NUTRACEUTICAL PROPERTIES OF THE MUSCADINE GRAPE (*VITIS ROTUNDIFOLIA*), *SORGHUM BICOLOR*, AND *POLYGONUM CUSPIDATUM*

by

EVE E. BRALLEY

(Under the guidance of Diane K. Hartle)

Abstract

Muscadine skins and seeds of two varieties were analyzed for total phenolic content and antioxidant ability. Extracts were then screened for abilities to directly inhibit hyaluronidase activity *in vitro.* Hyaluronidase is an enzyme responsible for the depolymerization of hyaluronic acid, a major component of the extracellular matrix of soft connective tissues including the synovial membrane and fluid. Its activity is implicated in inflammatory diseases such as osteoarthritis, facilitating the degradation of cartilage, and stimulating further inflammatory mediators. Similarly, a variety of sorghum brans versus other common grain brans were tested for total phenolic content, antioxidant activity, and anti-hyaluronidase activity *in vitro*. In other studies, extracts of muscadine skin, seed and pomace, and *Polygonum cuspidatum*, an herb rich in *trans*-resveratrol, were screened for anti-inflammatory activity *in vivo*. In the phorbol myristate (TPA) inflamed mouse ear edema model, markers of both acute and chronic inflammation were measured after treatment with each test extract. Finally, muscadine skin extracts were tested for the ability to decrease inflammation in the trinitrobenzene sulfonic acid (TNBS) colitis model in rats. **Results**: Hyaluronidase activity was inhibited by both muscadine seed and skin fractions. The seed fraction was 2-3 times more potent than skins on a wt/wt basis. Hyaluronidase activity in both correlated directly with total phenolics and antioxidant activity of

the extracts. Sorghum bran inhibited hyaluronidase more potently than other bran. Sumac sorghum bran had the greatest inhibitory activity. Inhibition again correlated with total phenolics and antioxidant activity in each bran. Commonly used wheat and rice bran had weak inhibitory activities relative to the high phenolic containing grain sorghum brans. In the TPA model of ear inflammation, extracts of muscadine skin, seed, and combination treatments significantly reduced ear edema, ear biopsy weight and polymorphonuclear infiltration compared to TPA vehicle control. *Polygonum cuspidatum* extract also inhibited both acute and chronic inflammation in the TPA model in a dose-dependent manner, and was more potent than *trans*-resveratrol when tested at comparable doses. In the TNBS model of colitis, muscadine-enriched diets decreased neutrophil invasion into the colonic tissue, edema and macroscopic scores. Inflammation in the colon was eliminated in rats receiving the muscadine enema treatment. In these, TNBS produced no significant changes in markers from healthy animals. **Conclusions**: Muscadine grape skin/seed, sorghum bran, and *Polygonum cuspidatum* extracts have high total phenolics, high antioxidant abilities, and are highly anti-inflammatory in both *in vitro* and *in vivo* screening systems. These qualities support use of these bioactive botanicals and/or their fractions in functional food, nutraceutical and cosmeceutical products.

KEY WORDS: Muscadine grapes, Sorghum grain, Polygonum cuspidatum, anti-inflammatory, nutraceuticals.

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B.S., The University of Georgia, 1998

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2007

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ACKNOWLEDGEMENTS

I owe thanks to many for help, guidance and encouragement throughout my degree program. First, I would like to thank my major advisor, Dr. Diane K. Hartle, for believing in me from the beginning. She took me under her wing with enthusiasm, and has been a valuable mentor in many ways including inspiring me with her vision. I hope to exude the strengths I see in her as a scientist and visionary in my future endeavors.

Although not 'officially' a major advisor, I feel fortunate that I have had essentially two mentors closely involved in my graduate career who foil each other so perfectly. Without Dr. Phillip Greenspan, I could not have achieved such an accomplishment in the lab. We have troubleshooting down pat, and it has come with lots of laughter. He has made coming to lab each day interesting, and forever full of learning experiences.

I would like to thank Dr. James L. Hargrove for not only playing an integral part in my starting this degree program, but for also being a wonderful editor and thinker. He is a joy to be around and a pleasure to work with.

I also sincerely thank my additional committee members, Dr. Opal Rebecca Bunce, and Dr. Roger Dean for your support by way of ideas, questions, suggestions, and a genuine care for the process.

I am grateful to the American Foundation for Pharmaceutical Education for their generous monetary gifts that have helped to support my education.

Special thanks go to my labmates Amy Burdette and Linda Duncan. Without you women in my life--our lunches at the Georgia Center, our talks about life and love, our genuine

friendships, carrying each other through good times and bad-- I know my time here wouldn't have been as meaningful. I look forward to many more years of lasting friendship.

I would also like to thank Joy Wilson and Mary Eubanks for their assistance during my graduate training. You have helped me so much, and always with a smile on your face. You two make this program run smoothly, and I hope you know how many people you help along the way.

I would also like to thank Michael Cook for his unconditional love, support, and encouragement he has given me. I thank him for the happiness he brings to my life, and for his gift of helping me keep it all in perspective.

Finally, I would like to express my deepest gratitude to my family for their continual love and support as I make my way through this life. I see the beauty of what love, hard work, and appreciation for life can do for the world. I look forward to continuing this journey with you.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

There is growing interest in the potential health benefits of diets enriched with fruit, vegetables, and whole grains. Many studies report an inverse association with intake and chronic disease development including cardiovascular disease, cancers, diabetes, and other inflammatory disease states [1-4]. Steinmetz and Potter reviewed 206 human epidemiologic studies and 22 animal studies looking at the relationship between fruit and vegetable consumption and the risk of cancer and reported a highly consistent protective effect [1]. Numerous studies have reported a protective effect of fruit, vegetable and whole grain intake against cardiovascular disease [5, 6]. The First National Health and Nutrition Examination Study (NHANES I) Epidemiologic Follow-up Study (n = 9,608) reported a strong inverse relationship between mortality from cardiovascular disease and fruit and vegetable consumption when just comparing 3 servings of vegetables and fruit per day to 1 serving [2]. Epidemiologic studies report that whole grain consumption is associated with decreased risk of heart disease and cancer possibly due to a combination of dietary fiber and phytochemicals in the bran fractions [3]. The overwhelming evidence that consumption of fruits, vegetables and whole grains is health-promoting is undisputed and these correlations have led to United States dietary guidelines that recommend 5-10 fruits and vegetables a day and replacement of decorticated grain products with whole grains.

The research presented in this dissertation focuses on the health-promoting, anti-inflammatory benefits of a fruit, a grain, and a medicinal herb: the muscadine grape (*Vitis rotundifolia)*, *Sorghum bicolor*, and *Polygonum cuspidatum*.

Muscadine Grapes

Muscadine grapes (*Vitis rotundifolia*) have been enjoyed for centuries for their taste and their use for wines, jams and sauces. The English explorer, Sir Walter Raleigh, discovered muscadines in America in 1584 describing them as being "on the sand and on the green soil, on the hills as on the plain, as well as on every little shrub…also climbing towards the top of tall cedars…in all the world like abundance is not to be found." The grape is native to southeastern United States but extends north to Delaware, and west along the Gulf of Mexico to eastern Texas. Georgia is its largest commercial producer with over 1100 acres. The grapes require a long growing season of 100 days to mature fruit. Grown in loose clusters of up to 40 grapes, the grapes ripen individually over an extended harvest period. The fruits are about 1-1.5 inches in diameter, 5-15 g, and have a thick, tough skin and several hard, oblong seeds. The *Vitis rotundifolia* species differ from *Vitis vinifera* (table grape) in many ways including the anatomy and morphology of the vine and fruit, the ability of latent buds to produce new shoots and roots, and the physical and chemical characteristics of the fruit, juice and wine [7]. Muscadine grapes also have an additional pair of chromosomes compared to table grapes ($2n = 40$ for muscadine grapes and $2n = 38$ for other grapes).

Only a small percentage of muscadines are marketed as fresh fruit, and 80% is processed for juice, wine, jellies and jams. Because of the thick, tough skins, there is a low juice yield leaving about 40-50% skins, pulp and seed (pomace) as a waste byproduct. More recently the industry has begun using the dried skin, seeds and pomace in food supplements and food products as a way to increase the market value per ton harvested, expand the uses of the muscadine beyond the traditional ones, and, coincidentally, decrease the cost of waste disposal.

Interest in the nutraceutical properties of muscadine grapes began because of their abundance of beneficial phytochemicals and their antioxidant capacity. Muscadine grapes have a high total phenolic acid content characterized mainly by high ellagic acid compounds, gallic acid, and anthocyanin 3,5-diglucoside concentrations [8-12]. Ellagic acid, myricetin, quercetin, kaempferol and *trans*-resveratrol are the most abundant phenolics in the skins, while gallic acid, catechin, epicatechin and oligomeric proanthocyanidins (OPC's) are abundant in the seeds [10]. The deep color of the grape skin in red, purple and black cultivars is attributed to anthocyanins that include delphinidin, cyanidin, petunidin, peonidin, and malvidin [8].

Biological activity of the Muscadine Grape

Anti-Cancer Activities

Yi et. al. studied the anticancer activities of muscadine grape crude extracts, and fractionated polyphenolics *in vitro* [13]. Cell-proliferation and apoptosis were measured after exposure of two colon cancer cell lines (HT-29 and Caco-2) to muscadine grape fractions. The crude muscadine extract (1-7 mg/mL) inhibited cancer cell proliferation by 50%, and the phenolic acid fractions were more potent with an IC_{50} of 0.5-3 mg/mL. DNA fragmentation, a marker of apoptosis, was increased 2- to 4-fold upon exposure to muscadine grape fractions, with the anthocyanin fraction being the most potent.

Aqueous extracts of muscadine pomace possessed significant anti-cancer activity *in vitro* by suppressing mutagenesis in *Salmonella typhimurium* cultures, stimulated by 2-aminoantracene, a potent mutagen [14]. Each extract tested had high antioxidant activity and also inhibited matrix metalloproteinase (MMP)-2 and -9 activities. MMPs are involved in angiogenesis, recruitment of growth factors, and tumor growth and establishment [15, 16].

Hudson, et. al. studied the chemopreventive properties of muscadine grape skin extract (GSE) against four human prostate cancer cell lines [17]. In each line, muscadine GSE inhibited prostate tumor cell growth and induced significant morphological changes in the cell. Apoptosis was induced by 24 hours. They compared GSE with resveratrol, and in each cell line, resveratrol inhibited prostate tumor cell growth, but the mechanism was different. Resveratrol, instead of inducing apoptosis, arrested the cell cycle in the G1 phase.

Anti-Diabetic Activities

Muscadine grape seed and skin extracts inhibited the formation of advanced glycation end (AGE) products *in vitro* [18]. AGE product formation has been linked to the pathogenesis of diabetes, and can lead to an enhanced formation of reactive oxygen species and activation of nuclear factor-κB (NF-κB) [19]. The muscadine seed extract was a more potent inhibitor of AGE product formation than the skin extract, and correlated with the total phenolic content in each extract.

Muscadine wine intake by diabetics with each meal for 28 days decreased several metabolic indicators associated with diabetic conditions including lower levels of blood glucose, insulin, glycated hemoglobin, sodium and chloride [20]. Red blood cell membrane saturated fatty acids were decreased and mono- and polyunsaturated fatty acids were increased by muscadine wine intake, indicating improved membrane fluidity. Liver function markers were also improved in diabetic patients consuming muscadine wine (150 mL/meal).

Anti-inflammatory Activities

Muscadine grapes have been show to be highly antioxidant as assessed by ORAC (oxygen radical absorbance capacity) and FRAP (ferric reducing antioxidant potential) assays [11, 12, 21]. In addition to the antioxidant potentional, our laboratory observed that muscadine extracts

have anti-inflammatory effects both *in vitro* and *in vivo* [22]. For example, the release of superoxide from phorbol myristate acetate-activated neutrophils was inhibited by the addition of muscadine skin extract. Secondly, the release of the cytokines TNF-α, IL-6, and IL-1β was inhibited with muscadine skin extract in lipopolysaccharide-activated peripheral blood mononuclear cells. In addition, rats fed a diet of 5% powdered muscadine grape skin had 50% less paw edema than control animals fed a regular chow diet when injected with carrageenan into the foot pad [22].

Sorghum Grain

Grain sorghum is native to Africa and has been eaten as a food grain since 6500 B.C.. Because of its drought-tolerant capabilities, it was domesticated around 2000 B.C. As a source of a reliable harvest in semi-arid regions, sorghum spread to similar regions of China and India [23]. To date, sorghum is the fifth in worldwide cereal grain production behind maize, wheat, rice, and barley with over 58,000,000 metric tons produced [24]. Sorghum was introduced to the United States in the 1700s by Benjamin Franklin. Currently the US cereal sorghum production ranks third behind maize and wheat, with 11,554,970 metric tons produced mainly in the Southern Great Plains region [24]. Although half of the sorghum grown worldwide is used for human consumption, the United States primarily uses it domestically for animal feed and ethanol, but it exports almost half of its yearly production. Sorghum grain consumption for human food has increased in recent years due to the need for those with Celiac disease or gluten allergy, to find a gluten-free flour source. Sorghum is also being used to produce fuel ethanol for industrial applications and automobile fuel.

The genus sorghum is genetically diverse with over 400 species. Some sorghum varieties have extremely high contents of phenolic compounds and antioxidant potential that aid in the natural defense against pest and diseases [25]. Sorghum phenolic compounds are mainly located in the bran fraction and fall into three categories: phenolic acids, flavonoids, and condensed tannins [26]. The phenolic acids are benzoic or cinnamic acid derivatives. The flavonoids are largely anthocyanins, flavan-4-ols, flavones, and flavanols [27-29]. Condensed tannins, or proanthocyanidins, are high molecular weight polymers of flavan-3-ols, usually catechin and epicatechin [30]. The high levels of phytochemicals in select sorghum bran indicate significant nutraceutical potential. Today, the high tannin varieties are only a small segment of the sorghum industry used as pollinators and forage. Commercially preferred sorghum varieties have been developed with reduced tannin content mainly because of the bitter flavor the tannin brings to the whole flour and bran and the fact that the phenolics decrease fermentation rate for the beer and ethanol industry.

Biological activities of Sorghum grain

Cardiovascular Disease (CVD) Protective

Cereal bran consumption is implicated in CVD risk reduction potentially due to the phytosterol, polyphenolic and fiber fractions. Cholesterol-lowering effects were seen in guinea pigs fed a low-tannin sorghum grain as 58% of the diet and the effect was greater than that produced by wheat, rolled oats, or pearl millet [31]. In a dose-dependant manner, hexane extracts of sorghum grain inhibited the enzyme 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase from rat liver microsomes [32]. In the same study, rats fed whole sorghum grain as 30% of the diet had an increased fecal bile acid secretion and an increase in HDL cholesterol, without a change in total cholesterol, implicating an overall anti-cholesterolemic effect. Burdette et. al. found that

hamsters fed a high cholesterol diet had a significant decrease in plasma total cholesterol and plasma triglyceride concentration when supplemented with 20% sorghum bran [33].

Aside from its cholesterol-lowering effects, sorghum grain may be potentially cardio-protective by way of maintaining red blood cell (RBC) integrity. Hemoglobin-catalyzed-oxidation of linoleic acid was inhibited in mullet fish fed a diet of high-tannin sorghum [34]. This inhibitory activity significantly improved RBC membrane integrity and blood-thinning ability. RBC hemolysis induced by H_20_2 was reduced, maintaining normal blood fluidity. The authors attributed these effects to the polyphenols present and their resulting anti-oxidant capabilities.

Anti-Diabetic Activites

Sorghum bran extracts inhibited the formation of advanced glycation end (AGE) products *in vitro* [35]. AGE product formation has been linked to the pathogenesis of diabetes, and can lead to an enhanced formation of reactive oxygen species and activation of nuclear factor-κB (NF-κB)[19]. The high total phenolic, and antioxidant sorghum bran extracts were more potent inhibitors of AGE product formation than the lower tannin sorghums. Wheat, rice, and oat bran did not significantly inhibit AGE product formation.

In numerous studies, high tannin sorghums have been reported to decrease weight gain in rats, pigs, rabbits and poultry [36-39]. While this is an undesirable characteristic in meat animal production, it may be desirable in the human diet to help reduce obesity, a risk factor for diabetes and heart disease. Tannins are large molecules that have a high binding affinity for proteins and may decrease the nutritive value of the foods. The tannins might also bind directly to digestive enzymes including sucrase, amylase, trypsin, chymotrypsin, and lipase [36, 40-42]. High tannin sorghums are slowly digested, thereby increasing the period of satiety or fullness after a meal.

Anti-Cancer Activities

Epidemiological studies from Africa, Russia, India, China, and Iran found that sorghum consumption consistently correlated with lower incidences of esophageal cancer whereas wheat and corn-flour consumption correlated with increased incidences [43, 44].

Grimmer et al reported that the polyphenol-rich fractions of sorghum grain acted as antimutagens when coincubated with standard mutagens [45]. In another *in vitro* study, sorghum tannins increased melanogenic activity, a process that protects against ultraviolet irradiation damage.

Anti-inflammatory Activities

Little research has been done to study the anti-inflammatory effects of sorghum bran. Recently, our lab reported inhibition of inflammation after phorbol-myristate acetate application to the mouse ear treated topically with sorghum bran extract [46]. Sorghum bran extract also inhibits TNF-α, IL-6, and IL-1ß release from human mononuclear cells activated with LPS [46].

Polygonum cuspidatum

Polygonum cuspidatum (PC), commonly called Japanese knotweed or Mexican bamboo, is a member of the polygonaceae family that is widely distributed in Asia and North America. In traditional Chinese medicine, PC is called Hu Zhang. It is used as an analgesic, antipyretic, diuretic, and an expectorant [47] in treatments for arthralgia, jaundice, amenorrhea, chronic bronchitis, and/or hypertension [47]. PCE (PC extract) is now in many nutraceutical product formulations because of its high concentration of *trans*-resveratrol, a polyphenolic *trans*-stilbene (3, 4'-5-trihydroxystilbene)[48, 49]. Other resveratrol analogs in PC root mainly include

resveratroloside (3, 5, 4'-trihydroxystilbene-4'-*O*-beta-D-glucopyranoside, piceid (3, 4'-trihydroxystilbene-3-beta-mono-D-glucoside) and piceatannol glucoside (3, 5, 3', 4'-tetrahydroxystilbene-4'-*O*-beta-D-glucopyransoide [50-52]. In addition, PC contains emodin and emodin 8-*O*-glucopyranoside.

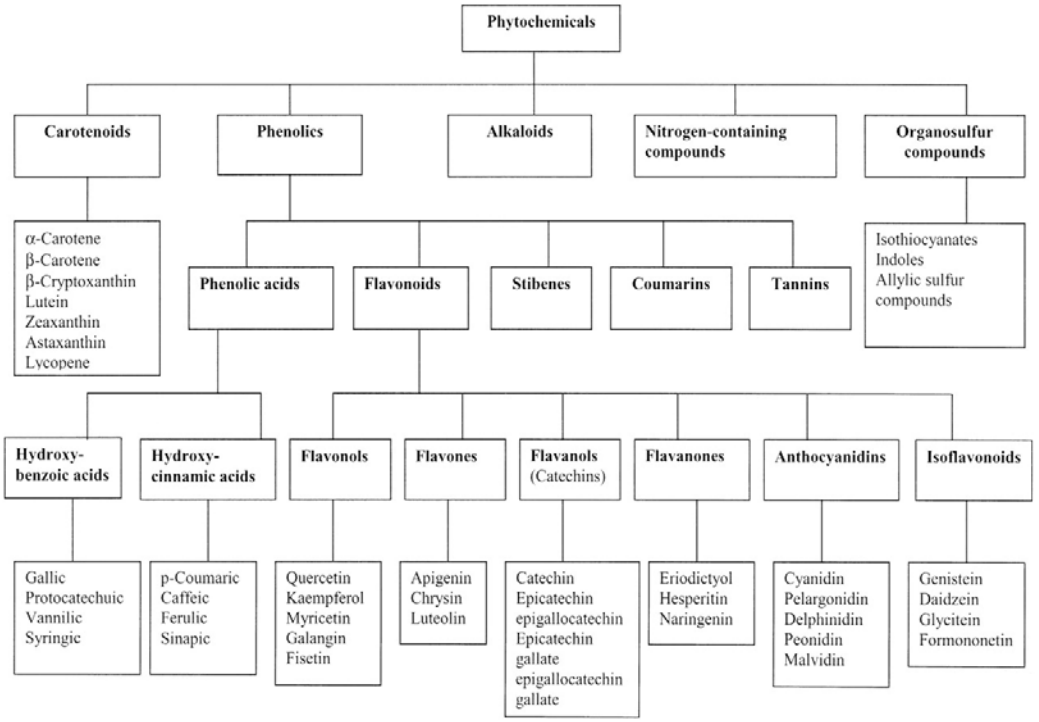
Biological activities of Polygonum cuspidatum

While most PC research to date has been done on individual chemical constituents PCE extracts are widely used in nutraceutical and cosmeceutical products. Biological testing of the PCE in test systems has yielded some interesting experimental data mostly from *in vitro* screening tests. PCE is suggested to be cardioprotective because it decreases cellular cholesterol ester content in HepG2 cells by inhibiting acyl-coenzyme A-cholesterol acyltransferase activity [53]. Resveratrol and emodin components isolated from PC are both protein tyrosine kinase C inhibitors [54, 55]. These kinases play an important role in regulation of cell growth and transformation and are therefore potential targets for anticancer agents. PCE exerts chemopreventive effects as demonstrated by its inhibition of growth of three prostate cancer cell lines [56]. PCE also possesses some anti-viral properties by inhibiting the production of the hepatitis B virus [57]. To date, there has been little *in vivo* testing of PCE.

Phytochemicals

Many phytochemicals are responsible for the health-promoting effects of diets rich in vegetable and fruit. These phytochemicals are grouped according to their chemical structures (Figure 1.1) [58-60].

FIGURE 1.1 Classification of dietary phytochemicals



Liu, R. H. J. Nutr. 2004;134:3479S-3485S

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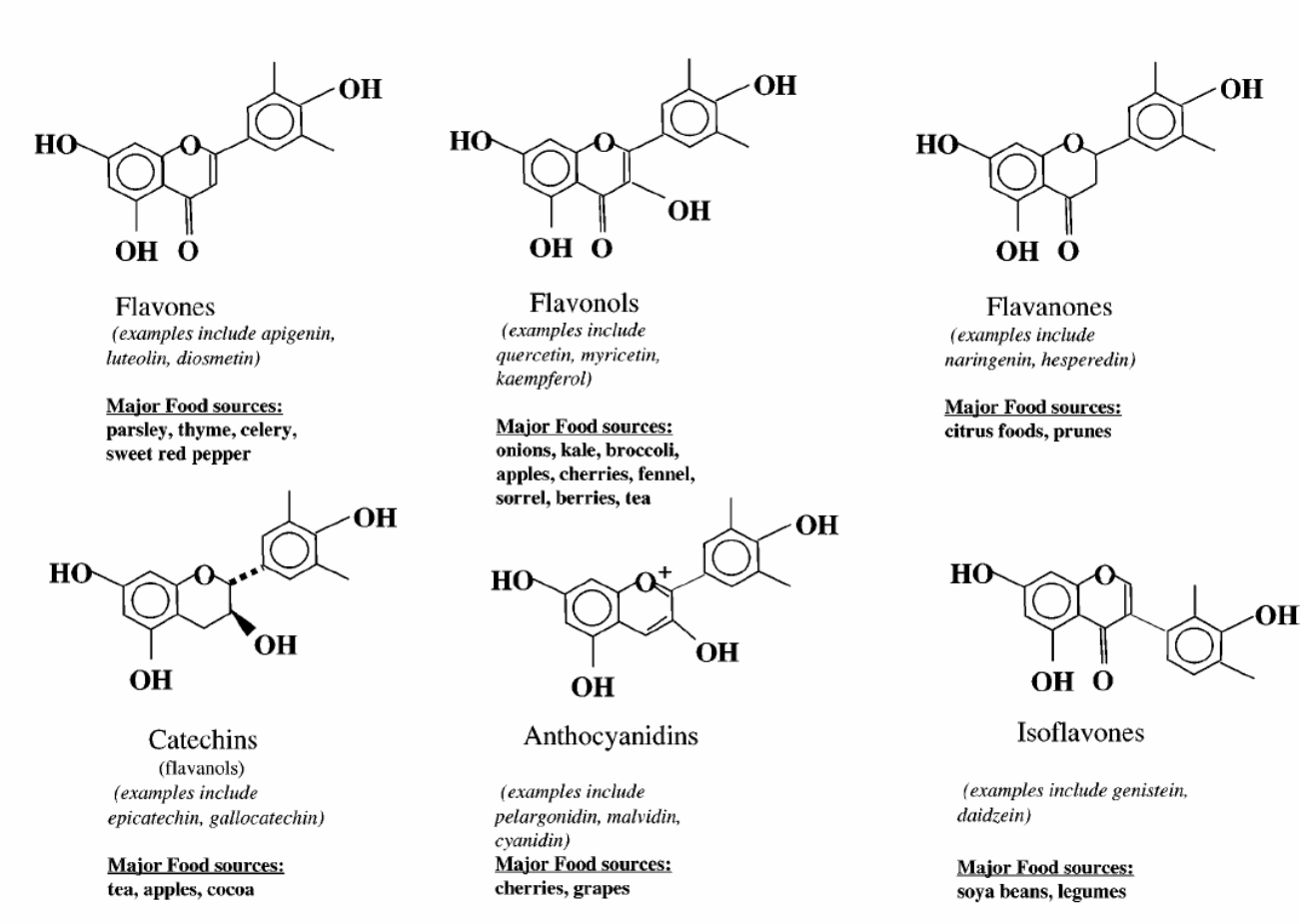
Flavonoids

Flavonoids are the most abundant group of plant polyphenols and provide much of the flavor and color of the food. There have been over 4000 structurally unique flavonoids identified in plants sources, and they are important components of plant physiology. They are light-responsive, and can control the level of auxin, a hormone responsible for plant growth and differentiation [61]. Flavonoids act as antifungal and antibacterial agents, and can act as anti-feedants to ruminants and other foraging animals and insects by making the plants distasteful [62, 63]. Pollination can be affected by inhibiting or stimulating the insects to feed [63]. Flavonoids can also chelate metals making it possible for plants to grow in contaminated soils, as well as protect against oxidant insult and UV radiation [64].

