NATURAL PRODUCTS AS POSSIBLE TREATMENTS OF TYPE II DIABETES MELLITUS AND ITS COMPLICATIONS

by

JOHNETTA L. FARRAR

(Under the Guidance of Phillip Greenspan)

Abstract

Diabetes mellitus is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin that is produced. Such a deficiency results in increased concentrations of glucose in the blood, which in turn damages several physiological systems, in particular blood vessels and nerves. Recently compiled data from the World Health Organization (WHO) show that approximately 150 million people have diabetes mellitus worldwide, and that this number may well double by the year 2025. Protein glycation, or the reaction of biological amines with reducing sugars to form a complex family of rearranged and dehydrated covalent adducts, is implicated in the formation of diabetic complications. Although some plant extracts have been shown to inhibit glycation, the effect of extracts of food products on protein glycation has not received significant attention. In this dissertation, the effect of ethanolic extracts of muscadine grapes, sorghum bran, and Japanese knotweed on protein glycation are investigated. A very high antioxidant capacity is common to these three products. These studies show that each of these strongly inhibit protein glycation. Possible mechanisms for this inhibition are scavenging of free radicals that are produced

in abundance in a hyperglycemic state during protein glycation or the complexing of metal ions that mediate the glycation reaction. This research, therefore, supports the rationale to incorporate muscadine grapes, sorghum bran, and Japanese knotweed into "functional foods" as a preventive of the complications of diabetes.

INDEX WORDS: muscadine grape, sorghum bran, Japanese knotweed, protein glycation, diabetes mellitus, Advanced Glycation Endproducts (AGEs)

NATURAL PRODUCTS AS POSSIBLE TREATMENTS OF TYPE II DIABETES MELLITUS AND ITS COMPLICATIONS

by

JOHNETTA L. FARRAR

B.S. Chemistry, Fort Valley State University, 2002

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

2006

Natural Products as Possible Treatments for Type II Diabetes Mellitus

by

Johnetta L. Farrar

Major Professor: Dr. Phillip Greenspan

Committee: Dr. Anthony Capomacchia
Dr. James Hargrove
Dr. Diane Hartle
Dr. Cecil Jennings

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2006

DEDICATION

To Mama – I wish you were here to see your dream materialize…

.

ACKNOWLEDGEMENTS

First and foremost, giving honor, glory and praise to my Lord and Savior Jesus Christ – without whom none of this would even be imaginable. I would like to thank my major professor, Dr. Phillip Greenspan, for endless weekends spent in the lab nurturing and guiding me throughout life as a graduate student. Without you, I would have long given up on this goal of being a "scientist." I wish you and your family nothing but success and happiness in the future.

To my committee, Drs. Capomacchia, Hargrove, Hartle, Jennings and Taylor - thanks so much for your time and guidance.

Michael – without you I am pretty sure I would still be enrolled as a graduate student. From your persistence to your contagious smile, I could not have done it without you.

Linda: "Quick, is Dr. Greenspan looking?? No – well give me a hug!" I don't know what I'll do when I'm unable to get those anymore. Thanks for your time and dedication in taking care of my "babies." You really improved their quality of life and made me feel a little less horrible. Thank you.

To my other family – Mr. Vernus, Mrs. Millie and Danielle: the way you accepted me as your own truly irradiates your Christian spirit. I know you were all meant to be a part of my life and can only hope that I have enhanced yours at least ***half*** as much as you have mine.

To the many friends that I have met along this journey – thank you for all of your various contributions of support.

Last but certainly not least, my family. Jay and Dee – I genuinely appreciate you allowing me to run to your family and home as a refuge. I may have completely lost it if I was not able to get away and visit you guys. Dee, it takes a Godly woman to be able to create an oasis, as you have, in the midst of chaos (and deal with my brother ;~). Daddy, you are truly one to be admired. You have shown the value of hard work and led by example versus "do as I say, not as I do." I could not have even dreamed this had it not been for you and your confidence in your baby girl…

Table of Contents

Acknowledgements……..v

Chapter

I Introduction and Literature Review …….....1

Diabetes Mellitus……1

Pathogenesis of Type II Diabetes Mellitus …….....2

Diabetic Complications …….....4

History of the Maillard Reaction ……4

Role of Oxidation in the Glycation Process ……6

Role of Methylglyoxal in Glycation ……...7

The Polyol Pathway ……8

Role of Oxidative Stress in the Pathogenesis of Diabetic Complications ……...10

Current Treatment of Type II Diabetes ……11

Current Non-Pharmacological Management of Type II Diabetes Mellitus ……13

Glycation Inhibitors ……..14

America's Premier Grape: The Muscadine ……...15

Sorghum Bran ……...16

Polygonum cuspidatum …….....17

Literature Cited ……19

II Inhibition of Protein Glycation by Skins and Seeds of the Muscadine Grape ……24

Abstract ……25

Introduction ……...26

Material and Methods ……...27

Results ……...30

Discussion ……32

Literature Cited ……36

Figures ……...39

III Novel Nutraceutical Property of Select Sorghum Brans: Inhibition of Protein Glycation

Glycation ……...44

Abstract ……45

Introduction ……...46

Material and Methods ……...47

Results ……...49

Discussion ……52

Literature Cited ……56

Tables ……....60

Figures ……...62

IV Resveratrol, a Major Constituent of *Polygonum cuspidatum*, is an Inhibitor of Protein Glycation ……..68

Abstract ……69

Introduction ……...70

Material and Methods ……...70

Results ……...72

Discussion ……73

Literature Cited ……76

Figures ……..78

V Conclusions……81

Chapter I – Introduction and Literature Review

Diabetes Mellitus.

Diabetes mellitus is a group of diseases characterized by high blood glucose levels resulting from defects in insulin secretion, insulin action, or both. Abnormalities in the metabolism of carbohydrate, protein, and fat are also present. Diabetics do not adequately produce or respond to insulin, a hormone produced by the β-cells of the pancreas; insulin is necessary for the use or storage of carbohydrates and fats. Without effective insulin action, hyperglycemia occurs and can lead to both the short-term and long-term complications of diabetes mellitus [1, 2].

There are two major types of this disease – Type I or insulin dependent diabetes mellitus (IDDM) and Type II or non-insulin dependent diabetes mellitus (NIDDM). Type I diabetes is an autoimmune disease that results in the destruction of pancreatic β-cells and insulin deficiency in the patient. Insulin is the storage and anabolic hormone of the body that is responsible for allowing target tissues to take up glucose [3]. Type I diabetes usually develops during childhood and only accounts for 5-10% of all diagnosed cases of diabetes [4]. The cause of Type I diabetes is not completely understood; however, environmental factors that could trigger the initiation of pancreatic β-cell destruction are thought to play a role. A genetic predisposition for the occurrence of this disease has been documented. Type II diabetes, conversely, is the most common form of the two types of diabetes. It accounts for about 90-95% of all diagnosed cases [4]. Type II diabetes is a progressive disease in which a person gradually forms a resistance to insulin [5]. In Type II diabetes, insulin is produced by the β-cells, but there is a lack of functional receptors to take up glucose for use by the cells.

Risk factors for Type II Diabetes include older age, obesity, a family history of diabetes, a prior history of gestational diabetes, impaired glucose homeostasis, physical inactivity, and race or ethnicity. Although approximately 80% of these with Type II Diabetes people are obese or have a history of obesity at the time of diagnosis, the disease can occur in individuals who are not obese, especially the elderly [6]. People with Type II diabetes can range from being predominantly insulin-resistant to predominantly deficient in insulin secretion with insulin resistance. Endogenous insulin levels may be normal, depressed, or elevated, but they are inadequate to maintain normal blood glucose levels.

