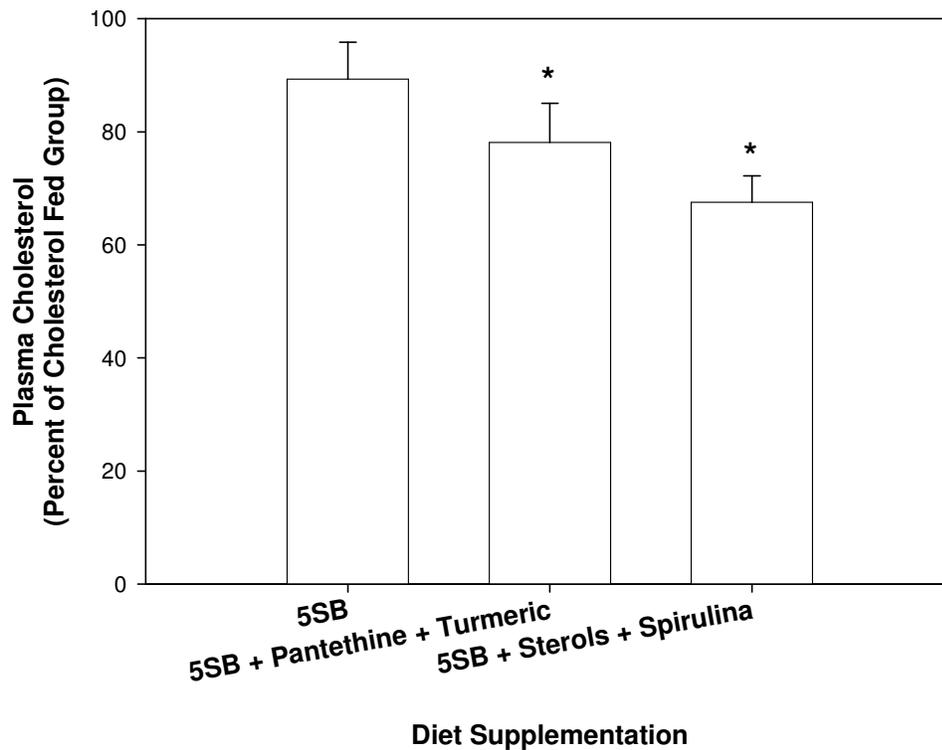


Figure 4.6. Effect of sorghum bran and different nutraceutical combinations on plasma total cholesterol concentration in the hypercholesterolemic hamster. Plasma total cholesterol concentration in hypercholesterolemic hamsters fed 5% (wt/wt) sumac sorghum bran (5SB) alone and two-ingredient nutraceutical formulations comprising 0.5% and 4% of the diet. Values are expressed as percentage (mean \pm SEM) of plasma cholesterol concentrations of animals fed a high fat diet. *Significantly different from the group receiving 5% coconut oil and 0.12% cholesterol, $P < 0.05$.

CHAPTER FIVE

CONCLUSIONS

The results presented in this dissertation demonstrate that varieties of sorghum bran can inhibit inflammatory processes and significantly lower plasma cholesterol in hypercholesterolemic hamsters. The anti-inflammatory effects of sorghum bran were investigated in LPS-stimulated mononuclear cells and in the TPA model of ear inflammation. Black sorghum bran extract significantly reduced the secretion of cytokines TNF- α and IL-1 from LPS-stimulated human peripheral mononuclear cells. Ethanolic extracts of black and sumac sorghum bran produced reductions in ear edema, measured by ear weight and ear thickness, and MPO activity, an indication of neutrophil involvement. No effect was observed with white or mycogen sorghum bran varieties or with extracts of rice, oat and wheat brans. The anti-inflammatory activity positively correlated with the high phenolic and antioxidant activity of black and sumac sorghum brans. Black and sumac sorghum bran extract did not affect COX-2 protein expression, an enzyme involved in prostaglandin biosynthesis.

We tested the cholesterol lowering capabilities of sorghum wax in hypercholesterolemic hamsters. Very long chain fatty acids and aldehydes, the major components of the wax fraction, have been shown by Cuban research institutions to lower cholesterol in experimental animals and humans. Sorghum wax failed to lower plasma and liver cholesterol in hamsters. These results are in agreement with numerous studies conducted by non-Cuban research facilities and we question the efficacy of the very long chain fatty acids and aldehydes. This finding is quite

timely because policosanol, a mixture of very long chain fatty alcohols, has recently been added to One-A-Day® Cholesterol Plus multivitamin.

The hypocholesterolemic action of sumac sorghum bran was evaluated in hamsters. Sorghum bran, a rich source of tannins and anthocyanins, significantly reduced plasma cholesterol and triglycerides in hyperlipidemic hamsters when incorporated at 20% of the diet. The addition of sorghum bran at 5 and 10% in the diets produced non-significant decreases of 9 and 11%, respectively. This lack of significant cholesterol-lowering effect is a result, to some degree, of high animal variability.

The effects of four nutraceuticals on plasma and liver cholesterol concentrations in hamsters were investigated. Significant reductions in plasma and liver total cholesterol concentrations and plasma triglycerides were observed with the combination of four natural components (blend of phytosterols, turmeric, *Spirulina*, and pantethine) in the hyperlipidemic hamster. The addition of sorghum bran to the formulation did not produce further reductions in plasma and liver cholesterol. Therefore, it was determined that the bulk of the cholesterol-lowering efficacy was attributed, not to sorghum bran, but to the four natural ingredients. These ingredients alone have all been previously reported to lower cholesterol, however, in combination, their additive pharmacological effects allowed for the administration of smaller doses than previously documented. These results should allow for the optimization of an inexpensive and efficacious cholesterol-lowering nutraceutical formulation.

The outcome of this research approach is the incorporation of select sorghum brans into the American diet or as a functional food, nutraceutical or cosmeceutical. These results suggest that consumption of rather small amounts of specific natural products may lower cholesterol and triglycerides and possibly reduce the incidence of cardiovascular disease. The bran fraction of

select sorghum grains, which are among the top five grains produced worldwide, may prove useful in lowering plasma cholesterol and ameliorating the inflammatory process in humans.