In the normal US diet, less than 1 g of flavonoids is generally consumed daily, with the highest fraction coming from cocoa, coffee, tea, beer and wine (420 mg). The estimated distribution of flavonoids in the diet is 44 mg from cereals, 79 mg from potatoes, bulbs and roots, 45 mg from peanuts and nuts, 162 mg from vegetables and herbs and 290 mg/day from fruits and juices [65]. Most flavonoids enter the diet as glycosides.

Of the six major classes of flavonoids (Figure 1.2), the botanicals in this dissertation mainly contain flavonols (quercetin, myricetin), flavanols (catechin, epicatechin) and the anthocyanidins (cyanidin, delphinidin, malvidin, petunidin, and pelaronidin), and to a smaller extent, flavones (apigenin, luteolin).

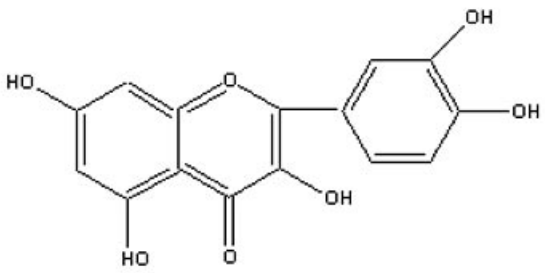
FIGURE 1.2 Major classes of Flavonoids



Ross JA, Kasum CM: Dietary Flavonoids: Bioavailability, Metabolic Effects and Safety. *Annual Rev of Nut* 2002; 22:19-34

Quercetin

FIGURE 1.3 Structure of Quercetin



Quercetin (3,3',4',5, 7-pentahydroxyflavone) is one of the most widely distributed flavonoids and is found in highest amounts in apples, tea, onions, red grapes, citrus fruits, broccoli and other leafy greens, cherries, and a variety of berries [66]. Quercetin is the aglycone form of other flavonoid glycosides such as rutin and quercitrin, but is found to be more biologically active. The biological activities are broad and include anti-cancer, antiviral, anti-inflammatory, and cardioprotective properties.

Quercetin was initially thought of to be mutagenic in initial *in vitro* assay systems [67, 68]. Consequently further study was done *in vivo*. FASEB reports from 17 feeding studies performed in rats, mice, and hamsters supplemented with 0.25 – 10% quercetin in the diet gave no evidence towards carcinogenicity with the exception of Ertuk et al. who found increased bladder tumors in rats [69, 70]. The National Cancer Institute screened over 200 naturally occurring flavonoids and quercetin was reported as increasing the lifespan of mice with P-388 leukemia [71]. Quercetin administered as 4% of the diet prevented nuclear injury in mice induced by an intrarectal dose of

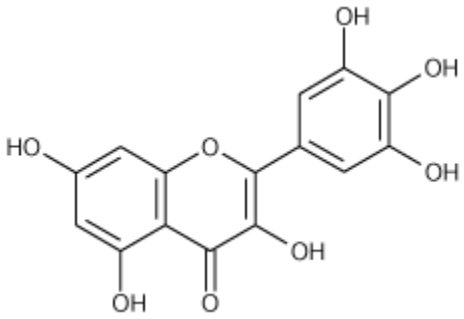
benzo[α]pyrene possibly by binding to the DNA [72]. In mice treated with phorbol myristate (TPA), a tumor promoter, initiation and promotion of skin carcinogenesis was blocked with quercetin topical administration [73]. Mammary tumor induction by the carcinogens dimethylbynzanthracene or *N*-nitrosomethyl urea was reduced by dietary quercetin with no toxicity or weight loss seen in the animals over a 20-week period [74].

Numerous antiviral activities of quercetin have been reported in such enveloped viruses such as herpes simplex 1, respiratory synctial, pseudo rabies, parainfluenza type 3 and Sindibis [75, 76]. Quercetin has been found to be able to bind to viral protein and interfere with viral nucleic acid synthesis [77]. Antiviral activity of interferon and 5-ethyl-2-deoxyuridine was enhanced by quercetin [78].

As an anti-inflammatory agent, quercetin was found to inhibit lipoxygenase (LOX) and cyclooxygenase (COX) enzymes resulting in subsequent inhibition of inflammatory mediator formation such as prostaglandins and leukotrienes [79]. Quercetin was also found to be a phospholipase A_2 inhibitor in human leukocytes thereby reducing arachidonic acid, the substrate for LOX and COX, release from cellular membranes [80]. The phorbol myristate-induced respiratory burst was reduced in neutrophils exposed to quercetin with a decreased production of superoxide anion and H_2O_2 [81, 82]. Nuclear Factor-κB (NF- κB) activation was inhibited in a dose-dependent manner by quercetin in rat hepatocytes stimulated with IL-1β [83]. The cardioprotective effects of quercetin can be attributed mainly to its antiplatelet aggregation ability [84], its antioxidant ability preventing the oxidation of LDL [85], and its anti-inflammatory ability.

Myricetin

FIGURE 1.4 Structure of Myricetin



Myricetin is one of the more abundant flavonoids found in the muscadine grape. It is a potent antioxidant [86]. In human lymphocytes, myricetin decreased oxidant-induced DNA strand damage [87]. Myricetin was found to be neuroprotective, acting as an antioxidant and reducing calcium-induced increase in oxidative metabolism of rat brain neurons [88]. In endothelial cells activated with TNF-α, myricetin suppressed the nuclear transcription factor, NF-κB, by inhibiting the kinase responsible for cleaving the inhibitory protein, IκB [89]. Myricetin was found to possess anti-diabetic properties by mimicking insulin. It can also stimulate lipogenesis and glucose transport in rat adipocytes [90]. This would facilitate glucose uptake in the absence of a fully functional insulin receptor.

Catechin/Epicatechin

FIGURE 1.5 Structures of Catechin and Epicatechin

Catechin and epicatechin are major chemical constituents in teas and wines and have been studied extensively for their health-promoting capabilities. Crespy and Williamson summarize numerous studies reporting the anti-cancer effect of catechins in *in vivo* models [91]. Catechins were found to be cancer-protective in organ systems including intestine, lung, liver, prostate, and breast.

The cardioprotective properties of the catechins are associated with their antioxidant ability. However, catechins have also been shown to increase the activity of superoxide dismutase, decrease plasma nitric oxide concentration, inhibit platelet aggregation, and decrease absorption of triglycerides and cholesterol *in vivo* [92, 93]. Catechin supplementation can decrease total cholesterol and plasma triglycerides, although there are some discrepancies among studies. Catechin can lower LDL cholesterol and increase HDL cholesterol in apolipoprotein-E deficient mice[94].

Catechins may be useful in treatment and prevention of type 2 diabetes. In studies with diabetic rats, green tea catechin decreased serum glucose, triglycerides, and plasma cholesterol levels [95-97]. In normal rats, green tea catechins decreased plasma insulin and triglyceride levels after a glucose tolerance test [98].

Other Phenolics

Oligomeric proanthocyanidins

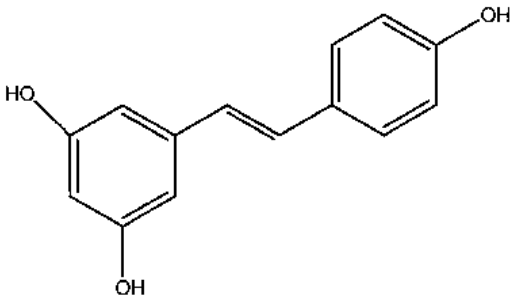
Oligomeric proanthocyanidins (OPCs) are high molecular weight polymers of basic flavanol units, often catechin and epicatechin, with an average polymerization between 4 and 11. Synonyms for OPCs include condensed tannins, procyanidins, leucoanthocyanins, and pycnogenols. In plants, they serve as an important protection against predators because of their astringent flavor. It is that same flavor that is enjoyed in wines, juices, and teas. OPCs are not readily digested in the small intestine, however the small amount that is absorbed into the bloodstream has been shown to significantly enhance plasma antioxidant capacity [99]. The biological activities of OPCs are broad and include antioxidant, anti-inflammatory, anticarcinogenic, and antiviral. A large percentage of OPCs continue, undigested, to the large intestine.

OPCs can inhibit lipid peroxidation, DNA fragmentation, and subsequent apoptosis in hepatic and brain tissue of mice [100]. Cardioprotective activities of OPCs include a vitamin E-sparing effect, stimulation of endothelium-dependent relaxing activity, iron and copper chelators to reduce the oxidative damage to the myocardium, and OPCs can decrease incidence of foam cells [101-103]. OPCs from grape seeds have been shown to inhibit peroxide formation by macrophages *in vitro* [104]. In animal studies, OPCs can inhibit the formation of the inflammatory cytokines IL-1ß, and TNF-α [105]. Numerous *in vitro* studies found OPCs are cytotoxic to a variety of cancer cell lines while remaining non-toxic to normal human cell lines [106]. OPCs inhibit tumor promotion and formation in the phorbol myristate induced skin tumor model [107]. In one human clinical trial, oral administration of OPCs decreased damage from excessive exposure to ultraviolet rays [108]. HIV infection was shown to be inhibited by OPCs *in vitro*, by preventing

binding of the HIV virus to cell receptor sites on normal white blood cells preventing infection [109].

Resveratrol

FIGURE 1.6 Structure of Resveratrol



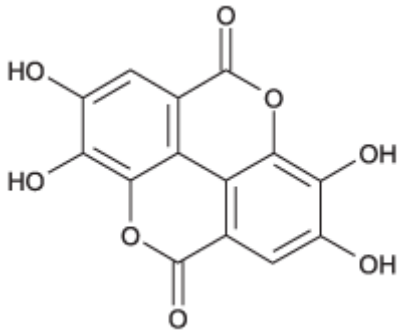
Resveratrol belongs to a class of polyphenols called stilbenes. In plants, resveratrol is useful as a response to stress, injury, fungal infection, and ultraviolet radiation. It is found most commonly in grapes, red wine, purple grape juice, peanuts, some berries, and *Polygonum cuspidatum.* The study of the potential health benefits of resveratrol has gained interest after speculation that it may be responsible for "The French Paradox". In France, the observation was made that mortalitity from coronary heart disease is low despite relatively high levels of dietary saturated fat and cigarette smoking. Regular consumption of red wine might provide protection from cardiovascular disease.

Trans-resveratrol and its derivatives have been studied extensively for their health promoting qualities, including its anti-inflammatory activity. Resveratrol and analogues of resveratrol all inhibit human TNF-α, and LPS-induced activation of NF-κB [110, 111]. Resveratrol can inhibit prostaglandin E_2 release from human peripheral blood leukocytes [112]. In a model of early colonic inflammation in rats, resveratrol significantly decreased elevated plasma levels of prostaglandin D_2 and decreased the expression of COX-2 [113]. Resveratrol also inhibits the TPA-

induced mouse dorsal skin inflammatory response mainly via NF-κB and activator protein-1 (AP-1) modulation [114, 115].

Ellagic Acid

FIGURE 1.7 Structure of Ellagic Acid



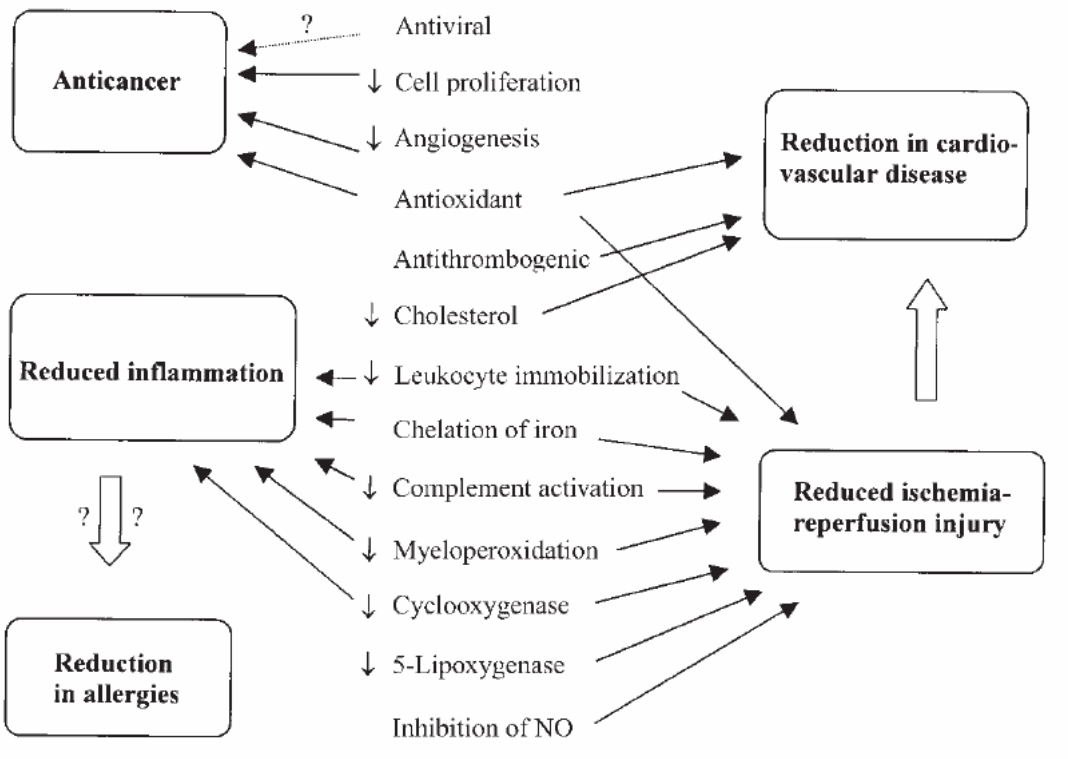
Ellagic acid is found in the blackberry, strawberry, raspberry, walnuts, pecans, green tea, bayberry, pineapple, pomegranate and muscadines. The muscadine is unique amongst other grape species in that it has a significant amount of ellagic acid. It can occur in its free form, as glycosides or as ellagitannins.

Ellagic acid possesses anticarcinogenic activities and has been shown *in vitro* to induce cell cycle arrest and apoptosis in a variety of cancer cell lines [116-119]. Lung and esophageal tumor promotion and formation in rats was inhibited by ellagic acid [120]. A United States patent was submitted relating the use of ellagic acid to the treatment of intestinal disorders such as constipation, heartburn, non ulcer dyspepsia, GERD, and/or esophagitis [121].

Summary of Phytochemical Biological Activities

Evidence of the health benefits of a diet enriched with fruits, vegetables and whole grains is large, and is due, in main part to its phytochemical constituents. The flow chart below summarizes the health-promoting effects of phytochemicals in the diet.

FIGURE 1.8 Possible mechanisms of action of dietary phytochemicals



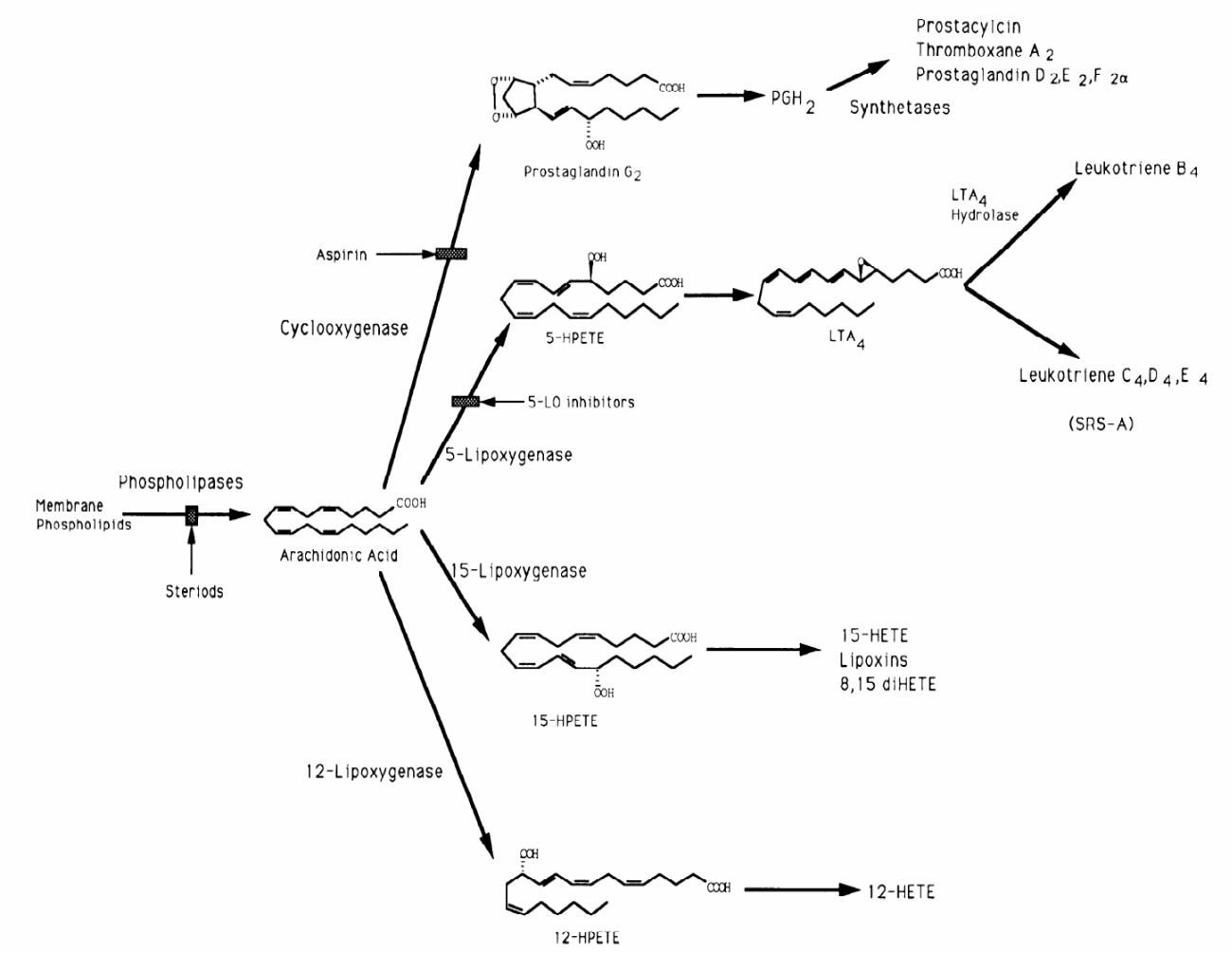
From: Nijveldt RJ, van Nood E, van Hoorn DEC, *et.al.*. Flavonoids: a review of probably mechanisms of action and potential applications. *American Journal of Clinical Nutrition* 2001; 74:418-423.

Inflammation and Arachidonic Acid Metabolism

Chronic systemic inflammation is a component of many age-related diseases such as Alzheimer's, arthritis, asthma, colitis, cancer, diabetes, heart disease, obesity, stroke and psoriasis [122, 123]. Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin 1β (IL-1β), and/or interleukin-8 (IL-8) can cause or contribute to the pathogenesis of these age-related diseases. Other inflammatory mediators such as prostaglandins and leukotrienes produced from the metabolism of arachidonic acid (AA) via cyclooxygenase (COX) and lipoxygenase (LOX) enzymes promote inflammation by recruitment of macrophages, neutrophils, and other leukocytes that release histamine and bradykinins [124].

AA is a 20-carbon fatty acid that is a major component of cellular membrane phospholipids. Inflammatory mediators stimulate a variety of G-protein-coupled receptors, and consequent signal transduction causes calcium mobilization and activates phospholipases. The phospholipases free AA from membrane phospholipids allowing the AA to be metabolized by either the COX pathway, or the LOX pathway (Figure 1.9).

FIGURE 1.9 Arachidonic acid metabolism



Sigal E: The molecular biology on mammalian arachidonic acid metabolism. *American Journal of Physiology, Lung Cellular and Molecular Physiology* 1991; 260: 13-28.

Prostaglandins elicit an inflammatory response by promoting vasodilation and increasing vascular permeability resulting in edema. They also potentiate the vascular permeability changes

elicited by bradykinin and histamine. Leukotrienes, specifically LTB_4, promote neutrophil chemotaxis and vascular adhesion molecules in the endothelium. Other leukotrienes can cause plasma leakage from postcapillary venules, enhance mucus secretion, and can act as eosinophil chemoattractants [125].

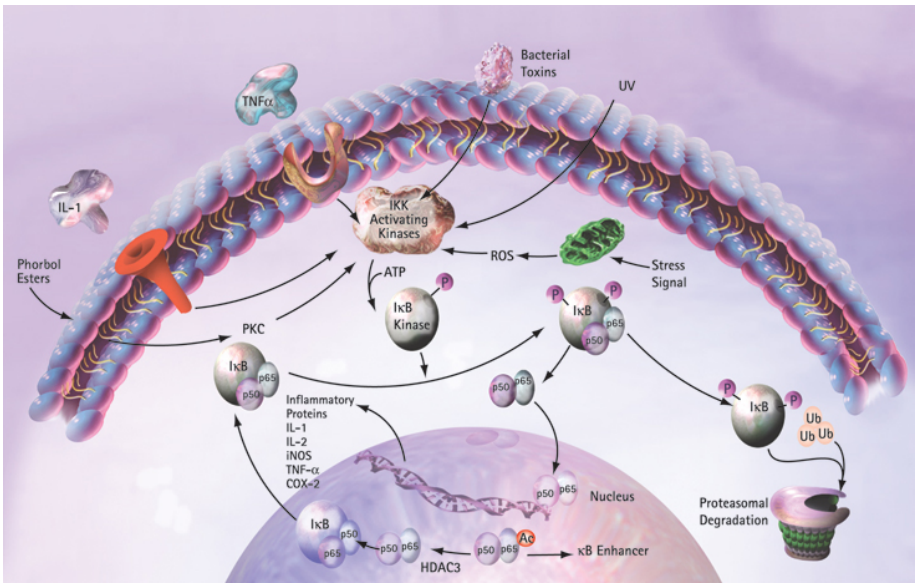
Polyphenols have been reported in numerous studies to inhibit phospholipase A_2 (PLA_2), COX and 5-LOX. In human and rat leukocytes, quercetin was found to inhibit PLA_2 [80]. Inhibition of COX and LOX enzymes by polyphenols can occur either directly, or at the transcriptional level via NF-κB inhibition. Phytochemicals that exhibit anti-COX and/or anti-LOX activity include apigenin, kaempferol, catechin, epigallocatechin gallate, resveratrol, genistein, quercetin, and myricetin [126-130].

NF-κB and Inflammation

Nuclear Factor-κB (NF-κB) is a nuclear transcription factor that is sequestered in the cytosol by its inhibitory protein, IκB. A variety of substances including bacterial toxins, free radicals, cytokines, ultraviolet radiation, and phorbol esters stimulate the activation of IκB kinases that phosphorylate IκB (Figure 1.10). Phosphorylation of IκB causes its degradation, and the p50 and p65 subunits of NF-κB are able to translocate into the nucleus where the induce transcription of a broad range of genes. NF-κB activation induces the expression of over 200 genes that range in function from suppression of apoptosis, induction of cellular transformation, proliferation, invasion, metastasis, and inflammation. Its activation has been linked to a number of diseases including cancer, atherosclerosis, myocardial infarction, diabetes, asthma, arthritis, and other inflammatory diseases [131]. Many phytochemicals have been shown to inhibit NF-κB activation including curcumin, ellagic acid, quercetin, resveratrol, caffeic acid, apigenin, and emodin [128, 132-

[134]. Activation of NF-κB and its subsequent transcription of a variety of inflammatory cytokines is hallmark in inflammatory diseases such as osteoarthritis.