The actual cause of NIDDM is unknown, but diet, lifestyle and genetic factors are thought to play a role. This disease may develop at any age and obesity is common in patients with diabetes of this type. Although the direct cause remains unknown, there are several symptoms that provide a clinical progression of the disease. These symptoms include frequent urination, excessive thirst, extreme hunger, unusual weight loss, increased fatigue, irritability, and vision problems [3].

Pathogenesis of Type II Diabetes Mellitus.

A large body of work done by many investigators over several decades has documented that Type II diabetes develops in obese persons when resistance to insulin action can no longer be compensated by insulin secretion. Hence, insulin resistance alone will not result in Type II diabetes without at least some degree of impaired ß-cell function. The relationship between insulin resistance and the amount of insulin needed to overcome this resistance is hyperbolic. This hyperbolic relationship is probably why people who are genetically predisposed to develop Type II diabetes maintain normal

blood glucose levels for many years. During the early prediabetic years, their insulin resistance is only mildly to moderately elevated and requires only modest increases in insulin secretion for compensation. Only when these patients enter the steep part of the insulin resistance – insulin secretion curve are they no longer able to recompense. This situation usually happens when they become older, physically inactive, or overweight [8].

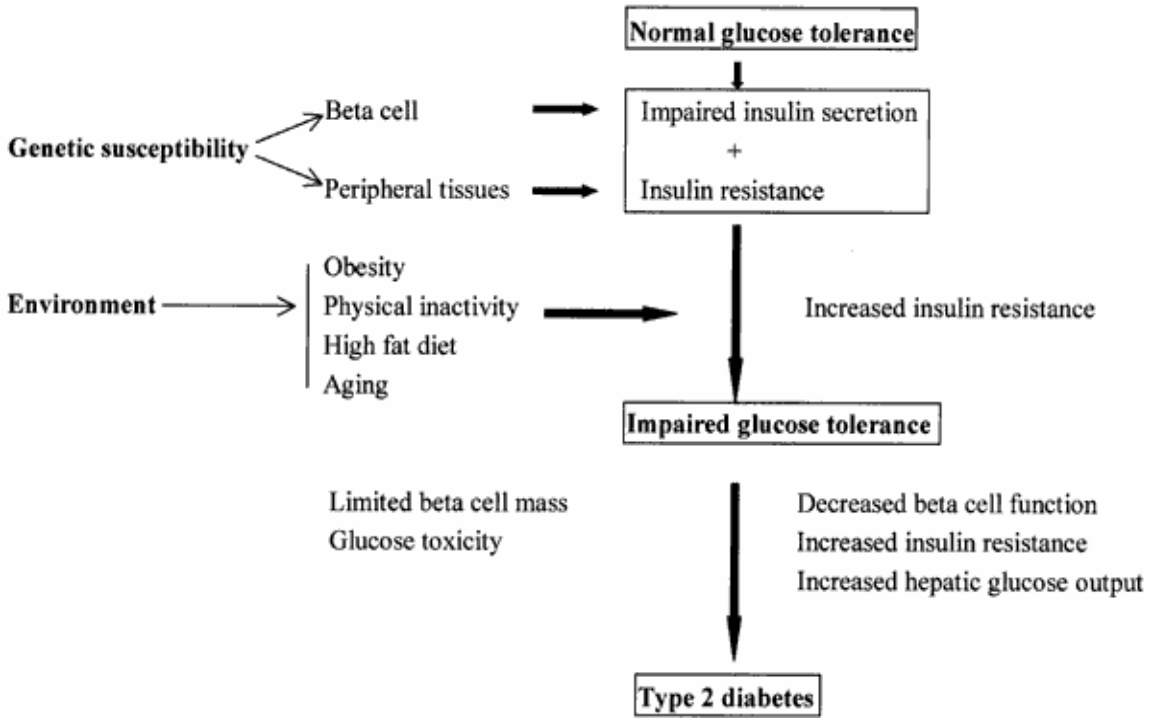

Figure 1. Cooper et. al., 2001

Diabetic Complications

Diabetes mellitus is a widespread disease and is one of the leading causes of blindness, kidney failure, heart attack, stroke, and amputation [9, 10]. Organs such as the lens, retina, and nerves are target organs for these diabetic complications [11]. These complications arise from chronic hyperglycemia that causes oxidative stress in tissues and results in damage to blood vessels and peripheral nerves [12].

Diabetes mellitus and its complications – cardiovascular disease, nephropathy, neuropathy, and retinopathy – are major public health problems that will assume epidemic proportions, as the population grows older. These disease processes can be slowed by early diagnosis and treatment [5]. Diabetes imposes a substantial cost burden to society and, in particular, to those individuals with diabetes and their families. Eliminating or reducing the health problems caused by diabetes through factors such as better access to preventive care, expanding diagnosis, increased intensive disease management, and the advent of new medical technologies could significantly improve the quality of life for people with diabetes and their families, while potentially reducing national expenditures for health care services [8]. In 2002 in the United States alone, with 18 million diagnosed with Type II diabetes, $132 billion was spent annually as health care costs or loss of revenue due to disability and low productivity [12].

History of the Maillard Reaction.

The Maillard reaction is a chemical reaction between an amino acid and a reducing sugar, that has been implicated in the progression of diabetic complications [14]. Described at the beginning of the 20th century by Louis Camille Maillard, a French

chemist, this reaction was first demonstrated when meat (protein) and sugar-containing (glucose) preparations where heated together. This reaction, "the preparation of melanoidins", occurs in ordinary cooking and was forgotten for a long time until it was rediscovered by food chemists during the 1950s because of its importance in food deterioration. In 1968, glycated hemoglobin A1C, a protein of the Maillard reaction became recognized as a reliable marker of long-term elevated glycemia in diabetic patients. This important discovery was followed by an avalanche of ever increasing output of research reports on the various aspects of the Maillard reaction [14]. Among other important results, the Maillard reaction was proposed as the molecular explanation of the Verzar phenomenon, the age-dependent increase of the cross-linking of collagen [15].

In this reaction, free amino groups of protein react slowly with the carbonyl groups of reducing sugars to produce Schiff base intermediates (Figure 3), which undergo spontaneous Amadori rearrangement to stable ketoamine derivatives [16]. The Amadori products then degrade into α-dicarbonyl compounds and deoxyglucosones. Schiff bases may also be fragmented to glyoxal species. These compounds are more reactive than the parent sugars with respect to their ability to react with amino groups of proteins**.**

Thus, the α-dicarbonyl compounds or α-ketoaldehydes are mainly responsible for forming inter- and intramolecular cross-links of proteins, known as advanced glycation end products (AGEs). The AGEs, which are irreversibly formed, accumulate with aging, atherosclerosis, and diabetes mellitus, especially associated with proteins, which are not subject to rapid turnover.

The α-dicarbonyl compounds are produced in a variety of ways. Fenton reaction-mediated oxidation of sugars, lipids, and proteins produces various α-dicarbonyl compounds. Accordingly, the transition metal ion-catalyzed oxidation of glucose is suggested to be a more important factor in glycation than the formation of the Amadori product of glucose itself. Another possible mechanism for the formation of these compounds is via α-ketoaldehydes, such as methylglyoxal, which are intermediates in biochemical pathways in both microorganisms and mammals**.**

Role of Oxidation in the Glycation Process.