FIGURE 1.10 NF-κB activation pathway

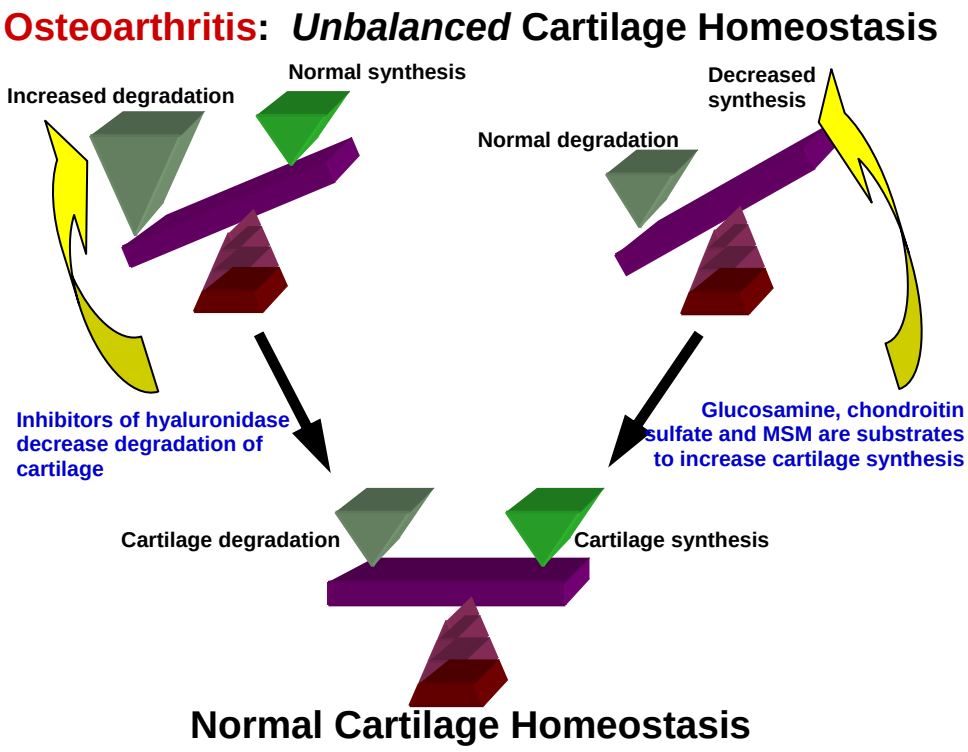


From: http://www.merckbiosciences.co.uk/sharedimages/calbiochem/IS_other_NK-kB_activation.jpg

Hyaluronidase and Osteoarthritis

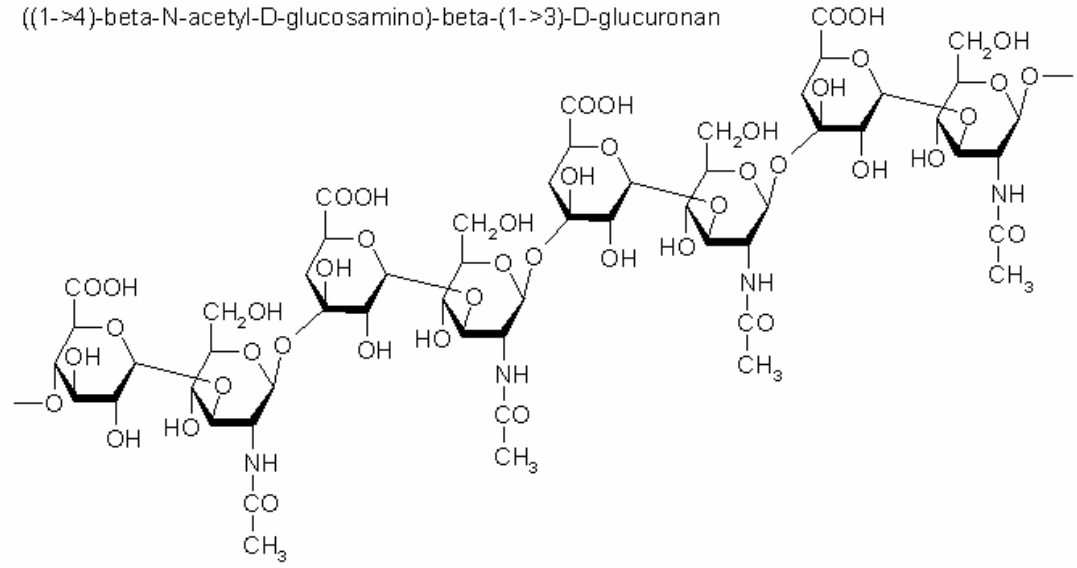
In the normal joint, cartilage is a dynamic tissue that maintains a delicate balance between synthesis and degradation, and is in a constant state of flux (Figure 1.11). Chondrocytes are the main regulatory cells, and are under the influence of circulating reactive oxygen species, prostaglandins, and cytokines. In pathogenic conditions such as osteoarthritis (OA), this balance is disrupted leading to cartilage degradation and subsequent loss of joint function due to a dramatic increase in inflammatory mediators, oxidative stress and degradative enzymes such as hyaluronidase.

FIGURE 1.11 Cartilage homeostasis



Hyaluronan (hyaluronic acid, HA), is a simple, linear glycosaminoglycan (GAG) that is found in almost all vertebrate organs, with the largest proportion found in the extracellular matrix of soft connective tissues including the synovial membrane and the synovial fluid[135]. It is composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, whose molecular size can reach 10^7 kDa.

FIGURE 1.12 Structure of hyaluronan



HA has several important structural and biological functions that appear to be dependent on its size[136]. High molecular weight (HMW) HA is responsible for tissue hydration, lubrication, structural integrity, free radical sequestration, and distribution of plasma proteins[135, 137]. It forms the backbone of cartilage to which large proteoglycan aggregates such as aggrecan attach and interact with collagen to aid in internal osmotic swelling pressure that maintains the load-bearing capacity of the joint tissue[138]. HA is synthesized in many cells including fibroblasts, endothelial cells and keratinocytes by hyaluronan synthase (HAS), an integral membrane protein, and is extruded into the extracellular space[139-141]. Low molecular weight (LMW) HA is typically fragments of HMW HA generated under conditions of inflammation, tumorigenesis, or tissue injury as a result of hyaluronidase activity[142] or oxidation[143].

Hyaluronidase (EC 3.2.1.35) is an enzyme that depolymerizes HA, and creates LMW HA fragments. It is found in mammalian organs and body liquids, leech heads, invasive bacteria, and venoms of bees, snakes, and scorpions[144]. It is important in degrading tissue and acts as a 'spreading factor' by accelerating venom absorption and diffusion into the tissues[145]. In the

human genome, there are six genes encoding for hyaluronidase[146]. The gene products have widely dispersed biological functions that are still under considerable debate. Hyal-1, Hyal-2 and Hyal-3 are expressed in synovocytes and chondrocytes and play an important role in cartilage development and destruction[147]. In the presence of IL-1 and TNF-α, Hyal-2 and Hyal-3 are both up-regulated, and are correlated with a decrease in HA concentration [147, 148]. In OA patients, a positive correlation was found between hyaluronidase activity and severity of the disease, C-reactive protein concentration and platelet count in the synovial fluid [149].

In a study reported by Ohno-Nakahara et. al., LMW HA fragments produced from hyaluronidase treatment of cultured chondrocytes up regulated gene expression of the CD44 receptor, a cell surface receptor for HA, and also matrix metalloproteinases (MMPs), the enzymes responsible for degradation of connective tissue matrices[150]. CD44 is linked to regulation of a variety of biological events including cell adhesion and trafficking, surface expression of other adhesion molecules, leukocyte activation, production of cytokines and chemokines, apoptosis, and proteinases translocation. Inflammatory cytokines such as TNF-α and IL-1β have been shown to up-regulate the expression of the CD44 receptor as well [151, 152]. Therefore, it is indicated that LMW HA fragments resulting from hyaluronidase activity can play an important role in cartilage destruction in pathological joints by accelerating the vicious circle of CD44 expression.

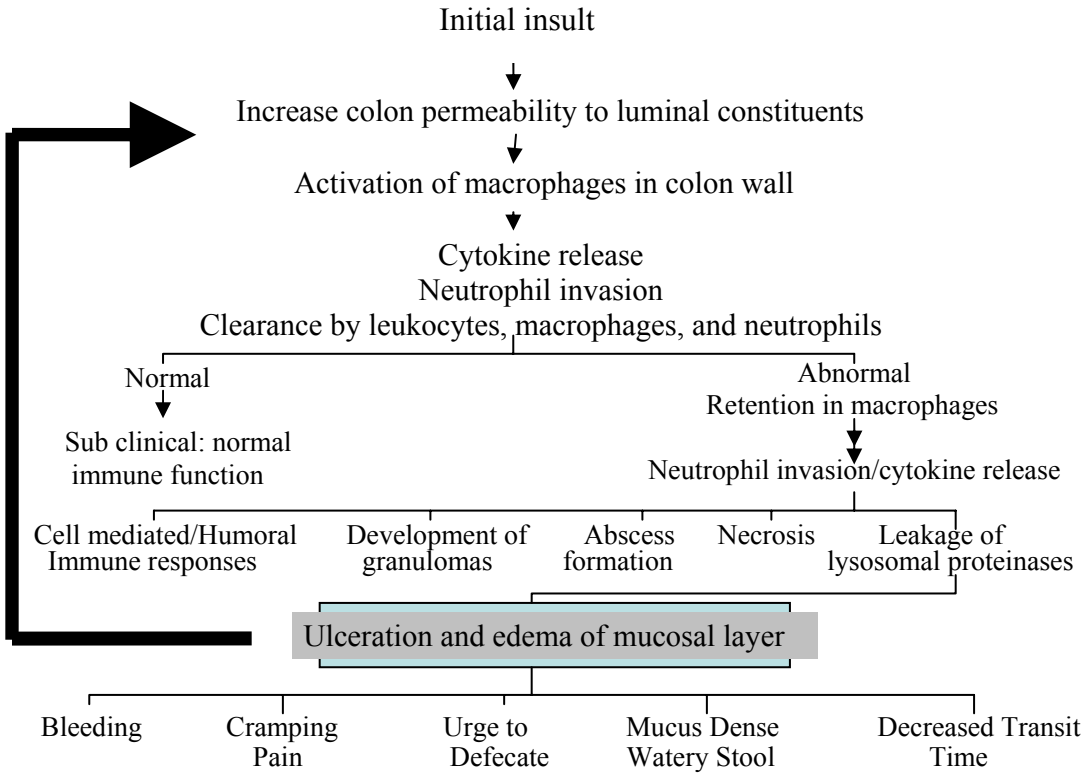
Various individual flavonoids inhibit hyaluronidase activity including condensed tannin, kaempferol, myricetin, quercetin, catechin and epicatechin [153-157]. Procyanidins have been shown to have anti-hyaluronidase activity as well as anti-collagenase and some anti-elastase activity [157].

Ulcerative Colitis

Ulcerative colitis (UC) is an Inflammatory Bowel Disease (IBD) that is the cause of illness for 1-2 million Americans. It is characterized by the ulceration of the innermost lining of the colonic mucosa, usually in the rectum and sigmoid colon and manifests as peri-anal irritations, fissures, hemorrhoids, fistulas, ulcers and abscesses. The origin of UC is unknown, but dietary, genetic, environmental, and immunological factors are all suggested causes [158]. Regardless of the lack of understanding of its etiology, there are many pathologic features seen in the progression of the disease. Among these include genetic predisposition, increase in inflammatory mediators, increased oxidative stress, imbalance in gut microflora, abnormal glycosaminoglycan content of the mucosa, and decreased oxidation of short chain fatty acids [158].

Primary lesions in UC begin with neutrophil invasion in areas of inflammation (Figure 1.13). Because the mucous layer is thinnest in the rectum and sigmoid colon, the disease is most pronounced in these areas. Tissue damage develops into small erosions and eventually ulcers caused by inflammatory cytokines released from leukocytes, macrophages and neutrophils. Further damage leads to abscess formation in crypts, necrosis, and ragged ulceration of the mucosal layer.

FIGURE 1.13 Pathology of Ulcerative Colitis



Disregulation of immune function is thought to be a major contributing factor in the progression of UC [159]. In the normal gut, the gastrointestinal tract acts as a barrier to foreign antigens from food and intestinal bacteria and their byproducts. The "controlled inflammation" that occurs is necessary to maintain mucosal homeostasis and eradicate the pathogens. Once the harmful antigen is gone, the local immune response must be attenuated, and this is done by either CD4+/CD25+ cells produced by the thymus, or by apoptosis of T lymphocytes, the major cell-mediated immune response cells [160, 161]. In a diseased colon, this attenuation of the immune response is absent, and a vicious cycle of inflammation occurs.

When exposed to exogenous antigens, a cell-mediated immune response is activated in the gut and T lymphocytes secrete specific pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) being the most abundant [162]. TNF-α amplifies the inflammatory response by auto-regulating its transcription and increasing IL-1ß and IL-6 mainly via activation of the nuclear-factor kappa B (NF-κB) pathway. TNF-α also increases the expression of adhesion molecules, and proliferation of fibroblasts [163, 164].

The exogenous antigens also directly stimulate production of cytokines such as IL-1, IL-6, and TNF- α from the intestinal epithelium [165]. These cytokines also amplify the immune response by enhancing the proliferation of T lymphocytes, facilitating cell-cell signaling, and promoting neutrophil infiltration into the inflamed tissue, a key stage in the inflammatory process [166]. Neutrophils reduce molecular oxygen to the superoxide anion radical. Through the enzyme myeloperoxidase (MPO), the potent cytotoxic oxidant, hypochlorous acid, is formed from hydrogen peroxide and chloride ions [167]. Cytokines also play a direct role in the inflammatory response by rapidly synthesizing and secreting reactive oxygen species, nitric oxide, leukotrienes, platelet-activating factor, and prostaglandins [168-171]. Cellular injury and necrosis are induced by reactive oxygen species by peroxidation of membrane lipids, protein denaturation, and DNA damage.

Current drug treatment includes corticosteroids and immunomodulators. Corticosteroids bind to the cytosolic glucocorticoid receptors and inhibit the arachidonic acid cascade, thus inhibiting activation of certain transcription factors such as IL-1, -6, and interferon-gamma. The therapy is designed for short-term, but if used chronically, side effects could include cataracts, osteoporosis, myopathy, and conditions associated with immune suppression and adrenal insufficiency. Immunomodulatory drugs mainly inhibit TNF-α activation thus inhibiting the T-

lymphocyte activity decreasing the cell-mediated immune response. Patients see a faster recovery rate than with corticosteroids and a longer remission, but the chance for serious side effects is increased. Immunomodulators promote activation of latent infections such as tuberculosis, and increase the vulnerability to active infections. There is a decreased immune vigilance, which can increase early tumor formation. Other side effects include demyelination and other neurological damage, aplastic anemia, intestinal perforations, and congestive heart failure.

Phytochemicals have been shown to modulate many metabolic pathways and signaling pathways that alter immune gene expression, mainly by inhibiting NF-κB. These modulatory effects may be especially important in the gastrointestinal tract because it is a focus of immunological defense and it is exposed to the highest concentrations of phytochemicals found anywhere in the body. The study of phytochemicals as a treatment for UC is not novel and is warranted due to the harsh side effects of current drug treatment. Dietary quercetin, a main phytochemical in the muscadine grape, was shown to ameliorate trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats by way of TNF-α induced NF-κB activation [172]. Resveratrol, another phytochemical in the muscadine grape, has been shown to inhibit TNF- α activation of NF-κB *in vitro* and is protective against TNBS-induced colitis due to impairment of neutrophil function, decrease in NF-κB activation, and stimulation of apoptosis in colonic cells [173, 174].

HYPOTHESES TESTED

In **Chapter Two** the following hypothesis was tested:

Muscadine skin and seed fractions will exhibit anti-hyaluronidase activity *in vitro*. Two varieties of muscadine grapes were tested, the Ison (purple) grape, and the Early Fry (bronze) grape. Inhibitory activity related to total phenolics and antioxidant ability, with the seed extracts of both varieties possessing the greatest inhibitory activity.

In **Chapter Three** the following hypothesis was tested:

Sorghum bran of several varieties will exhibit anti-hyaluronidase activity *in vitro*. The higher the total phenolic acid and antioxidant capacity bran will exhibit greater inhibitory activity. Sorghum bran will also be more potent inhibitors than the commonly used wheat and rice bran.

In **Chapter Four** the following hypothesis was tested:

Muscadine skin, seed, and pomace extract will inhibit acute and chronic inflammation associated with the topical application to mouse ears of phorbol myristate, a potent inflammatory agent and tumor promoter.

In **Chapter Five** the following hypothesis was tested:

Polygonum cuspidatum extract will inhibit acute and chronic inflammation associated with the topical application of phorbol myristate to mouse ears. *Polygonum cuspidatum* extract will be a more potent inhibitor of inflammation than a comparable dose of purified *trans*-resveratrol.

In **Chapter Six** the following hypothesis was tested:

Muscadine skin extract in the diet and/or administered intrarectally will decrease inflammation associated with the trinitrobenzene sulfonic acid colitis model *in vivo.*

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CHAPTER TWO

INHIBITION OF HYALURONIDASE ACTIVITY BY *VITIS ROTUNDIFOLIA* (MUSCADINE) BERRY SEEDS AND SKINS

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Accepted for publication by Pharmaceutical Biology, Vol. 45, issue 9, 2007 reprinted here with permission of publisher.

ABSTRACT

Hyaluronidase hydrolyzes glycosaminoglycans including hyaluronan (HA) in the extracellular matrix during tissue remodeling. Up regulation of hyaluronidase activity occurs in chronic inflammatory conditions, e.g., inflammatory joint disease. Prior work demonstrated that muscadine skin extracts possess significant *in vitro* and *in vivo* anti-inflammatory activities. In this study, we tested the ability of muscadine skin and seed extracts to inhibit hyaluronidase activity *in vitro*. Ethanol extracts (1:9 wt/vol 50% ethanol) were prepared from dried skins and seeds of Early Fry (bronze) and Ison (purple) muscadine varieties. Each extract inhibited hyaluronidase activity. Seed extracts were 2-3 times more potent per unit weight than skin extracts. The IC_{50}s of the Ison seed, Early Fry seed, Ison skin, and Early Fry skin were 0.3, 0.6, 1.0, and 1.0 mg/mL, respectively. Hyaluronidase inhibition correlated positively with total phenolic and FRAP (ferric reducing antioxidant power) values, but not with anthocyanin content. In addition to anti-inflammatory and antioxidant properties, the results of this study show that polyphenolics in muscadine grapes inhibit hyaluronidase. These three bioactivities are important for maintaining healthy connective tissue metabolism.

Key words: extracellular matrix, flavonoids, hyaluronan, hyaluronidase, inflammation, joint disease, muscadine, polyphenolics, *Vitis rotundifolia.*

Introduction

Recent research suggests that dietary phytophenolics are absorbed and have antioxidant and anti-inflammatory activities *in vivo* [1-4]. It is important to determine whether plant constituents can modulate the turnover of extracellular matrix during aging, inflammation, and joint disease. Balanced metabolism of the matrix glycosaminoglycan, hyaluronan or hyaluronate (HA), is particularly vital. HA contains repeating units of D-glucuronic acid and N-acetyl-D-glucosamine and is synthesized by hyaluronan synthase in fibroblasts, endothelial cells and keratinocytes. It is degraded by several enzymes in the hyaluronidase family (EC 3.2.1.35) [5-8]. HA polymers are important constituents of the extracellular matrix of connective tissues including cartilage and the synovial membrane and synovial fluid of joints [9].

HA has several important structural and biological functions that depend on its size, which can reach 10^7 kDa [10]. High molecular weight HA is responsible for tissue hydration, lubrication, structural integrity, free radical sequestration, and distribution of plasma proteins [9, 11]. Low molecular weight HA fragments are generated by hyaluronidase activity [12]. These smaller fragments act as signaling molecules that intensify the inflammatory process by upregulating CD44 receptors, various cytokines, matrix metalloproteinases, and suppressing proteoglycan sulfation [13, 14].

In connective tissue, rates of HA synthesis and degradation vary over time. An imbalance caused by increased degradation or decreased synthesis is seen in inflammatory situations such as osteoarthritis [15]. Imbalances can be attributed to increased oxidative stress, increased formation of inflammatory mediators, decreased production of HA, and increased degradation of HA [16]. Increased hyaluronidase activity contributes to degenerative changes in

connective tissues. The cells, enzymes and signaling pathways involved in these changes may be therapeutic targets for dietary, pharmaceutical and nutraceutical modulators.

The muscadine grape, or *Vitis rotundifolia* Michx (Vitaceae) is a unique plant of the southeastern United States that is tolerant to heat, humidity and various pests. Relatively few bioactivities of the muscadine have been reported. However, we observed that muscadine extracts have anti-inflammatory effects both *in vitro* and *in vivo* [17]. For example, the release of superoxide from phorbol myristate acetate-activated neutrophils was inhibited by the addition of muscadine skin extract. The release of the cytokines TNF-α, IL-6, and IL-1β was inhibited with muscadine skin extract in lipopolysaccharide-activated peripheral blood mononuclear cells. In addition, rats fed a diet of 5% muscadine grape skin showed 50% less paw edema than control animals fed a regular chow diet when injected with carrageenan into the foot pad [17]. Extracts of muscadine seeds and skins also inhibit topical inflammation of the mouse ear stimulated by phorbol myristate acetate [18].

The anti-inflammatory properties of muscadine grapes lead one to ask whether the flavonoids that are concentrated in the grape skin and seeds might affect other inflammatory processes. These parts of the grape are discarded during processing to make juices and wines, and it would be useful to identify activities that could add value to these by-products. Individual phytophenolics of several classes have been shown to have effects on enzymes regulating extracellular matrix metabolism including hyaluronidase [19-22]. In this work, we tested whether extracts of muscadine skins and seeds inhibit hyaluronidase activity.

Materials and Methods

Materials

Muscadine Products Corporation at Paulk Vineyards (Wray, GA) donated Ison and Early Fry muscadine grapes obtained at normal harvest (August-September). Type I-S hyaluronidase from bovine testis, hyaluronic acid from human umbilical cord, cetyltrimethylammonium bromide (CTAB) and 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), Folin-Ciocalteu reagent 2N, gallic acid (3,4,5-trihydroxybenzoic acid), were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of Muscadine Seed and Skin Extracts

Skins and seeds from Ison and Early Fry muscadine grapes were separated from the pulp. Skins and seeds were dried at 120°C in a Napco vacuum oven. The dried skins and seeds were ground in a coffee/spice mill for 60 sec. Phytochemicals were extracted from the dried powders by adding one gram of powder to 9 mL of 50% ethanol and stirring for one hour at room temperature. The mixtures were centrifuged (1500 rpm for 10 min) and the supernatants were used in this study.

Hyaluronidase Assay

Hyaluronidase activity was determined turbidimetrically by a method based on complex formation between hyaluronan and cetyltrimethylammonium bromide (CTAB) [23]. The complex provides a stable absorbance that is proportional to the amount of undigested hyaluronan remaining in solution as measured by the absorbance at 400 nm. Inhibition of hyaluronidase by plant extracts is indicated by a slower loss of absorbance compared to hyaluronidase alone.

The initial ethanolic extracts were diluted with acetate buffer (0.2 M sodium-acetate-acetic acid, pH 6.0, containing 0.15 M NaCl) to yield 1:100 extracts (dry wt/volume of diluent). Stock solutions of HA (0.5 mg/mL) and hyaluronidase (1 mg/mL) were made fresh in the acetate

buffer. The assay mixture contained acetate buffer, 200 µg hyaluronic acid, 30 µg hyaluronidase, and 0-70 µL of diluted muscadine seed or skin extracts in a final volume of 0.5 mL. The mixtures were incubated at 37° C for 15 min, and the reactions were stopped with the addition of 2 mL 2.5% CTAB in 2% NaOH (pH 12). All assays were performed in duplicate. After 10 min, the optical density of each sample was read at 400 nm (Beckman DU 650 Spectrophotometer) against a blank containing 0.5 mL acetate buffer and 2 mL CTAB. The concentration of HA without extract gave an absorbance of 0.8-1.2 units in the undigested reaction mixtures, and this decreased to 0.3-0.5 in the presence of 30 µg hyaluronidase. Polyphenolic compounds in the diluted extracts absorbed a small amount of light at 400 nm. The results have been corrected for this absorbance. Ethanol did not affect hyaluronidase activity at the dilutions used here.

Measurement of Total Phenolic Acid

Total phenolic acid content of each extract was measured by the method of Slinkard and Singleton [24] with minor modifications. Triplicate samples (20 µl) of each extract and each gallic acid standard were added to 1.58 mL of distilled water in a 3 mL, 10 mm polystyrene cuvette. 100 µl of 2 N Folin-Ciocalteu reagent was added and the sample was mixed well. Within 10 min, 300 µL of saturated sodium carbonate solution (200 g Na_2CO_3 in 1 L distilled water) was added and mixed. Samples were incubated for 2 h at room temperature. Absorbance was measured at 765 nm on a Beckman DU 650 Spectrophotometer. A phenolic standard curve was constructed using gallic acid (0-500 mg/L). Phenolic acid concentrations were expressed as gallic acid equivalents per gram of dried seed or skin powder.