Wolff and Dean (1987) demonstrated the importance of glucose autoxidation in glycation. They demonstrated that glycation, as determined both by fluorescence at the 350 and 415 nm wavelength pair and measuring the covalent attachment to glucose to

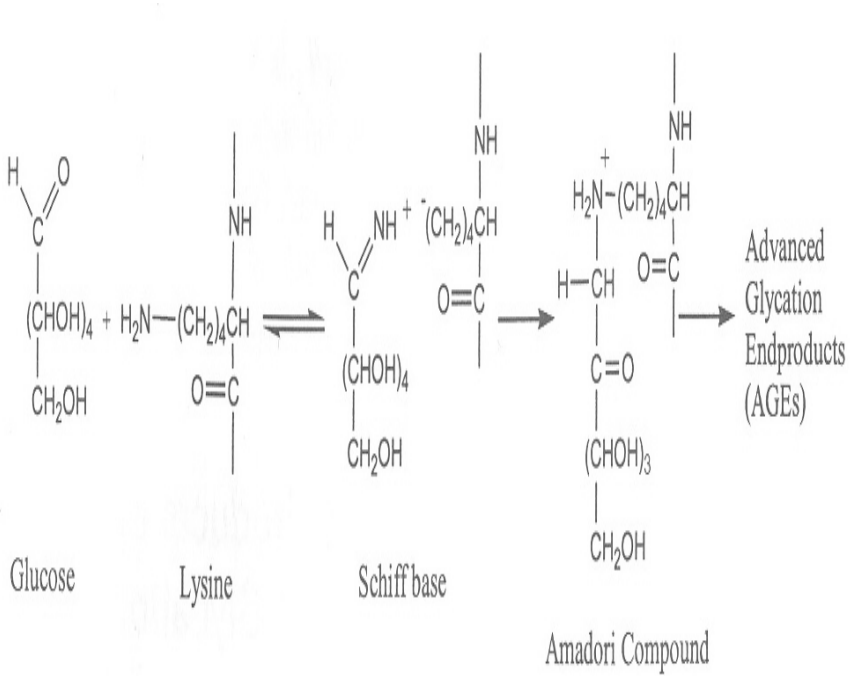

Figure 3. Schematic of the Maillard reaction.

albumin is a function of autooxidation. In the presence of a metal-chelating agent, diethylenetriaminepenta-acetic acid, keto-aldehyde production and glycation were inhibited by approximately 50%. There is an abundance of literature examining antioxidants, plant extracts and specific phenolic compounds inhibiting glycation. Vitamin E, a model antioxidant, inhibits both LDL and albumin glycation [17]. Another natural compound, turmeric, isolated from curcumin, is a potent inhibitor of AGE formation [18]. Flavonoids possess both antioxidant and AGE inhibitor properties; their potency functioning according to a structure-activity relationship [19].

The effects of antioxidants on AGE- related diabetic complications also have been studied in both animals and humans. Flavonoids decrease skin collagen-linked glycation-induced fluorescence in diabetic rats [20]). Vitamin E (800 mg/day) was reported to reduce AGE accumulation in arterial walls of diabetic patients [1]. A multi-center double blind study in 300 type II diabetic patients showed improvement of some clinical features of neuropathy after 3-week treatment of the antioxidant, lipoic acid [18].

Role of Methylglyoxal in Glycation.

Methylglyoxal (MG) also has received considerable attention as a common mediator to form AGEs. Methylglyoxal has been identified as a major intracellular reactive dicarbonyl intermediate originating from glycolysis; spontaneous dephosphorylation results in methylglyoxal formation. Methylglyoxal reacts with free lysine groups to form specific AGEs. It also reacts with free arginine groups to form hydroimidazolones. MG also has been found to be the most active dicarbonyl AGE-intermediate in the cross-linking of proteins, as well as generating reactive oxygen

species in the course of glycation reactions [21]. In patients with both Type I and Type II diabetes, the concentration of MG was increased at least two-fold [22].

The Polyol Pathway.

The polyol pathway was first discovered in seminal vesicles by Hers (1956) who demonstrated the conversion of blood glucose to fructose as an energy source of sperm cells [23]. In 1959, Van Heyningen proved that sorbitol could be found in the diabetic rat lens [24]. Van Heyningen's research lead to the recognition of the importance of the polyol pathway in the development of diabetic complications [23]. The polyol pathway (Figure 4) is an alternate pathway for glucose metabolism and converts glucose to fructose using two enzymes, aldose reductase and sorbitol dehydrogenase. Aldose reductase is the rate-limiting enzyme in this pathway and it catalyzes the conversion of glucose to sorbitol using NADPH as its cofactor [23]. Sorbitol is then oxidized to fructose by sorbitol dehydrogenase (SDH), which utilizes NAD^+ as its cofactor [25].

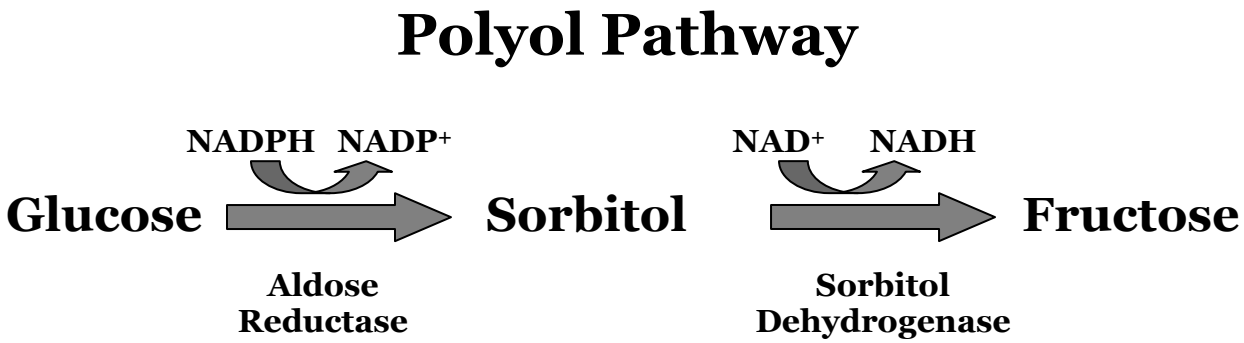

Figure 4. The Polyol Pathway

SDH activity was elevated in diabetic rats, which lead to increased fructose availability [26], with fructose being a ten-fold better substrate than glucose for glycosylation [27]. Amano, et. al. [28] suggests that SDH-mediated conversion of sorbitol to fructose and the resultant ROS generation may play an active role in the pathogenesis of diabetic retinopathy. Blockage of sorbitol formation by aldose reductase inhibitors is a therapeutic strategy for the treatment of early phases of diabetic retinopathies [28].

Role of Oxidative Stress in the Pathogenesis of Diabetic Complications.