Measurement of FRAP (Ferric Reducing Antioxidant Power)

The antioxidant potential in each sample was determined based on its ability to reduce ferric tripyridyl-s-triazine complex to its ferrous form as described by Benzie and Strain [25]. In a 3 mL, 10 mm polystyrene cuvette 10 µL of each sample or ferrous sulfate standard, 30 µl of distilled water and 300 µl of FRAP reagent were added. FRAP reagent was maintained at 37ºC and made by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL TPTZ solution (10 mM 2,4,6-tri[2-pyridyl]-s-triazine dissolved in 40mM HCl), and 2.5 mL of 20 mM ferric chloride solution. The solutions were incubated for six min, and then 0.34 mL of distilled water was added to each cuvette. The absorbance of the sample was read immediately at 593 nm. FRAP values were calculated from a standard curve of ferrous sulfate (0-1 mmol/L) and the seed and skin extract antioxidant power was expressed as mmol ferrous sulfate equivalents/100 g dry weight of fruit fraction.

Measurement of Anthocyanin Concentration

The method is based on the pH-dependent change in absorbance of cyanidin-3-glucoside, a major anthocyanin of muscadine grapes. At pH 1.0, anthocyanins exist in the highly colored flavilium form. Therefore, muscadine extracts were diluted in a potassium solution, pH 1 (62.5 mL 12 N hydrochloric acid, 3.7 g potassium chloride in 1 L of water). Samples were mixed thoroughly and incubated for 15 min. They were centrifuged to remove any precipitate, and absorbance was read at 510 nm. Total anthocyanin concentration was calculated based on Beer's Law using the molar extinction coefficient (29,600 M^{-1} cm^{-1}) of cyanidin-3-glucoside, and expressed in mg anthocyanin/g dried powder.

Results

Extracts of dried muscadine seeds and skins of Ison and Early Fry varieties each inhibited hyaluronidase (Table 2.1 and Figure 2.1). Inhibition per volume of extract was greater with

seeds than with the skins of both varieties, indicating that seeds contain more inhibitory compounds than skins when compared per g of initial dry weight. The IC_{50}s of the Ison seed, Early Fry seed, Ison skin, and Early Fry skin were 0.3, 0.6, 1.0, and 1.0 mg/mL, respectively (Table 1). This translates into extract dilution factors of 1:3000, 1:1700, 1:1000, and 1:1000, respectively.

The concentration of total phenolics per unit of dry weight was higher in extracts of seed powder compared to extracts of skin powder in both varieties (Table 2.1). Ison seeds contained the highest amount of phenolics. Antioxidant power (FRAP value) was also greater in the seeds than the skins in both varieties. Total phenolic content correlated directly with the measured FRAP antioxidant activities ($R^2 = 0.97$). As expected, the anthocyanin content was highest in the skins of the purple muscadine (Ison) variety and very low in the seeds of both grape varieties and skins of the Early Fry grape (Table 2.1).

Percent hyaluronidase inhibition correlated with total phenolic content ($R^2 = 0.99$) and FRAP value ($R^2 = 0.97$) of each muscadine fraction (Figure 2.2). Although the Ison skins had 7 times more anthocyanin content than Early Fry skins, there was no difference in anti-hyaluronidase activity between the two (Table 2.1).

Discussion

We tested the hypothesis that muscadine phytochemicals would inhibit hyaluronidase, an enzyme involved in normal tissue remodeling. An imbalance between synthesis of hyaluronan and its destruction by hyaluronidase contributes to joint disease and other types of inflammatory conditions. The results of our study show that skins and seeds of the muscadine grape contain compounds that inhibit hyaluronidase in vitro and have potential to modulate tissue metabolism when consumed as part of the diet. Hyaluronidase inhibition correlated positively with the total

phenolic and FRAP values. There was no correlation between inhibition and anthocyanin content. However, the anthocyanins are minor constituents of muscadine seeds and skins of the bronze varieties. The data indicate that several polyphenolic compounds inhibit hyaluronidase but do not exclude an inhibitory effect of anthocyanins.

Various individual flavonoids inhibit hyaluronidase activity including condensed tannin, kaempferol, myricetin, quercetin, catechin and epicatechin [19-22, 26]. Muscadine skins of the Early Fry and Ison varieties contain ellagic acid, myricetin, quercetin, kaempferol and resveratrol [27] while the seeds of each contain (-)-epicatechin, (-)-catechin and gallic acid as major phenolics [27]. Anthocyanins occur in both the skins and the seeds, but purple skins have a much higher content than bronze skin or the seeds of any variety (Pastrana-Bonilla *et al.*, 2003). Dominant species are delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin as 3,5-diglucosides [2, 28]. Ellagic acid, ellagic acid glycosides and ellagitannins are abundant in muscadine grape skins and seeds [29]. Ellagic acid glycosides include rhamnosides, xylosides and glucosides and the ellagitannins are of at least four different species (Lee *et al.*, 2005). Procyanidins have been shown to have anti-hyaluronidase activity as well as anti-collagenase and some anti-elastase activity [21]. Muscadine seeds had higher anti-hyaluronidase activity than muscadine skins compared per unit dry weight, but skins were as high as seeds when normalized on the basis of total phenolic content and FRAP value.

The balance between synthesis of HA and its degradation by hyaluronidase is a key feature of normal connective tissue [30]. During inflammation, the balance between synthesis and degradation of matrix components including collagen, glycosaminoglycans, and proteoglycans can be affected adversely. It has been hypothesized that hyaluronidase is secreted by inflammatory cells and by invasive bacteria such as streptococci [31, 32], which increases the

turnover of glycosaminoglycans in inflamed tissues [33]. Increased HA synthesis also occurs in pathological conditions and enhances infiltration of immune cells [34], thereby increasing potential for accelerated tissue damage. This occurs during autoimmune diseases such as colitis [35] and degradation of HA alters tissue integrity and decreases the integrity of epithelial barriers [36, 37]. HA prevents penetration of large macromolecules through the luminal capillary, arteriole and venule glycocalyces [38].

Phytochemicals modulate many metabolic pathways and signaling pathways that alter gene expression [39-41]. These modulatory effects may be especially important in the gastrointestinal tract because it is a focus of immunological defenses that can produce enteritis or colitis. The gastrointestinal tract is exposed to the highest concentration of phytochemicals found anywhere in the body. Muscadine phytochemicals are known to alter monocyte responses to inflammatory cytokines [17] and lymphocyte responses to lipopolysaccharide [42]. Muscadine phytochemicals have been reported to alter intestinal apoptosis and growth processes related to colon cancer [2, 4, 43]. Our results show that muscadine phytochemicals have the potential to inactivate hyaluronidase, which could modify tumor formation or invasiveness. For example, it has been postulated that hyaluronidase stimulates angiogenesis and facilitates tumor invasiveness [44]. Polyphenolic compounds also inhibit expression of matrix metalloproteinases that are involved in tumor invasiveness [45, 46].

Currently, treatment of osteoarthritis involves manipulating the balance of cartilage remodeling and destruction. This is done by injecting high molecular weight HA into joints, consuming nutraceuticals such as glucosamine sulfate and chondroitin sulfate to enhance glycosaminoglycan synthesis, and prescribing non-steroidal anti-inflammatory drugs. A fourth strategy that has not been fully employed is inhibition of hyaluronidase. Not only does

hyaluronidase degrade high molecular weight HA to cause structural breakdown, it also produces low molecular weight fragments that stimulate inflammation. The results of this study support the need for further studies to document *in vivo* enhancement of joint and cartilage health in humans who are compliant with regimens of drugs or food supplements.

Muscadine grape juice is sold commercially and is also used in the wine industry. The pomace (muscadine skins and seeds) contains most of the grape's polyphenolics and is a byproduct of the juicing process. Bioactivities found in pomace could add value that would benefit vineyard economies. The phenolics in pomace have anti-inflammatory activity and are known to inhibit hyaluronidase and matrix metalloproteinases [47]. Our data suggest that extracts of whole muscadine pomace should be tested in animal models for efficacy in relieving joint disease. We conclude that seeds and skins of both purple and bronze muscadine varieties significantly inhibit hyaluronidase activity. High antioxidant, anti-inflammatory and anti-hyaluronidase activities are a combination of activities that may prove useful in nutraceutical products supportive of balanced synthesis and degradation of extracellular matrix in inflammatory disease conditions.

Acknowledgments

We thank Mr. Chris Paulk of Muscadine Products Corporation for gifts of fresh muscadine grapes from Paulk Vineyards. This research was supported by USDA SBIR Phase II grant to Muscadine Products Corporation, Wray GA and Agricultural Experiment Station Hatch Project GEO00922.

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Tables and Figures

Table 2.1. IC50 values for inhibition of hyaluronidase, total phenolic acid content, anthocyanin content, and FRAP values of dried muscadine seeds and skins of the Ison (purple) and Early Fry (bronze) varieties.

	IC_{50}	Phenolic Acid	Anthocyanin	FRAP's
	mg powder/mL	(mg/g)	(mg/g)	(mmol/100g)
Ison Seed	0.3	153.3±1.2*	0.5±0.01*	71.01±6.3*
Early Fry Seed	0.6	95.4±4.4	0.4±0.02	48.85±7.5
Ison Skin	1.0	30.9±1.3	3.5±0.21	25.96±4.7
Early Fry Skin	1.0	25.2±1.8	0.4±0.04	16.41±2.0

* Results represent mean ± SEM.

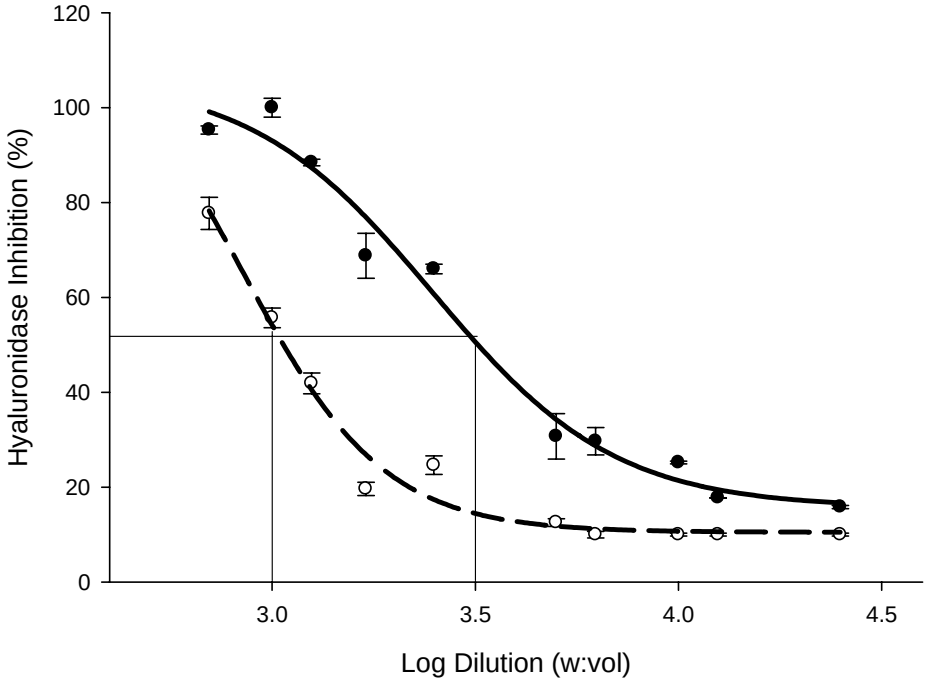

FIGURE 2.1 Inhibition of hyaluronidase by extracts of muscadine seeds and skins of the Ison variety. Solid line = Ison Seed, dashed line = Ison Skin.

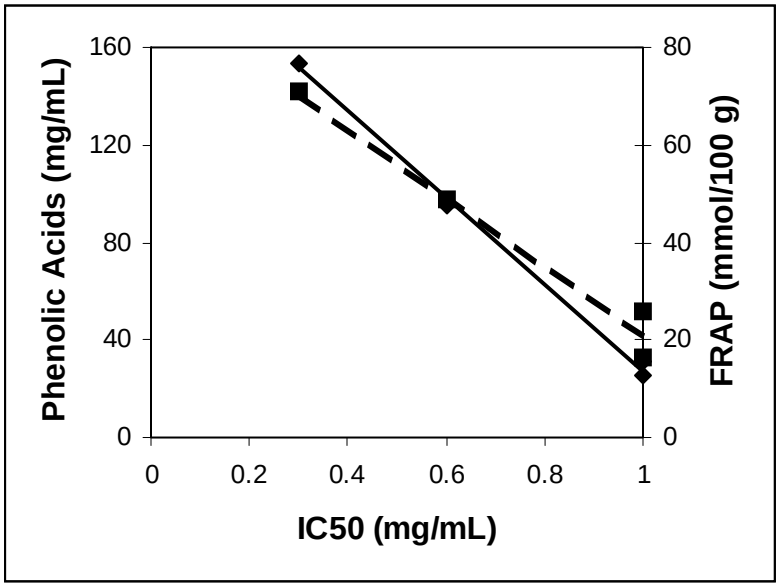

FIGURE 2.2 Correlation between IC50's for inhibition of hyaluronidase plotted versus content of phenolic acids (unbroken line) or FRAP values (dashed line) of muscadine extracts. IC50 is represented in mg of dried muscadine powder per mL in hyaluronidase assay. The correlation coefficients are $R^2 = 0.99$ for phenolic acid content and 0.97 for FRAP values.

CHAPTER 3

INHIBITION OF HYALURONIDASE ACTIVITY BY BRAN OF SEVERAL SORGHUM VARIETIES

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Submitted to the Journal of Medicinal Food.

ABSTRACT

Hyaluronidase hydrolyzes glycosaminoglycans including hyaluronan (HA) in the extracellular matrix during tissue remodeling. Up-regulation of hyaluronidase activity occurs in chronic inflammatory conditions, e.g., inflammatory joint disease. In this study, we tested the ability of brans from several grain sorghum varieties to inhibit hyaluronidase activity *in vitro*. Ethanol extracts (1:9 wt/vol 50% ethanol) were prepared from brans of six sorghum varieties. Each extract inhibited hyaluronidase activity in the order of Sumac >Shanqui Red >Black sorghum >Mycogen >Fontainelle >White sorghum. Hyaluronidase inhibitions correlated positively with total phenolic content and FRAP (ferric reducing antioxidant power) values for each bran extract. A variety of other common brans and whole grains were tested and compared with the grain sorghum brans. Commonly used wheat and rice bran had weak inhibitory activities relative to the high phenolic containing grain sorghum brans. Since hyaluronidase activity is important in conditions such as osteoarthritis and skin aging, these varieties deserve consideration for functional food, functional beverage, food supplement, nutraceutical and cosmeceutical product ingredients.

Key words: extracellular matrix, flavonoids, hyaluronan, hyaluronidase, inflammation, joint disease, Sumac sorghum bran, Shanqui Red, Black sorghum, polyphenolics.

Introduction

Population studies indicate that diets containing significant levels of phytochemicals and fiber are associated with lower incidence of numerous disease states [1, 2]. For example, whole grain consumption is associated with decreased incidence of heart disease, diabetes, and other chronic diseases. This is due to a combination of lower glycemic index and the full complement of dietary fiber and phytochemicals in the bran fractions [3, 4]. These correlations have led to United States dietary guidelines that recommend increased consumption of whole grains and decreased consumption of decorticated grain products that provide mostly high glycemic index starch.

Grain sorghum is a drought-tolerant food crop that can provide a reliable harvest in semiarid regions. It has been a staple part of the diet in parts of India, Africa, and China for millennia [5, 6]. Some sorghum varieties have extremely high contents of phenolic compounds and antioxidant potential that aid in the natural defense against pest and diseases [7]. These phenolic compounds are mainly located in the bran fraction [6]. Grain sorghum phenolic compounds fall into three major categories: phenolic acids, flavonoids and condensed tannins. The phenolic acids are benzoic or cinnamic acid derivatives. The flavonoids are largely anthocyanins, flavan-4-ols, flavones, and flavanols [8-10]. Condensed tannins, or proanthocyanidins, are high molecular weight polymers of flavan-3-ols, usually catechin and epicatechin [11]. Many commercially successful sorghum varieties have been developed with reduced tannin content. However, the high levels of phytochemicals suggest that select sorghum brans may have significant nutraceutical potential.

Enzymes and other regulatory molecules are possible targets for plant polyphenolics. One enzyme that is involved in tissue remodeling during inflammation is hyaluronidase, which degrades glycosaminoglycans including hyaluronan [12-15]. Hyaluronan polymers are important

constituents of the extracellular matrix of connective tissues, including cartilage, the synovial membrane and synovial fluid of joints [16]. Hyaluronan contains repeating units of D-glucuronic acid and N-acetyl-D-glucosamine. It is synthesized by hyaluronan synthase in fibroblasts, endothelial cells and keratinocytes and is degraded by a family of enzymes called hyaluronidases (EC 3.2.1.35). Many phytophenolics exert effects on enzymes regulating extracellular matrix metabolism, and these include hyaluronidase [17-20]. In this work, we tested the anti-hyaluronidase activity of multiple varieties of grain sorghum bran and correlated those activities with total phenolics and antioxidant content of the bran. The results provide rationale for development of select sorghum bran in the management of inflammatory diseases associated with elevated hyaluronidase activity.

Materials and Methods

Materials

Sorghum brans were gifts from Dr. Lloyd Rooney of Texas A&M University and Dr. Scott Bean, USDA, Manhattan, KS. The non-sorghum brans and grains were purchased commercially from the bulk food section of a health food store in Athens, Georgia. Type I-S hyaluronidase from bovine testis, hyaluronic acid from human umbilical cord, cetyltrimethylammonium bromide (CTAB) and 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), Folin-Ciocalteu reagent 2N, gallic acid (3,4,5-trihydroxybenzoic acid), were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of Bran Extracts

Phytochemicals were extracted from the dried powders by adding one gram of powder to 9 mL of 50% ethanol and stirring for one h at room temperature. The mixtures were centrifuged (1500 rpm for 10 min) and the supernatants were used in this study.

Hyaluronidase Assay

Hyaluronidase activity was determined turbidimetrically by a method based on complex formation between hyaluronan and cetyltrimethylammonium bromide (CTAB) [21]. The complex provides a stable absorbance that is proportional to the amount of undigested hyaluronan remaining in solution as measured by the absorbance at 400 nm. Inhibition of hyaluronidase by plant extracts is indicated by a slower loss of absorbance compared to hyaluronidase alone.

The initial ethanolic extracts were diluted with acetate buffer (0.2 M sodium acetate-acetic acid, pH 6.0, containing 0.15 M NaCl) to yield 1:100 extracts (dry wt/vol of diluent). Stock solutions of HA (0.5 mg/mL) and hyaluronidase (1 mg/mL) were made fresh daily in acetate buffer. The assay mixture contained acetate buffer, 200 µg hyaluronic acid, 30 µg hyaluronidase, and 70 µL of diluted bran extracts in a final volume of 0.5 mL. A dose-response assay was performed with sumac bran extract over a broad concentration range. The mixtures were incubated at 37° C for 15 min, and the reactions were stopped with the addition of 2 mL 2.5% CTAB in 2% NaOH (pH 12). All assays were performed in duplicate. After 10 min, the optical density of each sample was read at 400 nm (Beckman DU 650 Spectrophotometer) against a blank containing 0.5 mL acetate buffer and 2 mL CTAB. The concentration of HA without extract gave an absorbance of 0.8-1.2 units in the undigested reaction mixtures, and this decreased to 0.3-0.5 in the presence of 30 µg hyaluronidase. The absorbance of polyphenolic compounds in the diluted extracts at 400 nm was also determined and the results have been corrected for this absorbance. Ethanol did not affect hyaluronidase activity at the dilutions employed with these studies. All samples were run in triplicate.

Measurement of Total Phenolic Acid

Total phenolic content in each extract was measured by the method of Slinkard and Singleton [22] with minor modifications. Triplicate samples (20 µl) of each extract and each gallic acid standard

were added to 1.58 mL of distilled water in a 3 mL, 10 mm polystyrene cuvette. 100 µl of 2 N Folin-Ciocalteu reagent was added and the sample was mixed well. Within 10 min, 300 µL of saturated sodium carbonate solution (200 g Na_2CO_3 in 1 L distilled water) was added and mixed. Samples were incubated for 2 h at room temperature. Absorbance was measured at 765 nm in a Beckman DU 650 Spectrophotometer. A phenolic standard curve was constructed using gallic acid standards (0-500 mg/L) (Sigma Chemical Company, St. Louis, MO). Phenolic acid concentrations are expressed as gallic acid equivalents per gram of dry bran weight.

Measurement of FRAP (Ferric Reducing Antioxidant Power)

The antioxidant potential in each sample was determined based on its ability to reduce ferric tripyridyl-s-triazine complex to its ferrous form as described by Benzie and Strain [23]. In a 3 mL, 10 mm polystyrene cuvette 10 µL of each sample or ferrous sulfate standard, 30 µl of distilled water and 300 µl of FRAP reagent were added. FRAP reagent was maintained at 37ºC and made by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL TPTZ solution (10 mM 2,4,6-tri[2-pyridyl]-s-triazine dissolved in 40mM HCl), and 2.5 mL of 20 mM ferric chloride solution. The solutions were incubated for 6 min, and then 0.34 mL of distilled water was added to each cuvette. The absorbance of the sample was read immediately at 593 nm. FRAP values were calculated from a standard curve of ferrous sulfate (0-1 mmol/L) and the bran extract antioxidant power is expressed as mmol ferrous sulfate equivalents/100 g dry weight of bran.

Results

Effect of Grain Sorghum Bran Extracts on Hyaluronidase Activity

Sumac sorghum bran extract contained the most hyaluronidase inhibitory activity of any variety tested, and the inhibition occurred in a dose-response manner (Figure 3.1). An extract of 700 µg dry weight Sumac variety totally inhibited hyaluronidase. The IC_{50} was 90 µg dry weight of the bran per assay volume. Sorghum bran (Sumac variety) had a total phenolic content of 75.5 mg/g of dry weight and a FRAP value of 68.2 mg/g of dry weight.

Extracts of five other sorghum brans were also screened (Figure 3.2) for anti-hyaluronidase activity. Sumac sorghum had the highest anti-hyaluronidase activity. Total phenolic content and antioxidant activity correlated positively with anti-hyaluronidase activity (Figure 3.3). The order of potency for inhibition of hyaluronidase was: Sumac > Shanqui Red > Black sorghum >>> Mycogen > Fontainelle > White sorghum (Figure 3.2).

In all extracts, total phenolic content correlated directly with the measured FRAP value ($R^2 = 0.95$). Percent hyaluronidase inhibition correlated with total phenolic content ($R^2 = 0.97$) and FRAP value ($R^2 = 0.90$) (Figure 3.3).

Comparison of Whole Indian Millet, Whole Glutinous Indian Millet, Whole Flaxseed, Whole Quinoa, Wheat Bran and Rice Bran Extracts on Hyaluronidase Activity

The percent inhibition of hyaluronidase of these grains is reported in Figure 3.4. Indian millet, Glutinous Millet, Flaxseed and Quinoa showed moderate inhibition, similar to some of the less active grain sorghum extracts. Interestingly, two of the most common brans used in food products are wheat and rice bran. These had very low anti-hyaluronidase activities, phenolic content and FRAP values compared with brans from Sumac varieties of grain sorghum (Table 3.1).

Discussion

We tested the hypothesis that sorghum phytochemicals would inhibit hyaluronidase, an enzyme involved in normal tissue remodeling. A chronic imbalance between synthesis of hyaluronan and its destruction by hyaluronidase contributes to joint disease and other types of inflammatory conditions [24]. Inhibition of hyaluronidase activity is an important target for managing imbalances in hyaluronan metabolism. The results of our study show that high total phenolic/high FRAP sorghum bran extracts inhibit hyaluronidase *in vitro.* These brans were more potent on a wt/wt basis than other common brans (wheat and rice) and higher than millet and flaxseed whole grains.

HA has several important structural and biological functions that depend on its size, which can reach 10^7 kDa [25]. High molecular weight HA is responsible for tissue hydration, lubrication, structural integrity, free radical sequestration, and distribution of plasma proteins [16, 26]. Low molecular weight HA fragments are generated by hyaluronidase activity [27]. These smaller fragments act as signaling molecules that intensify the inflammatory process by up-regulating CD44 receptors, various cytokines, and matrix metalloproteinases, while suppressing proteoglycan sulfation [28, 29]. The balance between synthesis of HA and its degradation by hyaluronidase is a key feature of normal connective tissue [30]. During joint inflammation, the balance between synthesis and degradation of matrix components including collagen, glycosaminoglycans, and proteoglycans can be affected adversely. Inhibition of hyaluronidase activity could aid in the restoration of tissue homeostasis.

Various individual flavonoids inhibit hyaluronidase activity *in vitro* including kaempferol, luteolin, catechin and epicatechin; all are in the bran fraction of grain sorghum [9, 17, 18, 20, 31]. Sorghum bran's are a rich source of flavonoids, with anthocyanins being the major class

studied [9]. As main contributors to their color, the most common anthocyanins in sorghum are delphinidin, malvidin, pelargonidin, petunidin, and peonidin. Proanthocyanidins (OPCs), or condensed tannins, are potent inhibitors of hyaluronidase, collagenase and elastase, the three main enzymes regulating the structural components of the extracellular matrix [19]. Unlike the flavonoids, condensed tannin is not the main phenolic consituent in many brans. In this study, the sorghum brans with the greatest inhibitory capacity (Sumac and Shanqui Red) were the ones with the highest total phenolic values and antioxidant abilities. With the exception of black sorghum bran, the brans with the highest anti-hyaluronidase activity are also considered 'high tannin' sorghum brans.

OPCs are also found in abundance in grape seeds and pine bark and have been studied extensively for their health-promoting capabilities. OPCs have high antioxidant potential and have been show to inhibit chemically-induced lipid peroxidation, DNA fragmentation, and subsequent apoptosis in hepatic and brain tissue of mice [32]. As an anti-inflammatory agent, they have been shown to inhibit peroxide generation by macrophages *in vitro* [33]. *In vivo*, grape seed OPCs inhibit formation of proinflammatory cytokines, interleukins, and tumor necrosis factor-α upon stimulation in the carageenan-induced hind paw edema model [34].

Currently, treatment of osteoarthritis involves various combinations of anti-inflammatory agents to decrease inflammation with an increase of various substrates for glycosoaminoglycan synthesis, i.e., chondroitin sulfate, methylsulfonylmethane and glucosamine. Injections of hyaluronan are also approved for treatment of osteoarthritic joints. These injections provide temporary relief of pain in some patients. A complementary strategy is to inhibit excessive hyaluronidase activity. Inhibiting hyaluronidase activity would simultaneously decrease breakdown of high molecular weight glycosaminoglycan substrates and decrease formation of

lower molecular weight fragments that stimulate inflammation. The results of this study support the need to document the *in vivo* enhancement of joint and cartilage health in humans who are compliant with regimens of drugs or food supplements.

We conclude that high phenolic/high FRAP grain sorghum brans inhibit hyaluronidase activity to a significantly greater extent than wheat bran and rice bran. Previously we have reported that high antioxidant grain sorghum extracts are highly anti-inflammatory in a model of ear inflammation [35]. The combination of high antioxidant, anti-inflammatory and anti-hyaluronidase activities make selected grain sorghum brans attractive ingredients for nutraceutical and functional food and beverage products, as well as for food ingredients. The anti-hyaluronidase activity we demonstrated in this study supports further development work on products that will support cartilage and extracellular matrix health for numerous conditions *in vivo*.

In addition, hyaluronan is a naturally occurring polymer in the skin and is gaining increased popularity as a cosmeceutical in that it aids in skin regeneration and wound healing [36, 37]. Our previous work on the sumac sorghum brans indicated high anti-inflammatory topical activity[35]. We therefore predict that the properties of sorghum bran extracts may be useful in cosmeceutical products.

Acknowledgements

This work was supported by grant 58-5430-4-363 from the United States Department of Agriculture. Many thanks to Dr. Lloyd Rooney and Dr. Scott Bean for their donation of the various sorghum brans.

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Tables and Figures

TABLE 3.1 Total Phenolics and FRAP Values of Various Brans and Grains

Extract	Total Phenolics mg/g	FRAP Value mmol/100g
Sorghum Brans		
Sumac	75.5 ± 3.6	68.2 ± 5.6
Shanqui Red	50.1 ± 2.0	28.2 ± 0.8
Black Sorghum	27.6 ± 0.9	21.2 ± 0.5
Mycogen	7.8 ± 0.3	5.6 ± 0.4
Fontainelle	3.1 ± 0.3	2.4 ± 0.2
White Sorghum	3.9 ± 0.2	2.1 ± 0.1
Non-sorghum Grains		
Whole Indian Millet	7.8 ± 0.1	6.1 ± 0.3
Whole Glutinous Indian Millet	0.9 ± 0.1	0.3 ± 0.1
Whole Flaxseed	3.6 ± 0.1	3.9 ± 0.1
Whole Quinoa	2.1 ± 0.1	0.8 ± 0.1
Wheat Bran	2.2 ± 0.1	1.1 ± 0.1

Data represent mean ± SD of triplicate determinations.

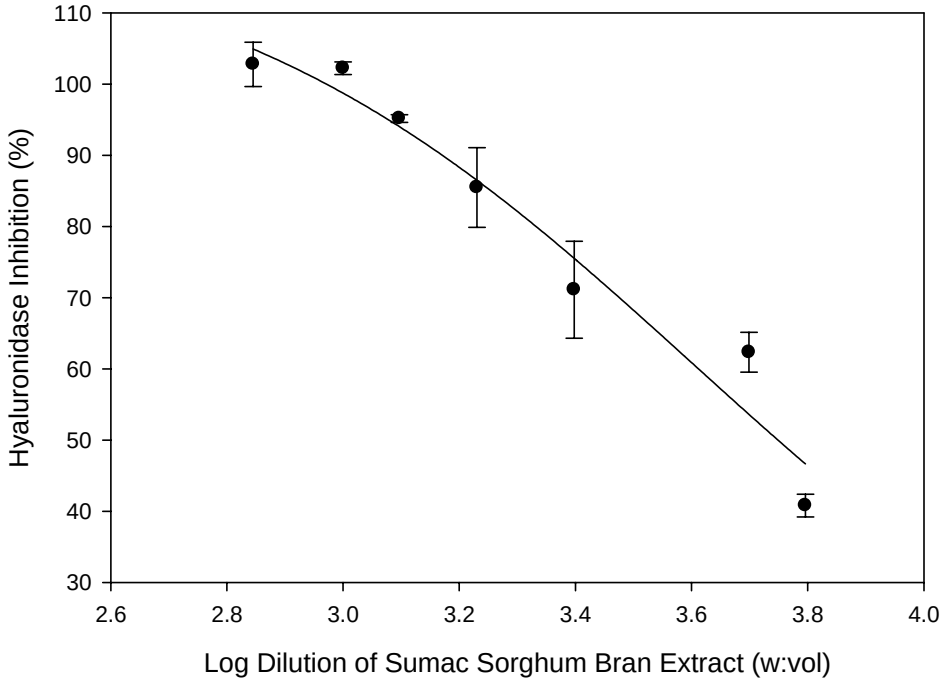

FIGURE 3.1 Inhibition of hyaluronidase activity by dilutions of Sumac sorghum ethanolic extract (w:vol). Results represent means ± SEM.

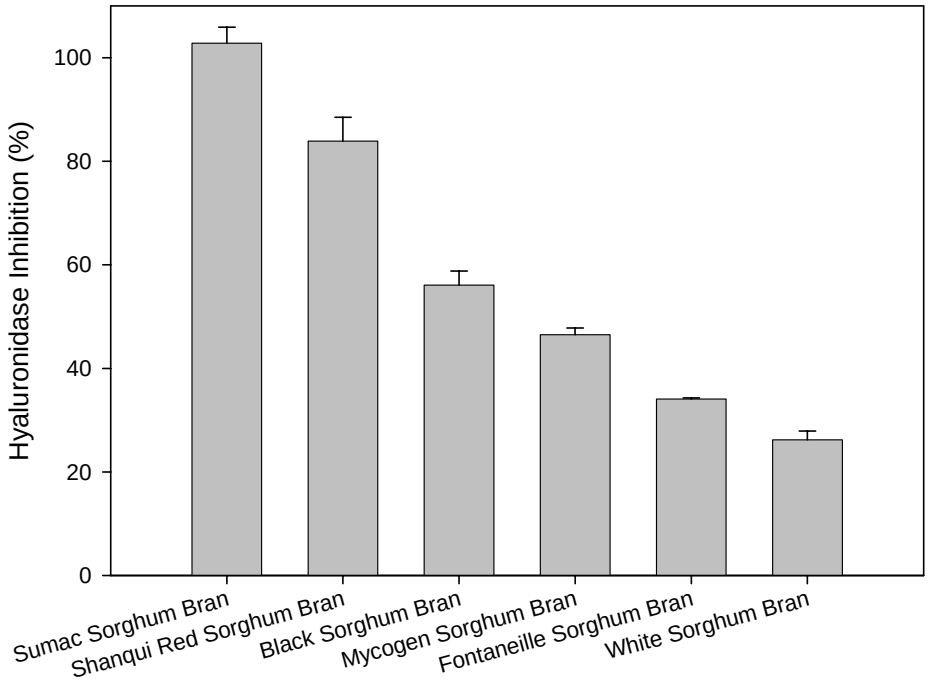

FIGURE 3.2 Inhibition of hyaluronidase activity by various grain sorghum brans (700 µg dry wt/assay volume). Results represent means ± SEM.

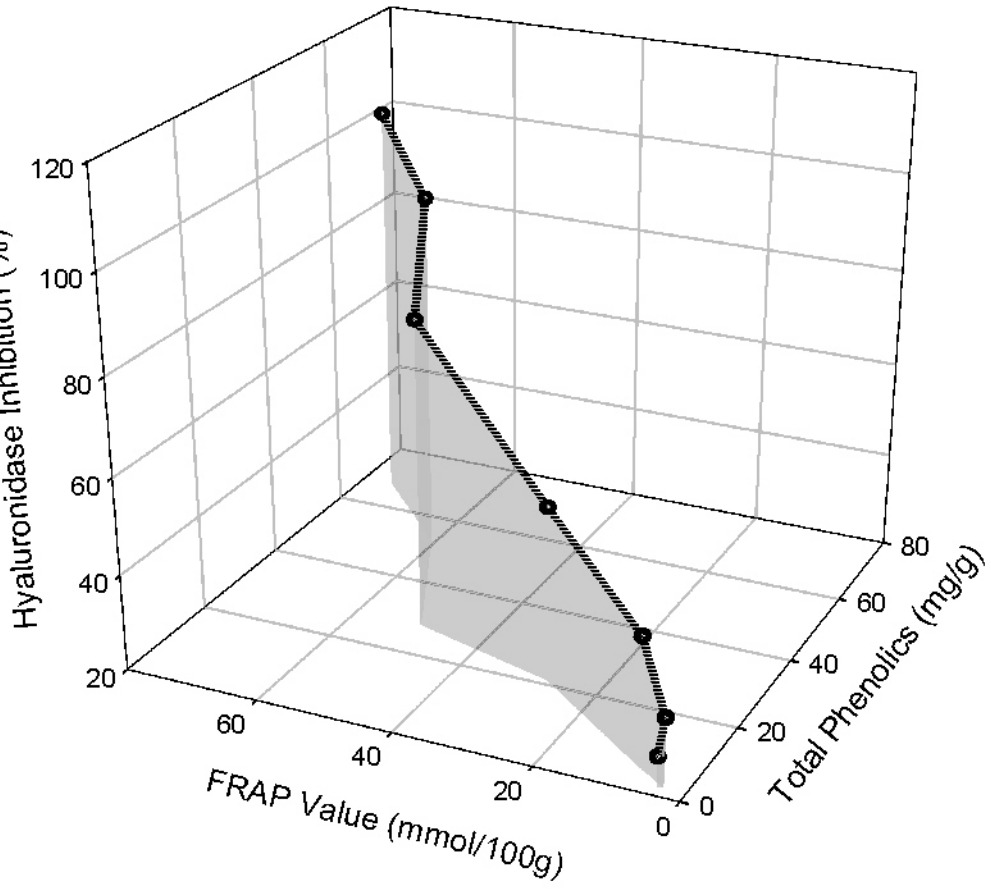

FIGURE 3.3 Correlation between hyaluronidase inhibition versus total phenolics and FRAP values in various sorghum grains.

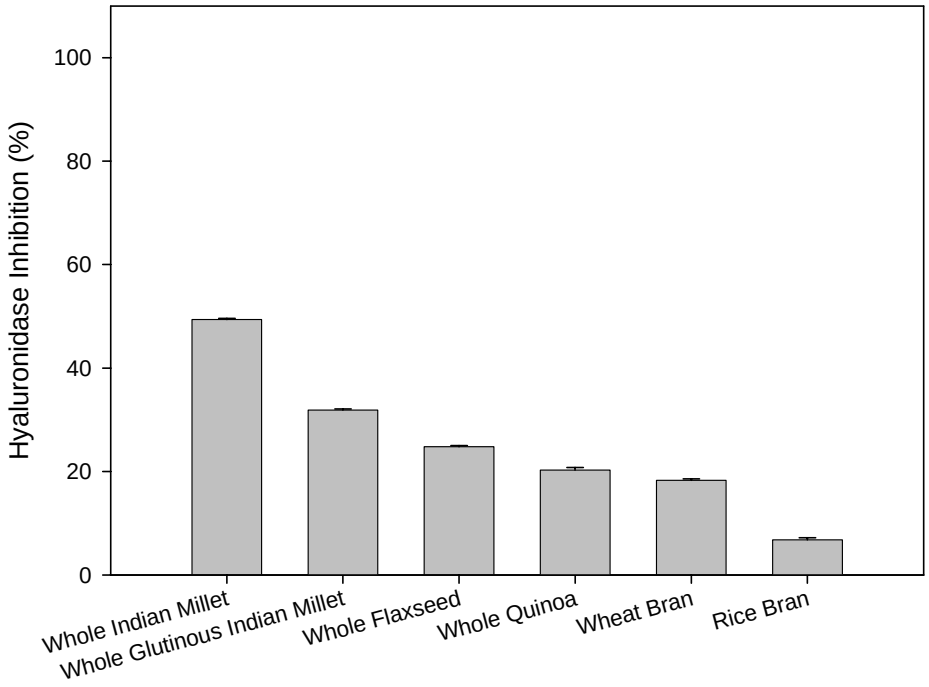

FIGURE 3.4 Inhibition of hyaluronidase activity by various whole grains or brans (700 µg dry wt/assay volume). Results represent means ± SEM.

CHAPTER FOUR

TOPICAL ANTI-INFLAMMATORY ACTIVITIES OF *VITIS ROTUNDIFOLIA* (MUSCADINE GRAPE) EXTRACTS IN THE TETRADECANOYLPHORBOL ACETATE (TPA) MODEL OF EAR INFLAMMATION

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Accepted to the Journal of Medicinal Food, reprinted here with permission from the editor

ABSTRACT

The ability of muscadine grape skin, seed or combined skin and seed extracts to inhibit mouse ear inflammation, edema, and polymorphonuclear (PMN) leukocyte infiltration was tested following topical application of 12-*O*-tetracecanoylphorbol-13-acetate (TPA). Ethanolic extracts of skins, seeds, or a combination of these from purple (Ison) cultivars were applied to both ears of female Swiss mice 30 min after TPA (2 μg/ear) administration. Control mice were treated with indomethacin or 50% EtOH vehicle 30 min after TPA. Ear thickness was measured before TPA and at 4 and 24 h post-TPA administration to assess ear edema. Ear punch biopsies were collected at 24 h and weighed as a second marker of edema. Myeloperoxidase (MPO, E.C. 1.11.1.7) activity was measured in each ear punch biopsy an index of neutrophil infiltration. Extracts of muscadine skin, seed, and combination treatments significantly reduced ear edema, ear biopsy weight and MPO activity compared to TPA vehicle control. There was no significant difference in anti-inflammatory activity of the skin and seed extracts. However, an additive effect was observed with the combination treatment that was statistically similar to the anti-inflammatory activity of indomethacin treatment. It can be concluded that muscadine skin, seed and combination skin/seed extracts exhibit significant topical anti-inflammatory properties.

Key words: anti-inflammatory, antioxidant, inflammation, edema, myeloperoxidase (MPO), polyphenols, nutraceutical, cosmeceutical.

Introduction

There is growing interest in potential health benefits of berries including various grapes, and anti-inflammatory properties are of special value. For example, consuming Concord grape (*Vitis labrusca*) juice increases plasma antioxidant capacity and decreases oxidation of plasma proteins in humans [1]. In rats, consumption of 10% grape juice improved performance in a water maze and increased dopamine release from the striatum, suggesting that age-dependent inflammation was reduced [2]. Beneficial effects of polyphenolic compounds extracted from highly colored berries, fruits and vegetables have been reported [3]. In this regard, the muscadine grape (*Vitis rotundifolia*) is native to the southeastern United States and there is considerable potential for extraction of biologically active compounds from its skin and seeds [4, 5].

Muscadine berries have thick, tough skins and several hard, oblong seeds. Their berries grow in loose clusters of up to 40 grapes, and ripen individually over an extended harvest period. *Vitis rotundifolia* is distinguished from *Vitis vinifera* by 20 pairs of chromosomes in the former vs. 19 pairs in the latter. Partly for this reason, botanists assign the grape to the genus, *Muscadinia* [6]. Muscadine grapes have a high total phenolic acid content mainly characterized by high ellagic acid compounds, gallic acid, and anthocyanin 3,5-diglucoside concentrations [7-11]. Ellagic acid, myricetin, quercetin, and kaempferol are the most abundant phenolics in the muscadine skin, while gallic acid, catechin, epicatechin and oligomeric proanthocyanidins (OPC's) are abundant in the seeds [9]. The muscadine is distinguished among other grapes by its high ellagic acid and ellagitannin concentration. The deep color of the grape skin in red, purple and black cultivars is attributed to anthocyanins that include delphinidin, cyanidin, petunidin, peonidin, and malvidin [8]. The muscadine also contains resveratrol derivatives. Current research on dietary polyphenols indicate many health benefits including antioxidant, anti-inflammatory,

and anticarcinogenic abilities [12-18]. The skins and the seeds are currently being used in food supplement products and extracts of these are in development for nutraceutical and cosmeceutical products.

Chronic systemic inflammation is a component of many age-related diseases such as Alzheimer's, arthritis, asthma, colitis, cancer, diabetes, heart disease, obesity, stroke and psoriasis [19, 20]. Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin 1b (IL-1β), and/or interleukin-8 (IL-8) can cause or contribute to the pathogenesis of these age-related diseases. Other inflammatory mediators such as prostaglandins and leukotrienes produced from the metabolism of arachidonic acid (AA) via cyclooxygenase (COX) and lipoxygenase (LOX) enzymes promote inflammation by recruitment of macrophages, neutrophils, and other leukocytes that release histamine and bradykinins [21]. The TPA model of ear inflammation is a screening test involving most of these processes and is useful for screening the efficacy and potency of topical anti-inflammatory and antioxidant abilities of plant extracts and phytochemicals [22]. In epidermal cell culture, TPA stimulates cell proliferation and increases the metabolism of AA to leukotrienes and prostaglandins [23]. Anti-inflammatory interventions may inhibit gene expression, alter the release or action of inflammatory cytokines, or inhibit COX and LOX enzymes [17, 21, 24, 25]. One of the early hallmarks of skin irritation and local inflammation is the thickening of the skin within 1-4 h due to processes including increased vascular permeability, edema and swelling within the dermis [25]. Secondarily, PMN leukocytes migrate to the dermis within about 24 h and may be quantified by the MPO assay. Dietary intervention for treatment of chronic inflammatory states is gaining interest due to unwanted side effects of many common anti-inflammatory drugs. Polyphenols

that are found in edible plant products such as fruits, vegetables, herbs and spices, and have been shown to inhibit the inflammation process [24].

Recently our laboratory examined a variety of anti-inflammatory activities of muscadine grape extracts using *in vitro* cell culture and *in vivo* models [17]. The release of superoxide from phorbol myristate acetate-activated neutrophils was inhibited by the addition of muscadine skin extract. The release of the cytokines TNF-α, IL-6, and IL-1β was inhibited with muscadine skin extract in lipopolysaccharide-activated peripheral blood mononuclear cells. In addition, rats fed a diet of 5% muscadine grape skin showed 50% less paw edema than control animals fed a regular chow diet when injected with carrageenan into the foot pad.

The present study tested whether muscadine skin, seed, or combined skin and seed extracts would have topical anti-inflammatory activities. This study was designed to test the ability of muscadine skin and seed extracts to inhibit inflammation, edema, and PMN leukocyte infiltration of the ear following topical application of TPA, a well-characterized model of topical inflammation [21, 22].

Materials and Methods

Materials

12-*O*-Tetradecanoylphorbol 13-acetate, hexadecyltrimethylammonium bromide, indomethacin (minimum 99% TLC), acetone, ethanol, hydrogen peroxide 30% w/w solution, 3 3', 5 5' tetramethylbenzidine dihydrochloride, *N, N*-dimethylformamide, Folin-Ciocalteu reagent, gallic acid, 10 mM 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), ferric chloride, ferrous sulphate and sodium acetate were all purchased from Sigma (St. Louis, MO).

Preparation of Muscadine Skin and Seed Extracts

Muscadine skin and seed powders of the Ison (purple) variety were prepared by drying separated skins and seeds at 120°C for 12 h in a forced-air pan dryer manufactured by Powell Manufacturing Company (Bennettsville, SC). The dried skins and seeds were ground in a Fitz Mill Comminutor Hammermill manufactured by the Fitzpatrick Company (Elmhurst, IL). Phytochemicals were extracted from the dried powders by adding one gram of powder to 3 mL of 50% ethanol and stirring for one hour. The mixtures were centrifuged (1500 rpm for 10 min) and the supernatants were used in the study. The combined extract was a 1:1 mixture of the separate skin and seed extracts.

Measurement of Phenolic Acid Content of Extracts

Total phenolic acid content of each extract was measured by the method of Slinkard and Singleton [26] with minor modifications. Extracts were diluted 1:9 (vol of extract/vol of water). Triplicate samples (20 µL) of each diluted extract were added to 1.58 mL of distilled water in 3 mL polystyrene cuvettes. 100 µL of Folin-Ciocalteu reagent was added and the sample was mixed well. Within 10 minutes, 300 µL of sodium carbonate solution (200 g Na_2CO_3 in 1 L distilled water) was added. Solutions were incubated for 2 h at room temperature. Absorbance was measured at 765 nm, and phenolic concentrations were calculated based on a gallic acid standard curve and expressed as mg phenolic acid per gram of dried powder.

Measurement of FRAP (Ferric Reducing Antioxidant Power)

The antioxidant activity in each sample was assayed in triplicate using the FRAP test [27]. Extracts were diluted further (1:9 vol of stock/vol of water). In a 3 mL polystyrene cuvette 10 µL of each sample, 30 µL of distilled water and 300 µL of FRAP reagent were added. FRAP reagent was made by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL TPTZ solution (10 mM 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) dissolved in 40mM HCl), and 2.5 mL of 20 mM ferric

chloride solution. The solutions were incubated at 37º C for six minutes, and then 340 µL of distilled water was added to each cuvette. The absorbance of the sample was read immediately at 593 nm. FRAP values were calculated from a standard curve of ferrous sulfate and expressed as mmol ferrous sulfate equivalents/100g dry weight of fruit.

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia and conducted according to IACUC guidelines. Female Swiss Webster mice weighing 22-25 g were housed in groups of 4 in large shoebox cages and were fed a standard diet *ad libitum* with free access to water. Animals were in the fed condition throughout the experiment. Photoperiods equaled 12 h of light and 12 h of darkness daily, with the environmental temperature maintained at 21°C.

TPA-induced Mouse Ear Edema

Edema was induced in both ears of each mouse by the topical application to both the inner and outer surfaces of 2 µg TPA dissolved in 20 µL of acetone. Additionally, one group was treated with acetone alone as a control (no TPA). Thirty minutes after the application of TPA, the inner and outer surface of each ear was treated with either 10 µL of ethanolic muscadine skin extract, muscadine seed extract, muscadine skin and seed extract (50%/50%), indomethacin (0.5 mg/ear dissolved in ethanol as an anti-inflammatory drug standard), or 50% ethanol (vehicle control). The thickness of the right and left ears was measured before TPA administration and after 4 h and 24 h using a micrometer (Mitutoya Series IP65). The micrometer was applied near the top of the ear just distal to the cartilaginous ridges. Ear punch biopsies were taken with a hole punch (6 mm in diameter), and were weighed to assess edema. A single investigator performed each measurement and punch to standardize the procedure.