Elevated levels of glucose in the blood or other body fluids cause oxidative damage, followed by an imbalance between the productions of reactive oxygen species and the antioxidant defense mechanisms present in biological systems [29]. Reactive oxygen species encompass a variety of diverse chemical species including superoxide anions, hydrogen peroxide, alkoxyl, peroxyl, hydroxyl radicals, and hypochlorous acid [28]. This presence of these species results in damage to various cell components and triggers activation of specific signalling pathways. AGE deposited in the arterial wall could generate free radicals capable of oxidizing vascular wall lipids and accelerate atherogenesis in hyperglycemic diabetic patients. These findings were confirmed recently in a study investigating the reaction of methylglyoxal (MGO) and amino acids such as alanine. In addition to the yellow fluorescent products formed, an indication of glycation, three types of free radicals species were generated. Their structures were identified using spectroscopy to be cross-linked radical cations, the methylglyoxal radical anions and superoxide radical anions. These reactions may contribute to the increased peroxidation of lipids in the arterial vessel wall and the pro-atherogenic state in diabetes.

In addition, structural and functional alterations in plasma and extracellular matrix (ECM) proteins occur in the diabetic state. Cross-linking of proteins and interaction of AGE with their receptors and/or binding proteins also can lead to enhanced formation of reactive oxygen species, which leads to activation of nuclear factor-κB (NF-κB) and release of pro-inflammatory cytokines, growth factors, and adhesion molecules. Collagen, a long-lived structural protein found in the ECM section of the kidney, experiences changes in elasticity, ionic charge and thickness when there is an AGE buildup [30].

Current Treatment of Type II Diabetes.

Exercise and dietary management for blood glucose control and weight loss often are first treatments for Type II Diabetes. These measures frequently are insufficient to bring blood glucose levels back to normal range. Administration of an oral medication to help decrease insulin requirements or cause an increase in the production of insulin is the next treatment option. Obese patients started on biguanides (for example, metformin), while nonobese patients are started on sulfonylureas (for example, glyburide or glipizide). If blood glucose levels can't be controlled with one medication, combination therapy such as a sulfonylurea and biguanide can be instituted. Other medications such as thiazolidinediones (for example, Actos or Avandia) or an alpha-glucosidase inhibitor (for example, Precose or Glyset) are also available.

Many individuals with type II diabetes will eventually require more than one medication to control their blood sugar levels. There are products on the market that combine both medications into a single tablet, which enables the patient to ingest one

tablet while getting the effects from both medications. These single tablet combinations are available for a sulfonylurea/biguanide and a biguanide/thiazolidineodione. If diabetes control cannot be maintained on oral medication, then insulin therapy may be started either alone or in combination with oral therapy. Medication works best when used in combination with appropriate meal planning and exercise. An optimum treatment for Type II diabetes does not exist.

Recently, the U.S. Food and Drug Administration (FDA) has approved two new medications (Symlin® and Byetta®) for the treatment of diabetes. Symlin® (pramlintide acetate) injection has been approved by the FDA and is structurally similar to the human hormone amylin, Symlin® is used in addition to insulin to help control blood sugar levels. By slowing down the movement of food through the stomach, sugar absorption into the blood is delayed, thus allowing for better blood glucose control.

Byetta® (exenatide) injection is a medication used to control blood sugar levels in Type II diabetics. Recently approved by the FDA in May 2005, Byetta is the first in a new class of drugs called incretin mimetics. When used together with insulin, Byetta® enhances glucose dependent insulin secretion from the beta cells in the pancreas, thus mimicking the body's natural response to glucose. As a result, more insulin is available in the body to help control blood sugar levels, especially after meals. Interestingly, current therapies of diabetes are directed to normalizing blood glucose concentrations. These remedies are not designed to block the complications of diabetes thought to arise from glycation and cross-linking of proteins.

Current Non-Pharmacological Management of Type II Diabetes Mellitus.

Diet has been recognized as a cornerstone in the management of diabetes mellitus. Currently, diet rich in fiber and low in fat, particularly saturated fatty acids, is recommended for the treatment of Type II diabetes to achieve better glycemic control and for lowering plasma LDL cholesterol. Further, there is growing interest in herbal remedies because of the side effects associated with the therapeutic agents (oral hypoglycemic agents and insulin) for the treatment of diabetes mellitus. Many traditional folk medicinal herb extracts have been used for the treatment of diabetes mellitus. However, most of them have exerted little or no effect on glycemic control in experimental studies, although some herbs possess hypoglycemic properties.

Cinnamon is one of the traditional folk herbs used in Korea, China, and Russia for diabetes mellitus. Cinnamon is the name given to the bark of the *Cinnamomi cassiae* (Lauraceae). Cinnamic aldehyde, cinnamic acid, tannin and methylhydroxychalcone polymer (MHCP) are its main components. Cinnamon extract decreases blood glucose in Wistar rats and can increase the insulin sensitivity and glucose uptake in adipocytes [31]. Turmeric, isolated from curcumin, was administered to diabetic animals and both blood glucose levels and blood cholesterol levels were significantly decreased [32]. An apparent blocking of enzymes, (i.e. sucrase and maltase) that convert dietary carbohydrates into sugar may be involved in lowering of blood sugar, whereas the decrease in cholesterol may be related to accelerated cholesterol turnover [3]. Recently, Banini et. al. (2006) reported a lowering of blood glucose, insulin, and glycated hemoglobin concentrations after diabetic patients drank muscadine wine for a four-week period [13].

Glycation Inhibitors.

In addition to the aforementioned natural products, other glycation inhibitors have been identified. Compounds such as aminoguanidine and taurine have shown to inhibit protein glycation both *in vivo* and *in vitro* [33]. Aminoguanidine (AG) is a prototype therapeutic agent for the prevention of formation of advanced glycation endproducts. It reacts rapidly with α, β-dicarbonyl compounds such as methylglyoxal, glyoxal, and 3-deoxyglucosone to prevent the formation of advanced glycation endproducts (AGEs). Most importantly, AG has been shown to inhibit the production AGEs and decrease the incidence of diabetic complications (such as retinopathy) in experimental animals. Aminoguanidine has not been employed in the treatment of diabetes; the safety of the drug has been questioned since it also inhibits catalase and increases the production of hydrogen peroxide in experimental systems [34].

Until now, research has shown that either specific compounds isolated from natural substances or plant extracts have been shown to inhibit protein glycation [35]. There has been a scarcity of data on the effect of ordinary foods or extracts of these foods on protein glycation. A water soluble and low molecular weight fraction of tomato paste has been shown to inhibit protein glycation [42]. Methanolic extracts of Finger millet and Kodo millet has also been shown to significantly lower the rate of collagen glycation [43]. In this dissertation, the effect of ethanolic extracts of muscadine grapes and sorghum bran on protein glycation is investigated. Both the muscadine grape and sorghum can be grown in hot, arid conditions and both contain extremely high content of antioxidants needed to survive the environment. *Polygonum cuspidatum*, (i.e. Japanese knotweed) is

also investigated in this dissertation for its ability to inhibit glycation; this specialty crop, currently grown in China contains extremely high amounts of resveratrol.

America's Premier Grape: The Muscadine

The muscadine grape (*Vitis rotundifolia*, bullace, scuppernong, southern fox grape) is native to the southeastern United States; it is found wild as far north as Delaware and as far west as Texas, but commercial production is limited to the Southeast [(38)]. Muscadines are very well adapted to the extreme heat of the southeast US and are have been cultivated since the 16th century. Muscadines are of importance to the economy of the southeastern US because the plant thrives in both the climate and soil conditions as well as maintaining a resistance to Pierce disease [36]. The vines may grow as tall as 100 feet in the wild, producing round fruits that range from one to one and a half inches in diameter. The fruit has a characteristic thick skin and may contain several seeds [37]. Muscadines range from bronze to dark purple to black in color when ripe. If the fruit is eaten raw, people often eat the fruit , but discard the skins and seeds; however, the highest medicinal content is found in the seeds and skins. Muscadines are also used in making juice, wine, and jelly.