Myeloperoxidase Assay

Tissue MPO activity was measured in biopsies from both ears 24 h after TPA administration using a method reported by Suzuki et. al. [28] and modified by De Young et. al. [29]. Each mouse ear biopsy (6 mm tissue punch) was placed in 0.75 mL of 80 mM phosphate-buffered saline (PBS) pH 5.4 containing 0.5% hexadecyltrimethyl-ammonium bromide (HTAB). Each sample was homogenized for 45 s at 4°C with a small sample laboratory Tissue Tearor Homogenizer Model 985-370 (Biospec Products, Bartlesville, OK). The homogenate was transferred to a microcentrifuge tube, and the vessel was washed with an additional 0.75 mL aliquot of the HTAB in PBS and added to the tube. The 1.5 mL sample was centrifuged at 12,000g for 15 minutes. 30 µL samples of the resulting supernatant were added to 96-well microtiter plate wells in triplicate. For the MPO assay, 200 µL of a mixture containing 100 µL of 80 mM PBS pH 5.4, 85 µL of 0.22 M PBS pH 5.4, and 15 µL of 0.017% hydrogen peroxide were added to each well. 20 µL of 18.4 mM tetramethylbenzidine HCl in 8% aqueous dimethylformamide was added to start the reaction. Plates were incubated at 37°C for 3 minutes, and then placed on ice. The reaction was stopped with the addition of 30 µL of 1.46 M sodium acetate, pH 3.0. MPO enzyme activity was assessed colorimetrically using Bio-Tek Microplate Reader (ELx 808) at an absorbance of 630 nm, and expressed as OD/biopsy.

Statistical Analysis

Data are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical evaluations were determined by one-way analysis of variance (ANOVA) with post-hoc tests for significance of differences by the Student-Newman-Keuls Method. Statistical significance was considered at $p<0.05$.

Results

The concentration of polyphenolic acids was higher in extracts of seed powder when compared to extracts of skin powder (Table 4.1). Total phenolic content correlated directly with the measured FRAP antioxidant activities ($R^2 = 0.989$).

At 4 h, treatment with muscadine skin extract, seed extract, and the combination skin and seed extract reduced ear edema by 29%, 32%, and 40%, respectively, compared to the TPA group (Figure 4.1). Indomethacin decreased edema by 58%, significantly more than the skin or seed extracts individually, but not significantly different from the combination skin and seed extracts. At 24 h, edema was reduced by 57%, 45%, 39%, and 39% respectively in the groups treated with indomethacin, skin extract, seed extract, and the combination extract.

Ear punch biopsies were weighed as an index of degree of edema associated with 24 h of inflammation (Figure 4.2). Indomethacin treatment significantly reduced ear biopsy weight compared to the group treated with TPA by 55%. Muscadine skin, seed and combination skin and seed extract each reduced ear biopsy weight compared to the control by 25%, 26% and 41%, respectively. There was not a statistically significant difference in ear biopsy weights between the groups treated with indomethacin and the combined seed/skin extracts ($p = 0.145$). There were no significant differences in ear punch biopsy weights from those treated with acetone and those untreated (7.9 mg).

Myeloperoxidase activity was measured in the ear punch biopsies taken at 24 h after TPA administration (Figure 4.3). Muscadine skin, seeds, and combination skin and seed extracts each inhibited MPO activity by 69%, 59%, and 62%, respectively, compared to the TPA + vehicle-treated ears. Inhibition of MPO activity by muscadine extracts was similar to inhibition by indomethacin (70%).

Discussion

Muscadine grapes and juices are normally ingested, and one might wonder why they should be tested in a model of ear inflammation. The rationale for this is two-fold: 1) the immune system defends all epithelia, and it is feasible that compounds may suppress or induce inflammation in more than one site. 2) It is easier to study anti-inflammatory properties of compounds on an exposed surface than an interior surface. In this regard, our results show that topical application of extracts of muscadine grape skins and seeds significantly inhibited three important processes related to the TPA-induced skin inflammatory response. Decreases were seen in acute (4 h) ear edema, the migration of PMN leukocytes to the dermis within 24 h, and chronic edema at 24 h. Inflammatory markers were significantly reduced in all treatment groups compared to the vehicle-treated TPA ears. Ear thickness and weight in the group treated with combined skin and seed extracts were not significantly different from ear thickness and weight in the indomethacin-treated group. Anti-inflammatory effects of combining the extracts of seeds and skin were additive because the individual extracts did not inhibit inflammation to the same extent as the combined extracts. The reason for this could be due to different phytochemical profiles in the skins and the seeds that allow for greater activity in combination. Ellagic acid, myricetin, quercetin, and kaempferol are the most abundant phenolics in the muscadine skin, while gallic acid, catechin, epicatechin and oligomeric proanthocyanidins (OPC's) are abundant in the seeds [9].

The efficacy of the polyphenolic acids in muscadine skin and seed extracts may differ with respect to inhibition of inflammation in this model. Per unit weight, the seeds have almost two-fold greater total phenolic content than the skins, however skin extracts inhibit inflammation as well as seed extracts. Seeds have high amounts of OPC's which are compounds of 4 to 11

repeating flavan-3-ol units. Transdermal penetration of the seed extract into the ear tissue might not be as effective because of the larger size of these molecules, which might decrease the concentration of active phenolic compounds within the tissue. Alternatively, the phenolic compounds in muscadine skins may be more potent inhibitors of inflammation than those found in the seeds.

TPA applied topically to mouse ears promotes a cascade of inflammatory process such as increased vascular permeability, edema, and mast cell infiltration. Mast cells react quickly in acute inflammatory responses, and release mediators such as histamine, serotonin, and tumor necrosis factor-α which increase vascular permeability and promote neutrophil influx [22]. TPA also causes the release of AA from phospholipid pools resulting in subsequent metabolism to inflammatory mediators. Phospholipase A_2 inhibitors have proven effective against leukocyte infiltration and edema in this TPA model of ear inflammation by inhibiting the release of AA from membrane stores [30]. Products of AA metabolism such as PGI2 and LTB4 interact to elicit vascular permeability leading to edema during the inflammatory response [21]. Therefore compounds inhibiting COX and LOX enzymes have been shown to inhibit TPA-induced inflammation [21].

Phytochemicals in muscadine grapes that exhibit anti-COX II or anti-LOX activity include caffeic acid and its derivatives, kaempferol, quercetin, myricetin, oligomeric proanthocyanidins, gallocatechin, epicatechin, piceid, resveratrol, and viniferin [31]. Caffeic acid has previously been studied in the TPA ear edema model and was found to be a weak inhibitor of TPA-induced inflammation and tumor promotion [32]. Flavonoids have also been shown to inhibit histamine release from human mast cells [33]. Chung et. al. reported that edema formation in this model may also be regulated by H_2O_2 generation [34], as evidenced by anti-inflammatory activity

of several antioxidant compounds against TPA-induced inflammation [35, 36]. Muscadine grapes have extremely high antioxidant capacities [10, 11, 37]. Recently our laboratory reported the ability of muscadine grape skin extract to inhibit the release of TNF-α, IL-1β and IL-6 from monocytes stimulated with lipopolysaccharide [17]. Stimulation by lipopolysaccharide is known to involve redox-sensitive steps that promote NF-κB activation [38]. Multiple phenolics in the muscadine skins and seeds have been shown to inhibit NF-κB activation [24]. Therefore the anti-inflammatory actions of the muscadine grape skin and seed against TPA-induced inflammation could be attributed to direct actions of the polyphenols and their indirect actions via modulation of gene transcription in multiple cell types.

There is considerable potential for muscadine bioactive compounds in functional foods and nutraceutical and cosmeceutical products. The present work shows that muscadine extracts can reduce the inflammatory response to TPA in skin. This suggests that the extracts would be useful for skin care products including moisturizing creams and anti-aging products. An example of a product that is used to support skin and gastrointestinal healing is *Aloe vera*. Aloe inhibits COX in skin and increases collagen biosynthesis and degradation [39]. However, aloe is also ingested and has proved useful in decreasing inflammation in ulcerative colitis [40, 41], suggesting that compounds that suppress inflammation in one epithelium often suppress it elsewhere owing to similarities of the epithelial and mucosal immune systems.

A unique feature of the muscadine grape skin compared to other grape species is its high concentration of ellagic acid [9]. Ellagic acid inhibits degradation of elastic fibers in dermal fibroblast cultures, which is consistent with use as an anti-aging cosmeceutical agent [42]. Ellagic acid is also a potent modulator of metabolic activation and detoxification of polycyclic aromatic hydrocarbon carcinogens in mouse keratinocytes [43]. Similarly, topical application of *vinifera*

grape seed proanthocyanidins protects against dermal inflammation caused by ultraviolet radiation in mice [44]. It is likely that anti-inflammatory phytochemicals in muscadine extracts would protect against exposure to environmental agents and ultraviolet radiation. While our laboratory has previously studied anti-inflammatory activities of muscadines in immune cell culture and in adjuvant-induced inflammation *in vivo* [17], the present data indicate that muscadine extracts are topical anti-inflammatory agents in the skin. It will be interesting to determine whether muscadine extracts may also reduce inflammation in the enteric mucosa.

Abbreviations Used

1. TPA: 12-*O*-tetradecanoylphorbol-13-acetate
2. PMN: Polymorphonuclear
3. MPO: Myeloperoxidase
4. TNF-α: Tumor Necrosis Factor – alpha
5. IL-6, -1β, -8: Interleukin-6, -1β, -8
6. AA: Arachidonic Acid
7. COX: Cyclooxygenase
8. LOX: Lipoxygenase
9. Indo: Indomethacin

Safety

The experimental protocols in the manuscript do not need special attention.

Acknowledgements

The authors wish to thank Muscadine Products Corporation and Paulk Vineyards, Wray, GA for supplying the muscadine grape skin and seed powder. The research was supported by a USDA

SBIR Phase I and Phase II Awards to Muscadine Products Corporations at Paulk Vineyards, Wray, GA.

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Tables and Figures

TABLE 4.1 Phenolic Acid and FRAP values of muscadine extracts.

Ison Grape Fraction	Phenolic Acid (mg/g)	FRAP Value (mmol/100g)
Seed	43.5 ± 0.1	45.4 ± 0.6
Skin	23.0 ± 0.9	19.4 ± 1.1
Skin and Seed	31.9 ± 0.1	28.2 ± 0.6

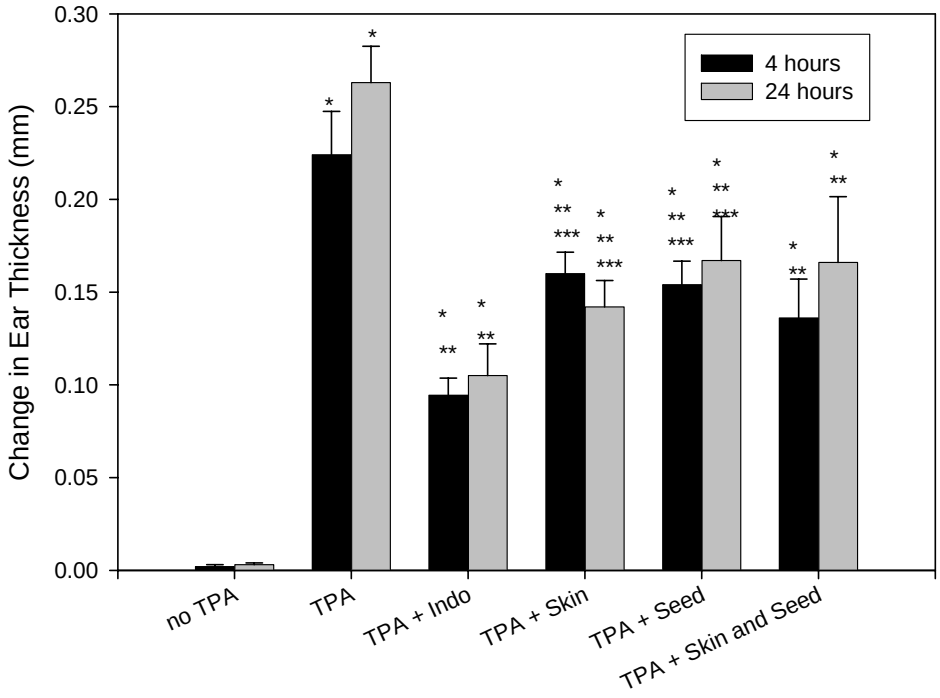

FIGURE 4.1 Change in ear thickness 4 and 24 h after TPA application. Ear thickness was measured with a digital micrometer 4 and 24 h after application of 2 µg TPA. Results represent means ± SEM. *p≤ 0.05 compared to no TPA, **p≤0.05 compared to vehicle treated TPA, *** p≤0.05 compared to indomethacin (indo).

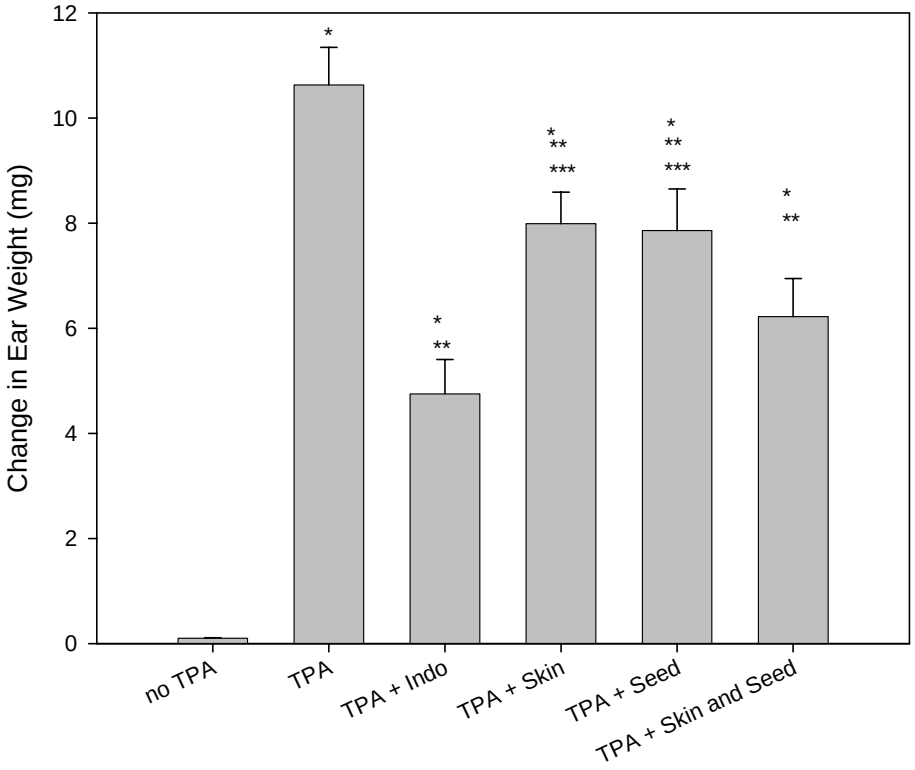

FIGURE 4.2 Change in Ear Punch Weight. Ear punches (6 mm diameter) were taken 24 h after TPA administration. Results represent means ± SEM. *p≤ 0.05 compared to no TPA, **p≤0.05 compared to vehicle treated TPA, *** p≤0.05 compared to indomethacin (indo).

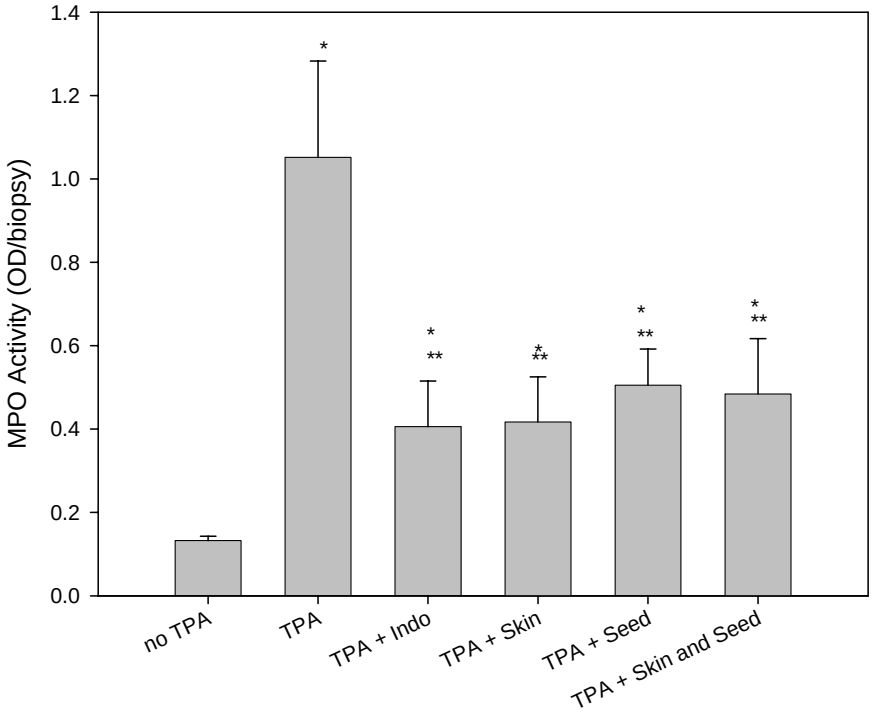

FIGURE 4.3 Myeloperoxidase activity. Myeloperoxidase activity (an index of neutrophil activation) was measured in ear punches 24 h after TPA administration. Results represent means ± SEM. *p≤ 0.05 compared to no TPA, **p≤0.05 compared to vehicle treated TPA.

CHAPTER FIVE

TOPICAL ANTI-INFLAMMATORY ACTIVITY OF *POLYGONUM CUSPIDATUM* EXTRACT IN THE TPA MODEL OF EAR INFLAMMATION

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Submitted to the Journal of Ethnopharmacology

ABSTRACT

The ability of *Polygonum cuspidatum* Sieb. et Zucc. (Polygonaceae) extract (PCE) to inhibit mouse ear inflammation, edema, and polymorphonuclear leukocyte infiltration was tested following topical application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). An ethanolic extract of commercial 200:1 PCE was applied to both ears of female Swiss mice 30 min after TPA (2 μg/ear) administration. Other groups examined consisted of mice treated with either the vehicle, or with a known anti-inflammatory agent, indomethacin (0.5 mg/ear), or with *trans*-resveratrol (0.6 mg/ear). Ear thickness was measured before TPA and at 4 and 24 h post-TPA administration to assess ear edema. Ear punch biopsies were collected at 24 h and weighed as a second index of edema. Myeloperoxidase activity was measured in each ear punch biopsy to assess the extent of neutrophil infiltration. Results: PCE treatment significantly reduced ear edema and MPO activity compared to the TPA control. The PCE response was dose-dependent and a dose of 2.5 mg PCE significantly inhibited all markers of inflammation to a greater extent than indomethacin (0.5 mg). *Trans*-resveratrol did not inhibit inflammation as well as PCE at comparable doses indicating that a combination of factors in PCE may positively contribute to its total activities. Conclusion: PCE exhibits significant topical anti-inflammatory properties.

Key words: Anti-inflammatory; Myeloperoxidase (MPO); Polyphenols; Nutraceutical; Resveratrol; Emodin.

Introduction

Polygonum cuspidatum (PC), commonly called Japanese knotweed or Mexican bamboo, is a member of the polygonaceae family that is widely distributed in Asia and North America. In traditional Chinese medicine, PC is called Hu Zhang. It is used as an analgesic, antipyretic, diuretic, and an expectorant [1] in treatments for arthralgia, jaundice, amenorrhea, chronic bronchitis, and/or hypertension [1]. PCE (PC extract) is now in many nutraceutical product formulations because of its high concentration of *trans*-resveratrol, a polyphenolic *trans*-stilbene (3, 4'-5-trihydroxystilbene)[2, 3]. Resveratrol and its related phytochemicals possess many biological activities, including anti-inflammatory, immunomodulatory, antioxidant, cardioprotective, chemopreventive, anti-bacterial, anti-fungal, and anti-viral effects [4-9]. Other resveratrol analogs in PC root mainly include resveratroloside (3, 5, 4'-trihydroxystilbene-4'-*O*-beta-D-glucopyranoside, piceid (3, 4'-trihydroxystilbene-3-beta-mono-D-glucoside) and piceatannol glucoside (3, 5, 3', 4'-tetrahydroxystilbene-4'-*O*-beta-D-glucopyransoide [10-12]. In addition, PC contains emodin and emodin 8-*O*-glucopyranoside.

Because of its broad ranges of use in traditional Chinese medicine, we became interested in studying PCE's anti-inflammatory properties. Increasing evidence implicates systemic inflammation as a major component of many seemingly unrelated chronic diseases, e.g., Alzheimer's disease, arthritis, colitis, cancer, diabetes, heart disease, obesity, stroke and psoriasis [13-15]. Anti-inflammatory interventions target production of inflammatory cytokines and the activities of COX and LOX enzymes. Polyphenolic phytochemicals from fruits, vegetables, herbs and spices, are known to exert diverse biological effects including the ability to inhibit multiple aspects of inflammation [16]. Diets enriched with anti-inflammatory phytochemicals are recommended for treatment of chronic inflammatory diseases.

Although many individual chemicals in PC have been studied for a variety of activities related to inflammation, the topical anti-inflammatory activity of PCE has not been reported. PCE is standardized to its *trans*-resveratrol content for use in nutraceutical and cosmeceutical products. This should not be interpreted to mean that *trans*-resveratrol is the only molecule of interest in the PCE; indeed, it represents less than 50% of the phytochemicals in most PCE preparations. Since PCE, not purified *trans*-resveratrol, is what is used as the ingredient of most topical products, PCE should be screened for its topical bioactivities. The present study tested whether PCE has topical anti-inflammatory activities in the well-characterized tetradecanoylphorbol acetate (TPA) induced mouse ear edema/inflammation screening model [17, 18]. This study was designed to test the ability of PCE to inhibit inflammation, edema, and PMN leukocyte infiltration of the ear following topical application of TPA.

Materials and methods

Materials

12-*O*-Tetradecanoylphorbol 13-acetate, hexadecyltrimethylammonium bromide, indomethacin (minimum 99% TLC), 3 3', 5 5' tetramethylbenzidine dihydrochloride, *N, N*-dimethylformamide, trans-3', 4', -5'-trihydroxystilbene (*trans*-resveratrol), Folin-Ciocalteu reagent, gallic acid, and 10 mM 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) were all purchased from Sigma (St. Louis, MO). *Polygonum cuspidatum* 200:1 powdered extract was purchased from Supplemental Health Formulations (Mayer, AZ).

Preparation of Polygonum cuspidatum extract (PCE)

PCE used in this study was a 200-fold concentrate prepared from PC root processed in India, but grown in China. Product specification chemical analysis from Supplemental Health Formulations reported that the emodin content was < 20 mg/gm and *trans*-resveratrol complex at

least 500 mg/g. As a quality control, we analyzed the extract by HPLC analysis using an Agilent 1200 system (Wilmington, DE). The details of the chromatography comprise the following: the column was a Phenomenex LUNA ODS2 (Torrance, CA), 150 mm by 4.6 mm, ID, 5 μm particle size; isocratic elution with a mobile phase consisting of 40:60 (v/v) acetonitrile:water; injection volume of 10 μl, flow rate of 1 ml/min and detection at 308 nm . The extract contained 48.4% *trans*-resveratrol.

The phytochemicals in the 200:1 PC powder were dissolved in 50% ethanol (1 part PC to 9 parts ethanol) and stirred for one hour at room temperature. The mixture was centrifuged (1500 rpm for 10 min, 4°C) and the supernatant was diluted for topical dose-response applications in this study.

Measurement of total phenolics of extracts

Total phenolic acid content of each extract was measured by the method of Slinkard and Singleton [19] with minor modifications. Triplicate samples of a 1:10 extract (wt/vol) (20 μL) were added to 1.58 mL of distilled water in 3 mL polystyrene cuvettes. 100 μL of Folin-Ciocalteu reagent was added and the sample was mixed well. Within 10 minutes, 300 μL of sodium carbonate solution (200 g Na_2CO_3 in 1 L distilled water) was added. Solutions were incubated for 2 h at room temperature. Absorbance was measured at 765 nm. Total phenolic acid concentration was calculated from a gallic acid standard curve (0-500 mg/L) and expressed as gallic acid equivalents per gram 200:1 PCE powder.

Measurement of FRAP (Ferric Reducing Antioxidant Power)

The antioxidant activity of a 1:10 (wt/vol) extraction was determined in triplicate by the FRAP method [20]. 10 μL of the sample or standard, 30 μL of distilled water and 300 μL of FRAP reagent were mixed. FRAP reagent was made by mixing 25 mL acetate buffer (300 mM, pH

3.6), 2.5 mL TPTZ solution (10 mM 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) dissolved in 40mM HCl), and 2.5 mL of 20 mM ferric chloride solution. The solutions were incubated at 37º C for six minutes then 340 µL of distilled water was added. The absorbance of the sample or standards was read immediately at 593 nm. FRAP value was calculated from a standard curve of ferrous sulfate (0-1 mmol/L) and the antioxidant power of the PCE was expressed as mmol ferrous sulfate equivalents/100g dry weight of the 200:1 PCE powder.

Animals

The present study was performed according to international and national rules considering animal experiments. It was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia and conducted according to IACUC guidelines. Female Swiss Webster mice weighing 22-25 g were housed in groups of 4 in large shoebox cages and were fed a standard diet *ad libitum* with free access to water. Each test group had 8 animals. Animals were in the fed condition throughout the experiment. Photoperiods equaled 12 h of light and 12 h of darkness daily, with the environmental temperature maintained at 21°C.

TPA-induced mouse ear edema

Edema was induced in both ears of each mouse by the topical application of a total of 2 µg TPA dissolved in 20 µL of acetone to both the inner and outer ear surfaces. Thirty minutes after the application of TPA, the inner and outer surface of each ear was treated (10 µL to each side) with 50% ethanolic solutions of PCE ranging from 0.075 – 2.5 mg PCE/ear, a 50% ethanol solution of trans-3', 4', -5'-trihydroxystilbene (0.62 mg/ear), indomethacin (0.5 mg/ear dissolved in 50% ethanol as an anti-inflammatory drug standard), or 50% ethanol (vehicle control). The thickness of each ear was measured using a micrometer (Mitutoya Series IP65) before and at 4 h and 24 h after TPA administration. The micrometer was applied near the top of the ear just distal to the

cartilaginous ridges. At 24 h each animal was sacrificed with CO_2 inhalation as per IACUC approved protocol. Ear punch biopsies (6 mm in diameter hole punch) were taken immediately, weighed, frozen and stored at -80°C. A single investigator performed all ear measurements and biopsies in order to standardize the procedure and reduce experimental error.

Myeloperoxidase assay

Tissue MPO (MPO, E.C. 1.11.1.7) activity was measured in biopsies taken from both ears 24 h after TPA administration using a method by Suzuki et. al. [21] and modified by De Young et. al. [22]. Each mouse ear biopsy was placed in 0.75 mL of 80 mM phosphate-buffered saline (PBS) pH 5.4 containing 0.5% hexadecyltrimethyl-ammonium bromide (HTAB). Each sample was homogenized for 45 s at 4°C with a small sample laboratory Tissue Tearor Homogenizer Model 985-370 (Biospec Products, Bartlesville, OK). The homogenate was transferred quantitatively to a microcentrifuge tube with an additional 0.75 mL HTAB in PBS. The 1.5 mL sample was centrifuged at 12,000 x g for 15 min, maintained at 4°C. Triplicate 30 µL samples of the resulting supernatant were added to 96-well microtiter plate wells. For the MPO assay, 200 µL of a mixture containing 100 µL of 80 mM PBS (pH 5.4), 85 µL of 0.22 M PBS (pH 5.4), and 15 µL of 0.017% hydrogen peroxide were added to each well. 20 µL of 18.4 mM tetramethylbenzidine HCl in 8% aqueous dimethylformamide was added to start the reaction. Microtiter plates were incubated at 37°C for 3 min, and then placed on ice. The reaction was stopped with the addition of 30 µL of 1.46 M sodium acetate, pH 3.0. MPO enzyme activity was assessed colorimetrically using a Bio-Tek Microplate Reader (ELx 808) at an absorbance wavelength of 630 nm. MPO activity was expressed as OD/biopsy.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical evaluations were determined by t-tests and one-way analysis of variance (ANOVA) with post-hoc tests for significance of differences by the Student-Newman-Keuls Method. Statistical significance was considered at $p<0.05$.

Results

Total phenolics and FRAP value

A 50% ethanolic extract (1:10 wt/vol) of the 200:1 PCE contained 188 mg total phenolics per gram of PCE. Antioxidant power based on the FRAP assay was 85 mmol ferrous sulfate equivalents/100g dry weight of PCE.

Ear edema

Ear edema was observed in all TPA-treated animals 4 h after treatment. Experimental groups showed significantly reduced ear edema compared to TPA treatment alone. PCE at 2.5, 1.25, and 0.3 mg per ear was as potent as indomethacin (0.5 mg/ear) in reducing edema (Figure 5.1). These treatments inhibited edema 61%, 55%, 52%, and 65% (IM), respectively compared to TPA treated with vehicle controls. In comparison, 0.62 mg of commercially purified *trans*-resveratrol inhibited edema by only 35%. At 24 h, all experimental groups had significantly reduced ear edema compared to TPA alone except PCE at 0.075 mg per ear and the *trans*-resveratrol-treated groups. PCE at 1.25, 0.3, and 0.15 mg per ear inhibited edema as well as IM (58%, 36%, 40%, and 45% (IM), respectively. PC, 2.5 mg per ear, was significantly more potent than indomethacin in reducing edema with a 73% reduction compared to the TPA treated vehicle control.

Ear punch biopsy weights were significantly lower in all PCE groups compared to the TPA-treated, vehicle control group (Figure 5.2). When comparing the activity of PCE and indomethacin, 2.5 mg of PCE reduced edema 80%, which was significantly greater than the reduction by 0.5 mg indomethacin (36%).

Myeloperoxidase activity

Myeloperoxidase activity was measured in the ear punch biopsies taken 24 h after TPA administration as an index of neutrophil infiltration (Figure 5.3). Biopsies from ears treated with indomethacin at 0.5 mg/ear and PCE at 1.25 and 2.5 mg/ear doses had significantly reduced MPO activity. The higher PCE dose (2.5 mg/ear) decreased MPO to 18% of the activity of the TPA-treated vehicle control group and was significantly greater at decreasing MPO activity than indomethacin. Indomethacin (0.5 mg/ear) and PCE (1.25 mg/ear) inhibited MPO to the same extent at 53% and 45%, respectively.

Discussion

Topical application of PCE significantly inhibited acute (4h) ear edema, chronic edema at 24 h, and the migration of PMN leukocytes into the dermis within 24 h as indicated by reduced MPO activity. Interestingly, PCE, at a dose of 2.5 mg/ear reduced edema and inhibited leukocyte infiltration to a greater extent than indomethacin (0.5 mg/ear). Indomethacin is a very potent and highly renal toxic NSAID drug with an LD_{50} of 50 mg/kg when given orally to mice based on a 14 day mortality response [23]. This translates to 1.25 mg indomethacin per 25 g mouse, just under the dose administered topically (1 mg/mouse). No significant toxicity has been shown for PCE in this bioequivalence range. Since the dose of indomethacin was a very high dose, the PCE used for topical anti-inflammatory activity is as active, without comparable toxicity.

PCE is widely used in nutraceutical products because of its high concentration of resveratrol and resveratrol derivatives. Knowing that PCE contains many bioactive phytochemicals in addition to *trans*-resveratrol we decided to examine any additive or synergistic effects by comparing it to purified *trans*-resveratrol. The certificate of analysis of the PCE powder, 200:1, reported a 50% *trans*-resveratrol content. The amount of *trans*-resveratrol used in the experiment (0.62 mg/ear) was equal to the amount of reported resveratrol in 1.25 mg of PCE, 200:1. Although *trans*-resveratrol did significantly inhibit the inflammatory markers of acute and chronic edema, it did so at a bioequivalence approximately equal to 0.075 mg PCE.

The TPA model of ear inflammation is useful for screening the efficacy and potency of prospective topical anti-inflammatory compounds or herbal extracts. In epidermal cell culture, TPA stimulates cell proliferation and increases the metabolism of arachidonic acid to leukotrienes and prostaglandins [24]. TPA applied topically to mouse ears promotes the cascade of events of the inflammatory process such as vascular permeability, edema, and mast cell infiltration. Mast cells react quickly and release mediators such as histamine, serotonin, and tumor necrosis factor-α during acute inflammatory responses. These mediators increase vascular permeability and promote neutrophil influx [17]. TPA also causes the release of arachidonic acid from membrane phospholipids, resulting in subsequent formation of inflammatory mediators. Phospholipase A_2 inhibitors have proven effective against both leukocyte infiltration and edema in the TPA model of ear inflammation by inhibiting the release of arachidonic acid from membrane stores [25]. Products of arachidonic acid metabolism such as PGI_2 and LTB_4 interact to increase vascular permeability leading to edema during the inflammatory response [18]. Therefore compounds inhibiting COX and LOX enzymes have been shown to inhibit TPA-induced inflammation [18]. Chung et. al. reported that edema formation in this model may also be

regulated by H_2O_2 generation [26], as evidenced by anti-inflammatory activity of several antioxidant compounds against TPA-induced inflammation [27, 28].

One of the early hallmarks of skin irritation and local inflammation in the TPA model is thickening within 1-4 h due to increased vascular permeability, edema and swelling within the dermis [29]. Secondarily, PMN leukocytes migrate to the dermis within 24 h and are estimated by the MPO assay. Both of these inflammatory processes were blocked by topical application of PCE in a dose-dependent manner.

While most PC research to date has been done on individual chemical constituents, PCE extracts are widely used in nutraceutical and cosmeceutical products. Biological testing of the PCE in test systems has yielded some interesting experimental data mostly from *in vitro* screening tests. PCE is suggested to be cardioprotective because it decreases cellular cholesterol ester content in HepG2 cells by inhibiting acyl-coenzyme A-cholesterol acyltransferase activity [30]. Resveratrol and emodin components isolated from PC are both protein tyrosine kinase C inhibitors [31, 32]. These kinases play an important role in regulation of cell growth and transformation and are therefore potential targets for anticancer agents. PCE exerts chemopreventive effects as demonstrated by its inhibition of growth of three prostate cancer cell lines [33]. PCE also possesses some anti-viral properties by inhibiting the production of the hepatitis B virus [34]. To date, there has been little *in vivo* testing of PCE. The present data represent topical anti-inflammatory activity of PCE *in vivo*.

The testing of PCE versus any single constituent both *in vivo* and *in vitro* becomes important when considering known additive and synergistic actions of phytochemicals in living systems. Although PC is processed to enrich the *trans*-resveratrol in PCE, we do not imply that all the anti-inflammatory activities are attributable to the resveratrol component. When the PCE

extract was compared directly to known *trans*-resveratrol, it appeared that there was more anti-inflammatory activity in the PCE extract. This comparison was performed to estimate relative activity of the PCE and to determine the effect of *trans*-resveratrol in the TPA ear edema model. In addition to 50% resveratrol, PCE extract contains other compounds, including emodin, resveratrol glycosides such as polydatin, and quercetin, with known anti-inflammatory activities and that these are biologically additive or synergistic with the activity of *trans*-resveratrol. There are ample reports in the literature of such interactions within biological systems *in vitro* [35, 36]. For example, in human leukemia cells, ellagic acid and quercetin interact synergistically with resveratrol to induce apotosis and cell cycle arrest [37].

Trans-resveratrol and its derivatives have been studied extensively for their health promoting qualities, including its anti-inflammatory activity. Resveratrol and analogues of resveratrol all inhibit human TNF-α, and LPS-induced activation of NF-κB [38, 39]. Resveratrol can inhibit prostaglandin E_2 release from human peripheral blood leukocytes [40]. In a model of early colonic inflammation in rats, resveratrol significantly decreased elevated plasma levels of prostaglandin D_2 and decreased the expression of COX-2 [41]. Resveratrol also inhibits the TPA-induced mouse dorsal skin inflammatory response mainly via NF-κB and activator protein-1 (AP-1) modulation [42, 43].

Emodin, an anthraquinone, is a major component in PCE. The emodin content in PCE is controlled during the processing to achieve a final content of $\leq$ 20 mg/g to reduce the risk of unpleasant gastrointestinal motility side effects of emodin in humans [44]. Emodin is a phytoestrogen, and an anti-viral, and anti-inflammatory agent [45]. It inhibits NF-κB activation and IκB degradation, and decreases gene expression of cell surface adhesion proteins in vascular endothelial cells [46]. Emodin also effectively inhibits gene expression for TNF-α, iNOS, and IL-

10 in RAW 264.7 macrophages by altering the intracellular location of NF- κB, and the production of IκB [47]. Thus, even though emodin levels have been reduced from levels in PC root, its continued presence in PCE may well contribute to the anti-inflammatory activity of PCE.

The present work demonstrated that 50% ethanolic solution of PCE has topical anti-inflammatory activity. At higher doses, PCE was more potent than the standard anti-inflammatory drug indomethacin at 0.5 mg/ear. *Trans*-resveratrol alone was not as potent as the PC extract, suggesting additive or synergistic effects of resveratrol with additional phytochemicals in the PCE. Because anti-inflammatory activity is a desirable property in a topical preparation, the present work done in vivo supports the use of PCE in cosmeceutical and dermatological products.

We tested PCE in a topical model of inflammation, but it is possible that PCE may have systemic anti-inflammatory activities. The rationale for this is two-fold: 1) the immune system defends all epithelia, and it is feasible that compounds may suppress or induce inflammation in more than one site. 2) It is easier to study anti-inflammatory properties of compounds on an exposed surface than an interior surface. PCE is currently sold commercially in nutritional supplements, and in traditional Chinese medicine, PCE is ingested orally. Further study is warranted to determine systemic anti-inflammatory properties of PCE *in vivo.*

Abbreviations used

1. TPA: 12-*O*-tetradecanoylphorbol-13-acetate
2. PMN: Polymorphonuclear
3. MPO: Myeloperoxidase

4. TNF-α: Tumor Necrosis Factor – alpha

5. IL-6, -1β, -8: Interleukin-6, -1β, -8

6. COX: Cyclooxygenase

7. LOX: Lipoxygenase

8. Indo: Indomethacin

9. PC: *Polygonum cuspidatum*

10. PCE: *Polygonum cuspidatum* extract

Acknowledgements

The authors are grateful to Ms. Linda Duncan for her technical assistance.

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Figures

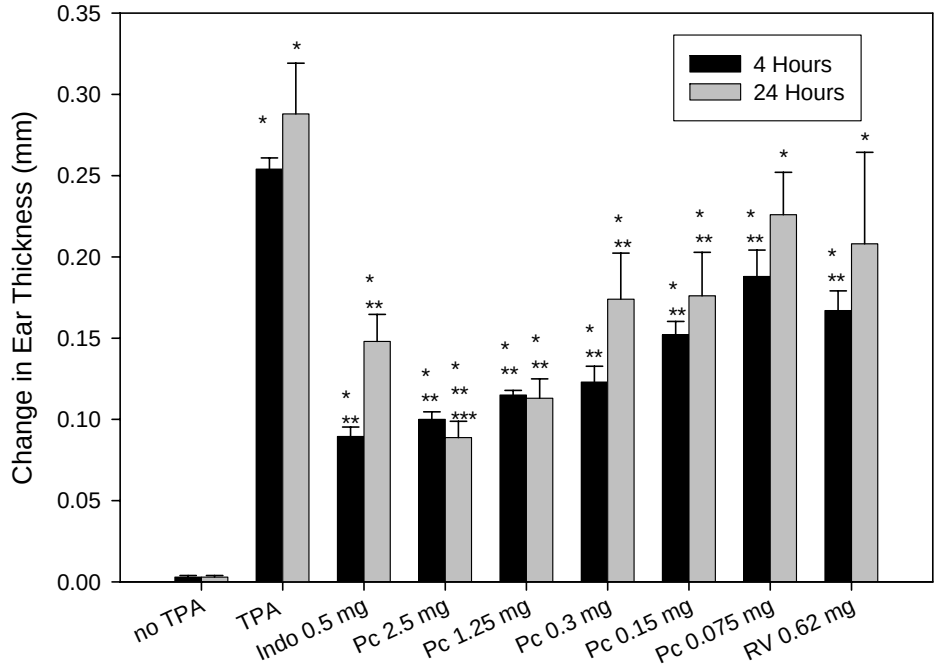

FIGURE 5.1 Change in ear thickness 4 and 24 h after TPA application. Ear thickness was measured with a digital micrometer 4 and 24 h after application of 2 µg TPA. Results represent means ± SEM. *p≤ 0.05 compared to no TPA, **p≤0.05 compared to TPA control, ***p≤0.05 compared to indomethacin (indo).

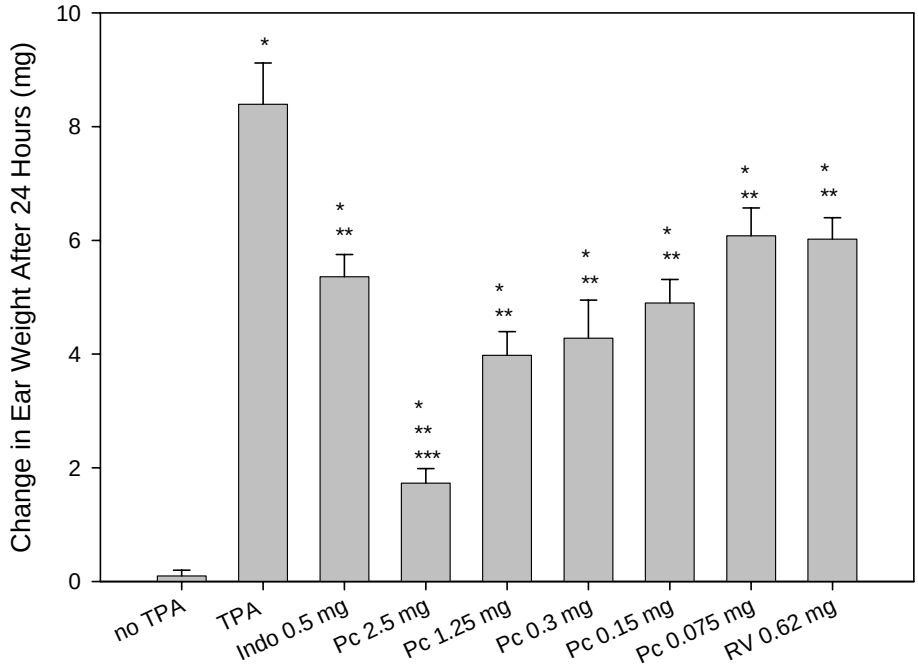

FIGURE 5.2 Change in Ear Biopsy Weight. Ear punches (6 mm diameter) were taken 24 h after TPA administration. Results represent means ± SEM. *p≤ 0.05 compared to no TPA, **p≤0.05 compared to TPA control, ***p≤0.05 compared to indomethacin (indo).

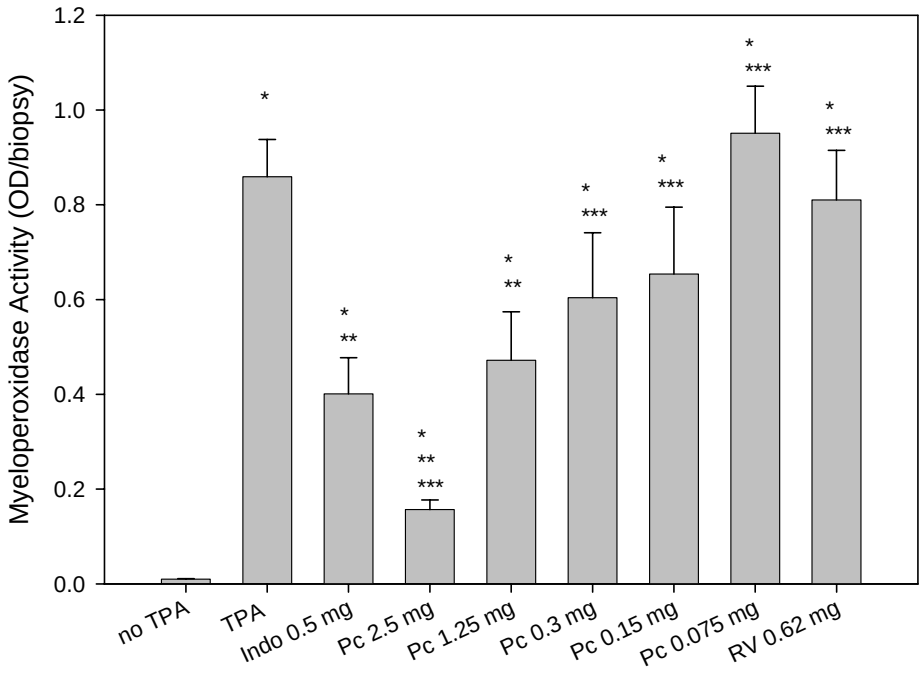

FIGURE 5.3 Myeloperoxidase activity. Myeloperoxidase activity (an index of neutrophil activation) was measured in ear punches 24 h after TPA administration. Results represent means ± SEM. *p≤ 0.05 compared to no TPA, **p≤0.05 compared to TPA control, ***p≤0.05 compared to indomethacin (indo).

CHAPTER SIX

ANTI-INFLAMMATORY EFFECTS OF MUSCADINE SKIN EXTRACT IN EXPERIMENTALLY-INDUCED ULCERATIVE COLITIS IN RATS

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ABSTRACT

TNBS (2,4,6-trinitrobenzene sulfonic acid)-induced colitis in rats reproduces macroscopic, histological and immunological hallmarks of human ulcerative colitis. TNBS binds to proteins initiating immune activation, infiltration, inflammation, edema and severe colon ulceration. Muscadine (*Vitis rotundifolia*) phytochemicals are markedly anti-inflammatory in both *in vitro* and *in vivo* tests. Hypothesis: Dietary muscadine skin powder in chow and extracts in drinking water, ± one enema with muscadine extract, will attenuate TNBS-induced colitis in rats. Experimental Design: Rats were pre-fed 5% muscadine skin powder in chow and muscadine skin extract in drinking water for 3 days before, and 7 days after TNBS treatment. Rats were administered TNBS intracolonically (120 mg/kg in 0.25 ml 50% EtOH). One group was given a muscadine extract enema (1 ml) 1 hr before TNBS. Control groups consisted of rats ± TNBS ± saline enema fed normal chow diets. Daily food intakes and body weights were recorded. Colon weights, colon lengths, macroscopic scores and myeloperoxidase activities (MPO, a marker of neutrophil invasion) were determined after sacrifice on Day 7. Results: Muscadine-enriched diets decreased neutrophil invasion into the colonic tissue, edema and macroscopic scores. Inflammation in the colon was eliminated in rats receiving the muscadine enema treatment. In these, TNBS produced no significant changes in markers from untreated controls fed normal chow. Conclusion: Muscadine phytochemicals exert a powerful protective effect in the TNBS model of colitis.

Introduction

Ulcerative colitis (UC) is an Inflammatory Bowel Disease (IBD) that is the cause of illness for 1-2 million Americans. It is characterized by the ulceration of the innermost lining of the colonic mucosa, usually in the rectum and sigmoid colon and manifests as peri-anal irritations, fissures, hemorrhoids, fistulas, ulcers and abscesses. The origin of UC is unknown, but dietary, genetic, environmental, and immunological factors are all suggested causes. Regardless of the lack of understanding of its etiology, there are many pathologic features seen in the progression of the disease. Among these include genetic predisposition, increase in inflammatory mediators, increased oxidative stress, imbalance in gut microflora, abnormal glycosaminoglycan content of the mucosa, and decreased oxidation of short chain fatty acids [1]. Primary lesions in UC begin with neutrophil invasion in areas of inflammation. Because the mucous layer is thinnest in the rectum and sigmoid colon, the disease is most pronounced in these areas. Tissue damage develops into small erosions and eventually ulcers caused by inflammatory cytokines released from leukocytes, macrophages and neutrophils. Further damage leads to abscess formation in crypts, necrosis, and ragged ulceration of the mucosal layer.

Major classes of drugs used to manage the disease include aminosalicylates, corticosteroids, and immunomodulators including tumor necrosis factor-alpha (TNF-α) receptor antagonists. Side effects from these drugs, however, can be quite severe.

The muscadine grape (*Vitis rotundifolia*) is a unique plant of the southeastern United States that is tolerant to heat, humidity and various pests. It is a rich source of biologically active constituents including ellagic acid, quercetin, resveratrol, and anthocyanins. We observed that muscadine extracts have anti-inflammatory effects both *in vitro* and *in vivo* [2]. Muscadine phytochemicals are known to alter monocyte inflammatory cytokines release upon stimulation

by lipopolysaccharide and also decrease the hydroxyl-radical release from activated neutrophils. In addition, rats fed a diet of 5% muscadine grape skin showed 50% less paw edema than control animals fed a regular chow diet when injected with carrageenan into the foot pad [2]. Extracts of muscadine seeds and skins also inhibit topical inflammation of the mouse ear stimulated by phorbol myristate acetate [3].

Because UC is a disease of inflammation, the anti-inflammatory properties of muscadine grapes lead one to ask whether the phytochemicals that are concentrated in the grape skin might affect the inflammatory processes involved in the pathology of UC. We have chosen to study the beneficial effects of muscadine grape skin on treatment of UC in the rat using the trinitrobenzene sulfonic acid (TNBS) rat model of inflammatory bowel disease [4]. TNBS is injected intrarectally and acts as a hapten to produce an intense local inflammatory response that exhibits many of the macroscopic, histological and immunological responses seen in UC. In this study, we are examining the effects of pre- and post-feeding 5% muscadine skin powder in the diet, the oral ingestion of a concentrated muscadine skin extract, and the administration of muscadine skin extract intrarectally.

Materials and Methods

Preparation of Muscadine Skin extract

Muscadine skin powder of the Ison (purple) variety was prepared by drying the separated skins at 120°C for 12 h in a forced-air pan dryer manufactured by Powell Manufacturing Company (Bennettsville, SC). The dried skins were ground in a Fitz Mill Communitor Hammermill manufactured by Fitzpatrick Company (Elmhurst, IL). In the muscadine treated animals, this powder was included as 5% of the chow.