Although sharing the genus *Vitis* with the other grapevine species, muscadines belong to a separate subgenus, *Muscadinia* (the other belonging to *Euvitis*). Because muscadines have 40 chromosomes, rather than 38, the grape is generally not cross-compatibile with other "*Vitis*" species, and most hybrids between the subgenera are sterile [38].

Muscadines contain vitamin C, one of the most potent antioxidant compounds, as well as a plethora of other compounds. These include catechin, epicatechin and gallic acid in the seeds. In the skins, ellagic acid, myricetin, quercetin and kaemferol are the predominant phenolic compounds. Ellagic acid is a phenolic compound that is unique to the muscadine. Anthocyanins are the major class of flavonoids found in the skin. The phenolics found in the seed, however, have a higher antioxidant capacity than other parts of the fruit. Muscadines rank extremely high in antioxidant capacity and compare favorably with raspberries, blackberries, and blueberries. The pulp has a high concentration of vitamin C [39]. The phenolics in the seeds have a higher antioxidant capacity than other parts of the fruit. [37].

Because the skins and seeds are not used in the juicing process, many vineyards have these leftover products that could be marketed. Many commercial vineyards have taken interest in developing both muscadine food supplements and nutraceutical products. Dried, powdered muscadine skin, seed, and pomace can be encapsulated and taken as a food supplement. Since most of the antioxidants are found in the skins and seeds, these capsules have a high nutraceutical value. These products have a long shelf life and obviously have a high concentration of naturally occurring antioxidant species.

Sorghum Bran.

Sorghum [*Sorghum bicolor* (L.) Moench] is the fifth leading cereal crop in the world and is used primarily in Asia and Africa as a food crop. The United States, however, uses sorghum mainly as a feed grain. Although all sorghums contain phenolic compounds, its genotype and the environment in which it is grown influence the amount present in any particular cultivar. In addition, these same factors affect the color,

appearance, and nutritional quality of the grain and its products. Experimental manipulation has led to sorghum brans that are either high in tannins or very low in these compounds. While tannins protect the plant against diseases, they also reduce digestibility of the plant's nutrients. Awika et. al. (2004) found that high tannin and sumac sorghum brans have a higher oxygen radical absorbance capacity, an antioxidant index, than common fruits [40]. In sorghum, the most common anthocyanin types are the 3-deoxyanthocyanidins and their derivatives. These anthocyanins, which include luteolinidin and apigeninidin, are not commonly found in higher plants. They lack a hydroxyl group at the C-3 position and are more stable in acidic solutions than the anthocyanins found in most food plants. This suggests that sorghum is potentially valuable source of natural food pigments. Although the benefits of dietary fiber and nutraceuticals consumption have been documented (decreased cardiovascular and cancer risk), the role of phytochemicals in the health benefits of whole grain has not been widely appreciated.

Polygonum cuspidatum.

Polygonum cuspidatum, otherwise known as Japanese knotweed, Mexican bamboo, etc. is a large, herbaceous, perennial plant, native to eastern Asia in Japan, China and Korea. A member of the family*Polygonaceae*, Japanese knotweed has hollow stems with distinct raised nodes that give it the appearance of bamboo, though it is not related to that. Japanese knotweed was first introduced to Europe and North America in the late nineteenth century for ornamental use, for planting to prevent soil erosion, and sometimes as a forage crop for grazing animals. Now, however, it is typically considered an invasive weed where it has been introduced, and is a frequent colonizer of roadsides and waste

places. It can be found in 39 of the 50 United States (PUSDA). The rapid growth of new shoots and leaves in the spring shades out any vegetation below, suppressing the growth of other plants, including established native species. Both Japanese knotweed and giant knotweed are sources of resveratrol.

Resveratrol (3,4′, 5-trihydroxystilbene) is a phytochemical found in many plants, mainly in grapes and in numerous types of wine as a *trans*- or *cis*-isoform. Resveratrol is a secondary plant metabolite belonging to the class of stilbenes and is found in relatively high concentrations in mulberries, grapes, peanuts, pine, and grapevine. [41]. Resveratrol inhibits lipid peroxidation mainly by scavenging peroxyl radicals and has a significant inhibitory effect on the NF-kB signaling pathway after cellular exposure to metal-induced radicals. The health benefits of red wine have been attributed, in part, to the presence of resveratrol in the product.

Literature Cited

1. Bonnefont-Rousselot, D., *[Antioxidant and anti-AGE therapeutics: evaluation and perspectives].* J Soc Biol, 2001. **195**(4): p. 391-8.
2. Buchanan, T.A., et al., *Preservation of pancreatic beta-cell function and prevention of type 2 diabetes by pharmacological treatment of insulin resistance in high-risk Hispanic women.* Diabetes, 2002. **51**(9): p. 2796-803.
3. Seely, R., *Anatomy & Physiology*. 6th ed. 2003, Boston: McGraw-Hill.
4. *Basics About Diabetes*. (2005) [cited; Available from: www.cdc.gov/diabetes.
5. Garber, A., *Endocrinology & Metabolism Clinics of North America: Type 2 Diabetes*. 2001, Philadelphia: W.B. Saunders Company.
6. Inzucchi, S.E., *Oral Antihyperglycemic Therapy for Type 2 Diabetes.* Journal of the American Medical Association, 2002. **287**(3): p. 360-72.
7. Hansen, B., et. al., *Insulin Resistance and Insulin Resistance Syndrome*. 2002, New York: Taylor & Francis.
8. Buchanan, T.A., et. al. , *Preservation of Pancreatic Beta-cell Function and Prevention of Type 2 Diabetes by Pharmacological Treatment of Insulin Resistance in High Risk Hispanic Women.* Diabetes 2002. **51**: p. 2796-2803.
9. Leslie, R., et. al., *Diabetic Complications*. 2004, London: Martin, Dunitz, Taylor & Francis.
10. Suzen, S. and E. Buyukbingol, *Recent studies of aldose reductase enzyme inhibition for diabetic complications.* Curr Med Chem, 2003. **10**(15): p. 1329-52.

11. Ueda, H., K. Kawanishi, and M. Moriyasu, *Effects of ellagic acid and 2-(2,3,6-trihydroxy-4-carboxyphenyl)ellagic acid on sorbitol accumulation in vitro and in vivo.* Biol Pharm Bull, 2004. **27**(10): p. 1584-7.

12. Sheetz, M.J. and G.L. King, *Molecular understanding of hyperglycemia's adverse effects for diabetic complications.* Jama, 2002. **288**(20): p. 2579-88.

13. Banini, A.E., et al., *Muscadine grape products intake, diet and blood constituents of non-diabetic and type 2 diabetic subjects.* Nutrition, 2006.

14. Monnier, V.M., et al., *Maillard reaction-mediated molecular damage to extracellular matrix and other tissue proteins in diabetes, aging, and uremia.* Diabetes, 1992. **41 Suppl 2**: p. 36-41.

15. Robert, L. and J. Labat-Robert, *The metabolic syndrome and the Maillard reaction. An introduction.* Pathol Biol (Paris), 2006. **54**(7): p. 371-4.

16. Lee, C., et. al., *Oxidation-Reduction Properties of Methylglyoxal-modified Protein in Relation to Free Radical Generation.* The Journal of Biological Chemistry, 1998. **273**(39): p. 25272-78.

17. Haidara, M.A., et al., *Role of oxidative stress in development of cardiovascular complications in diabetes mellitus.* Curr Vasc Pharmacol, 2006. **4**(3): p. 215-27.

18. Rahbar, S. and J.L. Figarola, *Novel inhibitors of advanced glycation endproducts.* Arch Biochem Biophys, 2003. **419**(1): p. 63-79.

19. Verzijl, N., et al., *Age-related accumulation of the advanced glycation endproduct pentosidine in human articular cartilage aggrecan: the use of pentosidine levels as a quantitative measure of protein turnover.* Matrix Biol, 2001. **20**(7): p. 409-17.

20. Odetti, P.R., et al., *Prevention of diabetes-increased aging effect on rat collagen-linked fluorescence by aminoguanidine and rutin.* Diabetes, 1990. **39**(7): p. 796-801.

21. Rahbar, S., et al., *Evidence that pioglitazone, metformin and pentoxifylline are inhibitors of glycation.* Clin Chim Acta, 2000. **301**(1-2): p. 65-77.

22. Yim, H.S., et al., *Free radicals generated during the glycation reaction of amino acids by methylglyoxal. A model study of protein-cross-linked free radicals.* J Biol Chem, 1995. **270**(47): p. 28228-33.

23. Yabe-Nishimura, C., *Aldose reductase in glucose toxicity: a potential target for the prevention of diabetic complications.* Pharmacol Rev, 1998. **50**(1): p. 21-33.

24. Van Heyningen, R., *Metabolism of xylose by the lens, 2. Rat lens in vivo and in vitro.* Biochem J, 1959. **73**: p. 197-207.

25. Vander Jagt, D.L., L.M. Deck, and R.E. Royer, *Gossypol: prototype of inhibitors targeted to dinucleotide folds.* Curr Med Chem, 2000. **7**(4): p. 479-98.

26. Latha, M. and L. Pari, *Effect of an aqueous extract of Scoparia dulcis on blood glucose, plasma insulin and some polyol pathway enzymes in experimental rat diabetes.* Braz J Med Biol Res, 2004. **37**(4): p. 577-86.

27. Hamada, Y., et al., *Role of polyol pathway in nonenzymatic glycation.* Nephrol Dial Transplant, 1996. **11 Suppl 5**: p. 95-8.

28. Amano, S., et al., *Sorbitol dehydrogenase overexpression potentiates glucose toxicity to cultured retinal pericytes.* Biochem Biophys Res Commun, 2002. **299**(2): p. 183-8.

29. Wu, C.H. and G.C. Yen, *Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts.* J Agric Food Chem, 2005. **53**(8): p. 3167-73.

30. Rahbar, S., et. al., *Novel Inhibitors of Advanced Glycation Endproducts.* Archives and Biochemistry and Biophysics, 2003. **419**: p. 63-79.

31. Kim, S.H., S.H. Hyun, and S.Y. Choung, *Anti-diabetic effect of cinnamon extract on blood glucose in db/db mice.* J Ethnopharmacol, 2006. **104**(1-2): p. 119-23.

32. Qin, B., et al., *Cinnamon extract (traditional herb) potentiates in vivo insulin-regulated glucose utilization via enhancing insulin signaling in rats.* Diabetes Res Clin Pract, 2003. **62**(3): p. 139-48.

33. Nandhini, A.T., V. Thirunavukkarasu, and C.V. Anuradha, *Stimulation of glucose utilization and inhibition of protein glycation and AGE products by taurine.* Acta Physiol Scand, 2004. **181**(3): p. 297-303.

34. Chang, K.C., et al., *Aminoguanidine prevents arterial stiffening in a new rat model of type 2 diabetes.* Eur J Clin Invest, 2006. **36**(8): p. 528-35.

35. Kim, H.Y. and K. Kim, *Protein glycation inhibitory and antioxidative activities of some plant extracts in vitro.* J Agric Food Chem, 2003. **51**(6): p. 1586-91.

36. Talcott, S.T. and J.H. Lee, *Ellagic acid and flavonoid antioxidant content of muscadine wine and juice.* J Agric Food Chem, 2002. **50**(11): p. 3186-92.

37. Pastrana-Bonilla, E., et al., *Phenolic content and antioxidant capacity of muscadine grapes.* J Agric Food Chem, 2003. **51**(18): p. 5497-503.

38. Hartle, D.K., Greenspan, P, Hargrove, JL, *Muscadine Medicine*. 2005, St George's Island: Blue Heron Nutraceuticals, LLC.

39. Yi, W., J. Fischer, and C.C. Akoh, *Study of anticancer activities of muscadine grape phenolics in vitro.* J Agric Food Chem, 2005. **53**(22): p. 8804-12.

40. Awika, J.M., L.W. Rooney, and R.D. Waniska, *Properties of 3-deoxyanthocyanins from sorghum.* J Agric Food Chem, 2004. **52**(14): p. 4388-94.

41. Kim, M.J., et al., *Protective effects of epicatechin against the toxic effects of streptozotocin on rat pancreatic islets: in vivo and in vitro.* Pancreas, 2003. **26**(3): p. 292-9.

42. Kiho, T., et al., *Tomato paste fraction inhibiting the formation of advanced glycation end-products.* Biosci Biotechnol Biochem, 2004. **68**(1): p. 200-5.

43. Hegde, P., G. Chandrakasan, and T. Chandra, *Inhibition of collagen glycation and crosslinking in vitro by methanolic extracts of Finger millet (Eleusine coracana) and Kodo millet (Paspalum scrobiculatum).* J Nutr Biochem, 2002. **13**(9): p. 517.

Chapter II - Inhibition of Protein Glycation by Skins and Seeds of the Muscadine Grape

---

[1]**Farrar, JL, Hartle, DK, Hargrove, JL, Greenspan, P*, to be submitted to the**

Journal of Agricultural and Food Chemistry

***Abstract*.**

The formation of advanced glycation end (AGE) products, mediated in part by oxidative processes, has been linked to the pathogenesis of diabetic complications. In this study, the effects of seed and skin extracts of the muscadine grape on AGE product formation were examined. Seeds and skins were extracted (10% w/v) with 50% ethanol and incubated at 37°C with a solution containing 250 mM fructose and 10mg/ml albumin in 200 mM potassium phosphate buffer, pH 7.4. After 72 hours, fluorescence was measured at the wavelength pair of 370 and 440 nm as an index of the formation of AGE products. Both seed and skin extracts were found to be efficacious inhibitors of AGE product formation. A 1:300 dilution of the seed extract decreased fluorescence by approximately 65%, whereas muscadine grape skin extract produced a 40% lowering. This difference correlates with the greater antioxidant activity found in muscadine seeds in comparison to skins. Gallic acid, catechin and epicatechin, the three major polyphenols in the seeds, all decreased the AGE product related fluorescence by 40% at a concentration of 100 μM. Neither muscadine seed extract nor skin extract inhibited the methylglyoxal-mediated glycation of albumin. These results suggest that consumption of the muscadine grape may provide protective effects against the development of diabetic complications.

KEYWORDS: muscadine grape; advanced glycation end products (AGEs); diabetic complications; antioxidant activity, oxidative processes

Introduction.