The phytochemicals were extracted from the dried powders in order to be suspended in the drinking water and administered intrarectally by adding 100g skin powder to 900 ml of 50% ethanol and stirring for one hour. The mixture was centrifuged (1500 rpm for 10 minutes) and the supernatant was removed. To remove the ethanol and to concentrate the phytochemicals, the extract was heated to 65°C and stirred until the volume was reduced by 50%. Extracts were standardized based on total phenolic content.

Experimental Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia and conducted according to IACUC guidelines. Male Wistar rats weighing 200 g were ordered from Harlan Laboratories (Indianapolis, Indiana) and were placed individually in single wire hanging cages. Photoperiods equaled 12h of light and 12 h of darkness daily, with environmental temperature maintained at 21°C. Rats were pre-fed for 3 days with either control chow or control chow containing 5% muscadine skin powder. All rats were fasted 24 hours before the induction of colitis but were allowed access to water throughout.

Induction of Colitis

Colitis was induced using according to the procedure described by Whittle, et.al.[5]. Rats were anesthetized with 0.075 ml/100g of a ketamine, acepromazine, xylazine (3:2:1 v:v:v) mixture (Fort Dodge Animal Health/Wyeth). Once sedated, 0.12mg/g TNBS (Sigma, St. Louis, MO) dissolved in 50% ethanol was administered intrarectally via a 2 mm outside diameter flexible plastic tube, in a volume of 0.25 ml. The catheter was inserted 8 cm into the colon, and the TNBS was administered in a constant flow as the catheter removed. Sham animals received a

0.25 ml saline intracolonic injection following the same procedure. After the intracolonic injections, animals were maintained in a Trundleberg position for 15 min.

One experimental group of animals received an enema of muscadine skin extract 1 h before TNBS administration. The animals were sedated as described above and a catheter was inserted 8 cm into the colon. One ml of the concentrated muscadine skin extract was irrigated into the colon within 15 seconds.

Experimental groups each had 8 animals and consisted of:

1. Saline injection (no TNBS), normal chow
2. TNBS injection, normal chow
3. TNBS injection, 5% muscadine skin in the diet and 10% concentrated extract in the drinking water
4. Saline enema, TNBS injection, normal chow
5. Muscadine extract enema, TNBS injection, 5% muscadine skin in the diet and 10% concentrated extract in the drinking water.

The animals were checked daily for behavior, food intake, stool consistency, and body weight. Seven days after TNBS administration, the rats were sacrificed via carbon dioxide inhalation.

Assessment of Colitis

The entire colon of each animal was removed, slit longitudinally, and gently rinsed with ice-cold PBS buffer, pH 7.0 to remove any feces. The colon was weighed, and length was measured. Macroscopic scoring was performed by a single investigator and was assessed based on the colitis score described in Table 1. The colon was then cut up and snap frozen in liquid nitrogen and stored at -80°C until used for biochemical assay.

2.5 Assessment of neutrophil infiltration (myeloperoxidase(MPO) activity)

MPO activity was assessed in full-thickness tissue samples from macroscopically inflamed areas, or comparable locations in the control rats by a method described by Grisham et. al. [6]. It was the most distal region of each colon that was used to assess neutrophil invasion. Tissue samples (200mg/4ml) were placed in an ice-cold solution of hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in a 50 mM potassium phosphate buffer, pH 6.0) and were homogenized with a Tissue Tearor Homogenizer Model 985-370 (Biospec Products, Bartlesville, OK) three times for 30 sec each. The homogenizer was then rinsed with 2 ml HTAB buffer. The pooled homogenate and washes were sonicated for 10 sec. The sample was then freeze-thawed three times (-80 °C/37 °C) and then centrifuged at 4,000 x g for 25 minutes at 4 °C. To a cuvette, 69 µl of the supernatant was added to 2 ml of a 50 mmol/L phosphate buffer (pH 6.0) containing 0.167 mg/ml *O*-dianisidine hydrochloride and 0.0005% hydrogen peroxide. The change in absorbance over two minutes was measured at 460 nm. One unit of MPO activity is defined as that converting µmol of hydrogen peroxide to water in 1 minute at 22 °C. Results were also normalized based on MPO units per mg protein as assessed by the Bradford Protein Assay (Bio-Rad). All other chemicals were purchased from Sigma, St. Loius MO).

Results

A thickening and a shortening of the colon is a hallmark characteristic of inflammation in the TNBS model of ulcerative colitis. Normal chow fed, TNBS injected animals had a 70.4% increase in colon weight compared to the colon weight of a healthy animal (Figure 6.1). The control animals given an additional saline enema had a 72.8% increase in colon weight. The increase was reduced to only 42.6% in the animals fed the muscadine diet, and was reduced to 25.7% in those given an additional muscadine extract enema. Inflammation due to TNBS

reduced the colon length by 33.8% compared to healthy animals (Figure 6.2). Control animals given an additional saline enema had a 25.5% reduction in colon length. Animals fed the muscadine diet only had a 7.8% reduction in colon length, and those given an additional muscadine extract enema had no significant difference in colon length compared to the healthy animal.

Gross morphological damage scores were 54.5% and 81.8% lower in the muscadine fed, and additional muscadine extract enema groups, respectively, than in the TNBS, chow fed control group (Figure 6.3, Figure 6.4).

MPO activity was measured as a marker of neutrophil invasion into the colon tissue. The saline enema treated group inhibited activity by 25.2% compared to the TNBS, chow fed control animal (Figure 6.5). The muscadine fed group and the muscadine extract enema group inhibited MPO activity by 60% and 82.1%, respectively.

Discussion

Results support the hypothesis that muscadine grape skin in the diet and/or muscadine skin extract injected intrarectally act as anti-inflammatory agents in the colon by decreasing markers of inflammation including colon weight, macroscopic scoring and neutrophil invasion. To elucidate the mechanism of action, a further understanding of the pathophysiology of UC is required.

Disregulation of the immune function is thought to be a major contributing factor in the progression of UC [7]. In the normal gut, the gastrointestinal tract acts as a barrier to foreign antigens from food and intestinal bacteria and its byproducts. The "controlled inflammation" that occurs is necessary to maintain mucosal homeostasis and eradicate the pathogen. Once the harmful antigen is gone, the local immune response must be attenuated, and is done so by either

CD4+/CD25+ cells produced by the thymus, or by apoptosis of T lymphocytes, the major cell-mediated immune response cells [8, 9]. In a diseased colon, this attenuation of the immune response is absent, and vicious cycle of inflammation occurs.

When exposed to exogenous antigens, a cell-mediated immune response is activated in the gut and T lymphocytes secrete specific pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) being the most abundant [10]. TNF-α amplifies the inflammatory response by increasing transcription of itself, and IL-1ß and IL-6 mainly via activation of the nuclear-factor kappa B (NF-κB) pathway. TNF-α also increases the expression of adhesion molecules, and proliferation of fibroblasts [11, 12].

The exogenous antigens also directly stimulate production of cytokines such as IL-1, IL-6, and TNF- α from the intestinal epithelium [13]. These cytokines amplify the immune response by enhancing the proliferation of T lymphocytes, facilitating cell-cell signaling, and promoting neutrophil infiltration into the inflamed tissue, a key stage in the inflammatory process [14]. Neutrophils reduce molecular oxygen to the superoxide anion radical and through the enzyme MPO, the potent cytotoxic oxidant, hypochlorous acid, is formed from hydrogen peroxide and chloride ions [15]. Cytokines also play a direct role in the inflammatory response by rapidly synthesizing and secreting reactive oxygen species, nitric oxide, leukotrienes, platelet-activating factor, and prostaglandins [16-19]. Cellular injury and necrosis are induced by reactive oxygen species by peroxidation of membrane lipids, protein denaturation, and DNA damage.

Current drug treatment includes corticosteroids and immunomodulators. Corticosteroids bind to the cytosolic glucocorticoid receptors and inhibit the arachidonic acid cascade, thus inhibiting activation of certain transcription factors such as IL-1, -6, and interferon-gamma. The therapy is designed for short-term, but if used chronically, side effects could include cataracts,

osteoporosis, myopathy, and conditions associated with immune suppression and adrenal insufficiency. Immunomodulatory drugs mainly inhibit TNF-α activation thus inhibiting the T-lymphocyte activity decreasing the cell-mediated immune response. Patients see a faster recovery rate than with corticosteroids and a longer remission, but the chance for serious side effects is increased. Immunomodulators promote activation of latent infections such as tuberculosis, and increase the vulnerability to active infections. There is a decreased immune vigilance, which can increase early tumor formation. Other side effects include demyelination and other neurological damage, aplastic anemia, intestinal perforations, and congestive heart failure.

Phytochemicals have been shown to modulate many metabolic pathways and signaling pathways that alter gene expression, mainly by inhibiting NF-κB. These modulatory effects may be especially important in the gastrointestinal tract because it is the largest body surface in contact with the outside world. It is the site of the mucosal immune system, and the gut-associated lymphoid tissue is the largest lymphatic organ in the body. It is also exposed to the highest concentrations of phytochemicals found anywhere in the body. The study of phytochemicals as a treatment for UC is not novel but is warranted due to the harsh side effects of current drug treatment. Dietary quercetin, a main phytochemical in the muscadine grape, was shown to ameliorate TNBS-induced colitis in rats by way of TNF-α induced NF-κB activation [20]. Resveratrol, another phytochemical in the muscadine grape, has been shown to inhibit TNF- α activation of NF-κB *in vitro* and is protective against TNBS-induced colitis due to impairment of neutrophil function, decrease in NF-κB activation, and stimulation of apoptosis in colonic cells [21, 22].

Our previous *in vitro* work shows that muscadine grape skin phytochemicals inhibit several processes related to the pathology of UC. In human mononuclear cells stimulated with muscadine grapeskin extract inhibited the release of TNF-α, IL-1β and IL-6, three of the main cytokines involved in UC progression. In phorbol-myristate acetate activated neutrophils, muscadine grape skin extract inhibited the superoxide respiratory burst [2]. Muscadine grape skin extracts have also been shown to be highly anti-oxidant [23]. *In vivo* work demonstrated that topical application of extracts of muscadine skins to the mouse ear inhibited the inflammatory process associated with phorbol-myristate acetate administration including edema, and neutrophil infiltration [24].

Muscadine grape skin phytochemicals exert highly anti-inflammatory and anti-oxidant properties and can be useful, non-toxic treatments in diseases of inflammation including ulcerative colitis.

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Tables and Figures

Table 6.1 Criteria and score for gross morphological damage.

Colitis Score	Gross Morphology
0	No Damage
1	Localized hyperemia, but no ulcers
2	Hyperemia and ulceration in one site
3	Two or more sites of ulceration and/or inflammation
4	Two or more major sites of inflammation with ulceration extending > 1 cm along the length of the colon

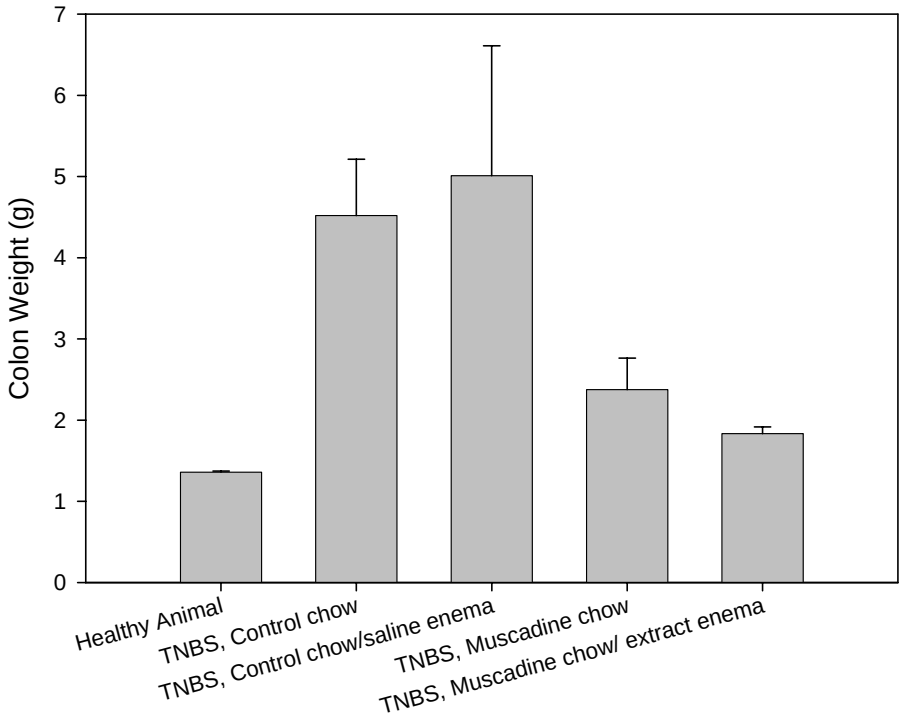

FIGURE 6.1 Colon weight as an index of inflammation and edema.

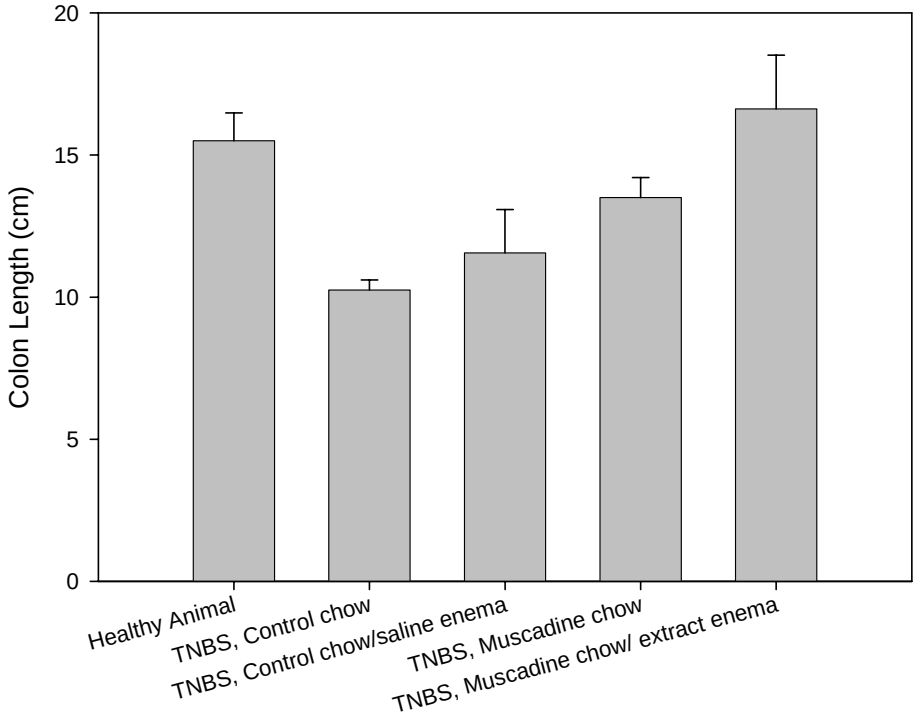

FIGURE 6.2 Colon length as an index of inflammation and edema.

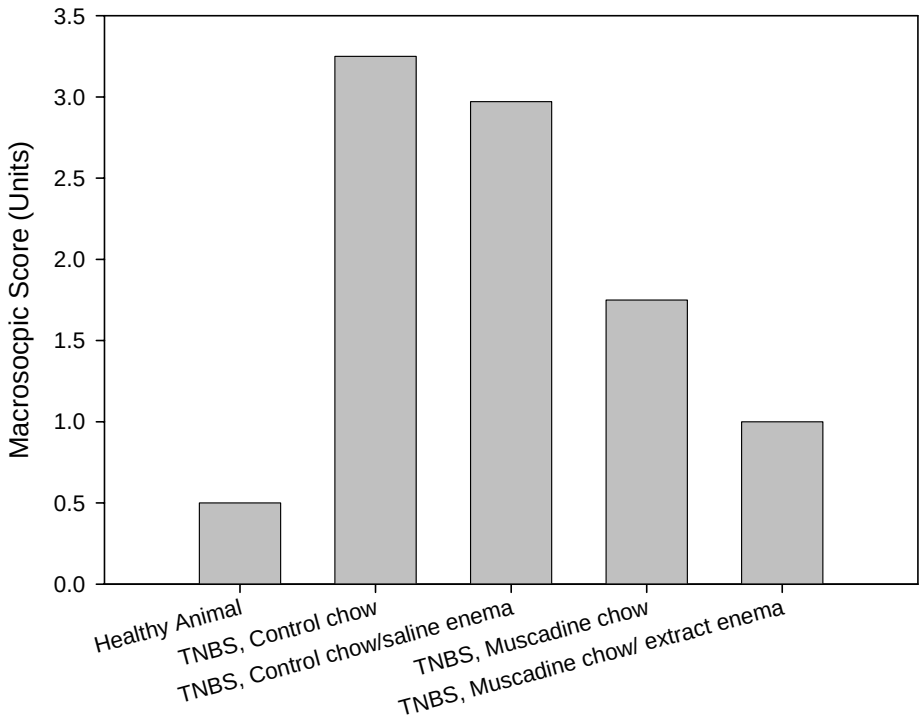

FIGURE 6.3 Macroscopic scores of rat colon slit longitudinally as an index of inflammation. Scores followed the guidelines as described in Table 6.1, and were performed by a single investigator.

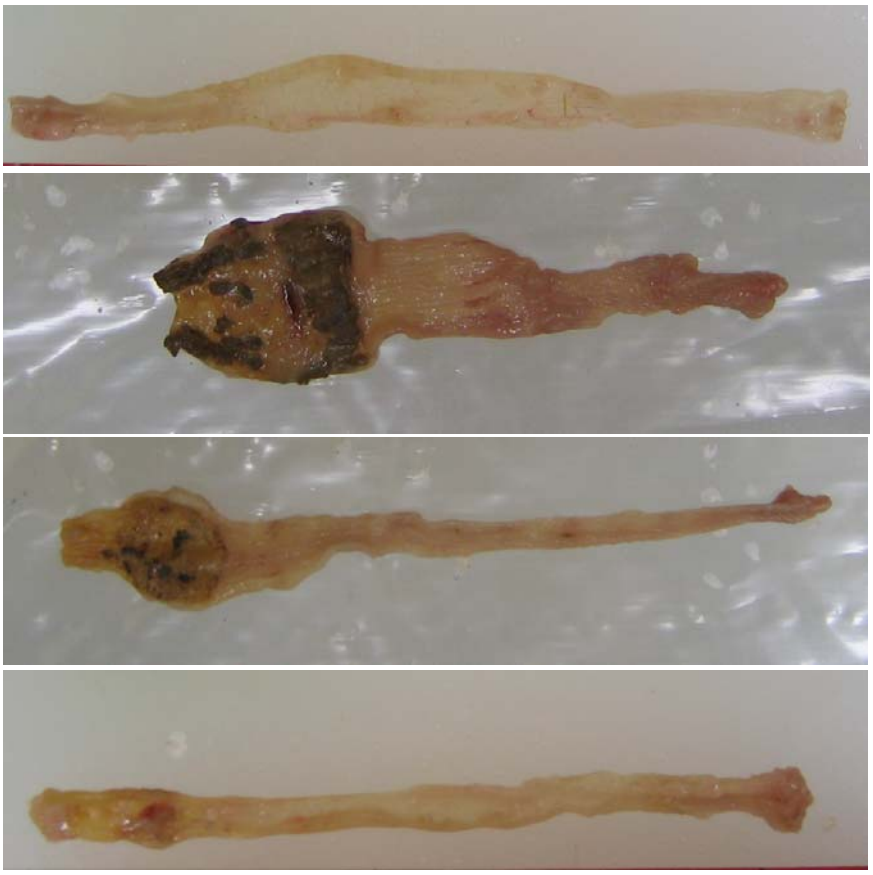

FIGURE 6.4 Gross morphological changes seen in the TNBS model of ulcerative colitis.

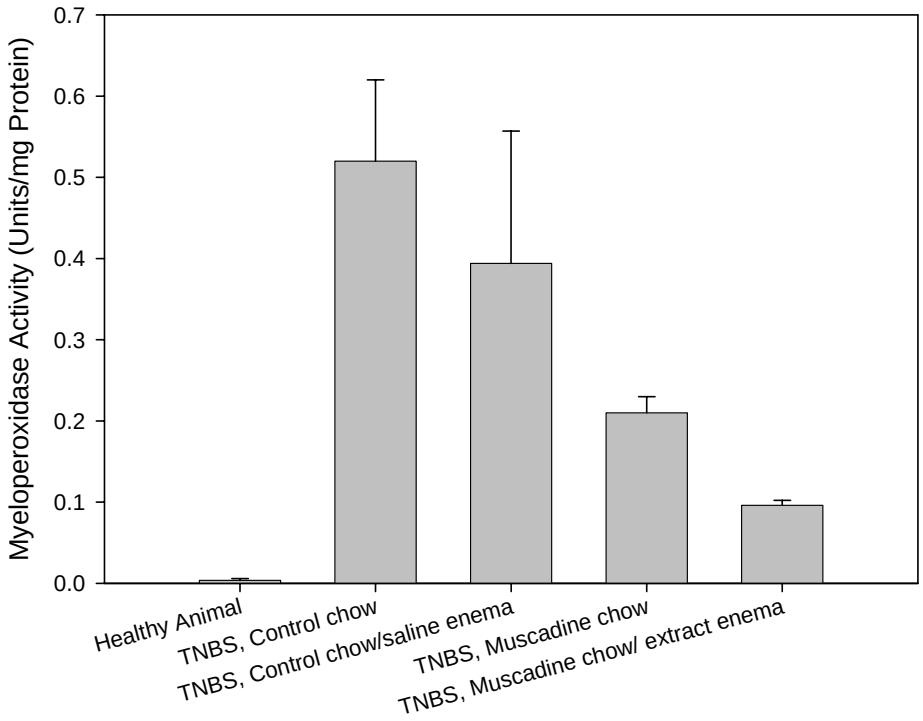

FIGURE 6.5 Myeloperoxidase activity as an indicator of neutrophil invasion in the colonic tissue.

CHAPTER SIX

CONCLUSIONS

The work in Chapter Two tested the hypothesis that muscadine skin and seed fractions would inhibit hyaluronidase activity *in vitro.* Both Ison (purple) and Early Fry (bronze) grape skin and seed extracts inhibited hyaluronidase. Inhibitory activity correlated with total phenolic content and antioxidant activity. Hyaluronidase inhibition could be an important strategy in treatment and prevention of osteoarthritis. Not only does hyaluronidase degrade high molecular weight hyaluronan to cause structural breakdown in the joint tissue, it also produces low molecular weigh hyaluronan fragments that stimulate inflammation. The results of this study support the need for further studies *in vivo* to determine the effect of muscadine skin and seed fractions on joint and cartilage health.

In Chapter Three, the hypothesis was tested that sorghum bran of several varieties will inhibit hyaluronidase activity *in vitro*. All sorghum brans tested inhibited hyaluronidase *in vitro*, and inhibitory activity correlated with total phenolic content and antioxidant activity. Sumac sorghum bran possessed the greatest inhibitory activity, and inhibition was dose-dependent. Commonly consumed wheat bran and rice bran had weak inhibitory activities relative to sorghum bran, which had much higher content of phenolic compounds. The results of this study support the need for further studies *in vivo* to determine the effect of sorghum bran on joint and cartilage health.

In Chapters Four and Five, inhibition of skin inflammation by muscadine grape fractions and *Polygonum cuspidatum* extract was tested. The model of inflammation used in these chapters resulted from application of a defined irritant called phorbol myristate to the mouse ear.

Extracts of muscadine skin, seed and combination treatments significantly reduced inflammatory markers compared to the control. An additive effect was observed with the combination treatment that was statistically similar to the anti-inflammatory of indomethacin, the control drug treatment. *Polygonum cuspidatum* extract inhibited acute and chronic inflammation in a dose-dependent manner, and was a more potent inhibitor of inflammation than a comparable dose of purified *trans*-resveratrol. These results represent some of the first anti-inflammatory tests of muscadine extracts and Polygonum cuspidatum in a live animal as compared to cell culture or other tests *in vitro*.

Finally, results in Chapter Six supported the hypothesis that muscadine skin extract in the diet and/or administered intra-rectally would decrease severity of ulcerative colitis in rats. Muscadine enriched diets decreased several markers of inflammation associated with this model. Inflammation in the colon was eliminated in rats receiving the muscadine enema treatment.

The botanicals of this dissertation come from muscadine grapes, Sumac sorghum bran, and the root of Japanese knotweed. Tests showed that each kind of extract is highly anti-oxidant and anti-inflammatory in several model systems. Because of the broad range of biological activities of the phytochemicals, these botanicals would be excellent sources for use in functional foods, functional beverages, food supplements, nutraceuticals, and cosmeceutical product ingredients. Further studies are called for in order to investigate whether bioactive compounds from muscadine grape pomace and sorghum bran have benefits in other inflammatory conditions such as development of the metabolic syndrome or specific cancers.