Among the major risks of type II diabetes mellitus are chronic problems affecting multiple organ systems that eventually affect patients with poor glycemic control. Many problems in the kidneys, eyes and nervous system arise from damage to blood vessels [1]. These angiopathies may be divided into those arising from large and small blood vessels. Interestingly enough, small vessel disease is minimized by tight blood glucose control, but large vessel disease remains unaffected [2].

Glycation is a spontaneous non-enzymatic aminocarbonyl reaction between reducing sugars and long-lived proteins and lipids. These chemical damages are detectable in the form of advanced glycation endproducts (AGE) [3, 4]. Both reactive oxygen species and reactive α-dicarbonyl intermediates are generated during the glycation process. In this reaction, free amino groups of protein react slowly with the carbonyl groups of reducing sugars to produce Schiff base intermediates, which undergo spontaneous Amadori rearrangement to stable ketoamine derivatives [5]. The Amadori products then degrade into α-dicarbonyl compounds and deoxyglucosones. These compounds are more reactive than the parent sugars with respect to their ability to react with amino groups of proteins. Thus, the α-dicarbonyl compounds or α-ketoaldehydes are mainly responsible for forming inter- and intramolecular cross-links of proteins, known as advanced glycation end products (AGEs) [3]. These processes produce structural and functional alterations in plasma and extracellular matrix (ECM) proteins, specifically, from cross-linking of proteins and interaction of AGE with their receptors

and/or binding proteins. Such interactions lead to an enhanced formation of reactive oxygen species with successive activation of the nuclear factor-κB (NF-κB) cascade and release of pro-inflammatory cytokines, growth factors, and adhesion molecules [6]. These processes increase the rate at which atherosclerosis develops.

Inhibition of the glycation process both *in vitro* and *in vivo* has been observed with antioxidants such as vitamin E [7], plant extracts [8] and isolated phenolic compounds [9]. In this communication, the effect of ethanolic extracts of the muscadine grape on the glycation of albumin is examined. The muscadine grape (*Vitis rotundifolia*) is native to the southeastern United States [10]. Having been cultivated since the 16th century, the grapes are very well adapted to the heat of the southeast and are more prevalent than any other grape variety in this climate. The grape possesses one of the highest antioxidant capacities of any fruit [11]. The seeds of the muscadine have a greater antioxidant capacity than the skins. The results presented demonstrate that both muscadine skins and seeds can significantly inhibit the non-enzymatic glycation of albumin.

Materials and Methods.

Chemicals.

Bovine serum albumin (BSA) (Fraction V, Essentially Fatty Acid Free, D- (-) fructose, Chelex 100 (sodium form), Folin-Ciocalteu reagent, methylglyoxal solution, gallic acid, epicatechin and catechin, and TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) were

purchased from Sigma Chemical Company (St Louis, MO). Muscadine grape seed and skin were a gift of Muscadine Product Corporation, LLC (Wray, GA).

Preparation of Muscadine Extracts.

Muscadine grapes, from the Ison variety, were pressed for juice production and deseeded [11]. The grape skins were dried at 50°C for 12 hours in a forced-air pan dryer manufactured by Powell Manufacturing Company (Bennettsville, SC). The dried skins were ground in a Fitz Mill Comminutor Hammermill manufactured by the Fitzpatrick Company (Elmhurst, IL). To prepare the muscadine seed extract, dried muscadine seed was made into a powder using a commercial coffee grinder. The seeds and skins were extracted 1:10 (w/v) with 50% ethanol for 2 hours at room temperature with periodic vortexing. The extract was then centrifuged to remove the precipitate and filter sterilized to obtain the final extract.

Total Phenolic Content.

Phenolic content of samples was determined with the Folin-Ciocalteu method as described by Singleton [12]. Gallic acid was employed as the standard. Absorbance was measured at 765 nm in a Beckman DU 600 series spectrophotometer. Results are expressed as milligrams of gallic acid per gram of pulverized extract.

Ferric Reducing Antioxidant Protein (FRAP).

FRAP values were determined with a modified version of the Benzie and Strain method [13], with ferrous sulfate as the reference standard. Absorption was measured at 593 nm in a Beckman DU 600 series spectrophotometer. The FRAP assay is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex to the ferrous form. The

results are expressed as millimoles of ferrous sulfate formed per 100 grams of dry weight of grape skin or seed.

Albumin Glycation.

The fluorescence assay to determine the glycation of albumin was performed as described by McPherson, et. al. [4] Bovine serum albumin (BSA; 10 mg/mL) was incubated with D- (-) fructose (250 mM) in potassium phosphate buffer (200 mM; pH 7.4; 0.02% sodium azide) in a 5% carbon dioxide incubator at 37ºC for 72 hours. The buffer was treated with Chelex 100 prior to use. Various concentrations of the extracts were added to the 3-ml incubation mixture. To control for the ethanol present in the extract, control incubations were incubated in the presence of the appropriate concentration of ethanol. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm using a Perkin-Elmer LS 55 Luminesence Spectrometer. Slit widths were set at three nanometers. Values were corrected for the intrinsic fluorescence of muscadine seed and skin extracts.

Modification of Albumin by Methylglyoxal.

Bovine serum albumin (100 μM) was incubated with 1 mM methylglyoxal in 0.1 M sodium phosphate, pH 7.0 according to the method of Lee [5]. The buffer was treated with Chelex prior to use. After 96 hours, the fluorescence was measured using the wavelength pair of 350 and 409 nm. Values were corrected for the intrinsic fluorescence of muscadine seed and skins extracts.

Statistical Analysis.

Experiments were performed in triplicate. Values are expressed as mean ± SEM. Data within skin and seed groups were analyzed using one-way analysis of variance (ANOVA) and multiple comparisons were performed employing the Duncan's Multiple Range test. $P < 0.05$ was considered statistically significant.

Results.

Effect of Muscadine Grape Skin and Seed Extracts on Albumin Glycation.

Both muscadine seeds and skins were extracted with 50% ethanol (10% w/v) and the phenolic content of the extracts was ascertained. Muscadine seeds had a phenolic content of (38.7 mg/g), which was significantly greater than muscadine skins (20.0 mg/g), in agreement with the results of Pastrana-Bonilla and colleagues [11]**.** The effect of muscadine seeds and skin extracts on the glycation of albumin was examined at three different dilutions of the extract. Control incubations of fructose and albumin resulted in significant albumin glycation; the relative fluorescence intensity was found to be approximately 180 units. When fructose and albumin were incubated with 1:300 and 1:600 dilutions of muscadine seeds and skins, a significant concentration dependent decrease in fluorescence intensity was observed. Conversely, the 1:1200 dilution was not significantly different from the fluorescence intensity observed from the control incubation. A significant concentration-dependent decrease in fluorescence intensity was also observed with muscadine seed extracts (Figure 2). In contrast to that observed with muscadine grape skins, the muscadine grape seed extract at a dilution of 1:1200 produced a significant inhibition in albumin glycation.

Effect of Muscadine Grape Seed Phenolic Compounds on Albumin Glycation.

Three of the major monomeric phenolics present in the muscadine seed fractions are catechin, epicatechin and gallic acid [14]. The effect of these three compounds at two different concentrations on the glycation of albumin was examined. As seen in Figure 3, all three phenolic compounds inhibited the glycation of albumin. Catechin and epicatechin produced the greatest inhibition, an approximate 65% inhibition of glycation was observed at a concentration of 100 μM. In contrast gallic acid was the weakest inhibitor of glycation; 100 μM gallic acid produced an approximate 40% decrease in the extent of albumin glycation.

Effect of Water and Ethanolic Extracts of Muscadine Grape Seeds on Albumin Glycation.

In this experiment, muscadine grape seeds were extracted with 50% ethanol or distilled water. After the two-hour incubation with periodic vortexing, the phenolic content of the extracts was determined. The ethanolic extract had a phenolic content of 2.4 mg/mL while the content of the aqueous extract was 1.6 mg/ml. The antioxidant property of the extracts was determined employing the FRAP assay. In agreement with phenolic content, the ethanolic extract has a greater FRAP value (27.8 mmoles/100 g vs. 23.5 mmoles/100 g, respectively). The fluorescence intensity of the glycation of albumin was measured in the presence of two dilutions (1:300 and 1:600) of the muscadine grape seed extracts. As seen in Figure 4, both ethanolic and aqueous extracts at both dilutions inhibited the glycation of albumin as evidenced by a significant decrease in fluorescence. The ethanolic extract inhibited glycation to a greater extent than the corresponding

aqueous extract, in agreement with the greater phenolic concentration found in the ethanolic extracts.

Effect of Muscadine Seeds and Skins on Methylglyoxal Induced Albumin Fluorescence.

Methylglyoxal, an important intermediate in the autooxidation of reducing sugars, can readily glycate proteins [16]. As seen in Figure 5, methylglyoxal, when incubated with albumin, produced significance fluorescence at the wavelength pair of 350 and 409 nm, indicative of albumin glycation. When two dilutions (1:100 and 1:200) of muscadine seed and skin ethanolic extracts were incubated with methylglyoxal, a significant decrease in fluorescence intensity was not observed. These results indicate that muscadine seed and skin extracts do not inhibit all pathways of protein glycation.

Discussion.

Results presented in this communication suggest that phenolic compounds in both the seeds and skins of the muscadine grape inhibit the protein glycation, a reaction thought to be responsible for diabetic complications [18]. This reaction has been shown to be inhibited by a variety of antioxidant compounds [9] and therefore it was not unexpected that high in phenolic muscadine extracts inhibit the formation of glycated albumin. Hyperglycemia induced oxidative damage may be prevented by the antioxidant defense mechanisms present in biological systems [15, 19].

The total phenolics content of the muscadine extracts differ between the skins and seeds. The phenolics present in seeds, catechin, epicatechin and gallic acid, all inhibited albumin glycation by fructose (Figure 2). The results agree with those reported by Wu and Yen [19] for the inhibition of catechin and epicatechin of glucose mediated albumin glycation. However, the phenolics present in the skin are different than those in the seed. The skins have high levels of ellagic acid, myricetin, quercetin, and kaempferol [11]. Although not performed in this study, the previous report of Wu and Yen showed that quercetin and kaempferol were potent inhibitors of glucose mediated albumin glycation. These results shown in this study demonstrate that individual phenolics have distinct inhibitory properties on protein glycation; this agrees with previous published findings indicating that phenolic-induced inhibition correlated with antioxidant properties [15].

Methylglyoxal has been shown to be an important intermediate in the autooxidation of reducing sugars and can readily glycate proteins [16]. Protein glycation by methylglyoxal is a nonenzymatic modification whereby arginine and lysine side chains of proteins participate in forming a heterogeneous group of advanced glycation end-products [17]. It is interesting to note that while muscadine grape seed extracts strongly inhibited fructose mediated albumin glycation, the extract did not significantly inhibit methylglyoxal mediated albumin glycation. This result suggests that the antioxidants present in the muscadine seed extract do not possess significant inhibitory activity of ketoaldehyde-induced glycation. Wu and Yen demonstrated similar results with both catechins; this flavonoid significantly inhibited glucose mediated glycation, but have no effect on methylglyoxal mediated albumin glycation. This suggests that

muscadine seed extracts were inhibiting the glycation process prior to the non-enzymatic formation of methylglyoxal.

The glycation of albumin mediated by the autooxidation of reducing sugars is dependent on the presence of metal ions in the incubation solution [20]; chelating metal ions results in significant decrease in glycation. Flavonoids possess free radical-scavenging activity and this may be the mechanism by which the muscadine grape skins and seeds inhibit protein glycation. Certain flavonoids, such as quercetin, are capable of complexing metal ions directly [21].

Many studies in the literature demonstrating that certain plant extracts [22] and spice extracts (garlic, *Cassia tora* [23]) and individual phenolic compounds inhibit protein glycation [15]. This report is the first to demonstrate that an extract from a commonly edible grape or berry can significantly inhibit this important reaction that has been implicated in the pathogenesis of diabetic complications. Previous reports have showed that a water-soluble extract of tomato paste [25] and methanolic extracts of Finger and Kodo millet can inhibit protein glycation [26].

The possibility that these muscadine products may have a pharmacological effect is dependent on the absorption of phenolic compounds in the gastrointestinal tract. As recently reviewed, bioavailability of certain phenolic compounds is significant, with approximately 40% of the dose being excreted in the urine [24].

Since both muscadine seed and skin fractions come from a generally recognized as safe (GRAS) fruit, there is no toxicity associated with these products. These fractions may prove useful for nutraceutical and functional food/beverage products to treated type

II diabetic patients. In the future, these nutraceutical products may be employed to prevent or delay the onset of diabetic complications.

Literature Cited

1. Jawa, A. and V. Fonseca, *Cardiovascular effects of insulin sensitizers in diabetes.* Curr Opin Investig Drugs, 2006. **7**(9): p. 806-14.
2. Haidara, M.A., et al., *Role of oxidative stress in development of cardiovascular complications in diabetes mellitus.* Curr Vasc Pharmacol, 2006. **4**(3): p. 215-27.
3. Ahmed, N., *Advanced glycation endproducts--role in pathology of diabetic complications.* Diabetes Res Clin Pract, 2005. **67**(1): p. 3-21.
4. McPherson, J.D., B.H. Shilton, and D.J. Walton, *Role of fructose in glycation and cross-linking of proteins.* Biochemistry, 1988. **27**(6): p. 1901-7.
5. Lee, C., et al., *Oxidation-reduction properties of methylglyoxal-modified protein in relation to free radical generation.* J Biol Chem, 1998. **273**(39): p. 25272-8.
6. Iwashima, Y., et al., *Advanced glycation end product-induced peroxisome proliferator-activated receptor gamma gene expression in the cultured mesangial cells.* Biochem Biophys Res Commun, 1999. **264**(2): p. 441-8.
7. Packer, L., E.H. Witt, and H.J. Tritschler, *alpha-Lipoic acid as a biological antioxidant.* Free Radic Biol Med, 1995. **19**(2): p. 227-50.
8. Kim, H.Y. and K. Kim, *Protein glycation inhibitory and antioxidative activities of some plant extracts in vitro.* J Agric Food Chem, 2003. **51**(6): p. 1586-91.
9. Bousova, I., et al., *Evaluation of in vitro effects of natural substances of plant origin using a model of protein glycoxidation.* J Pharm Biomed Anal, 2005. **37**(5): p. 957-62.

10. Hartle DK, G.P., Hargrove JL, *Muscadine Medicine*. 1st ed. 2005, St George's Island: Blue Heron Nutraceuticals, LLC. 132.

11. Pastrana-Bonilla, E., et al., *Phenolic content and antioxidant capacity of muscadine grapes.* J Agric Food Chem, 2003. **51**(18): p. 5497-503.

12. Singleton, V.L.R. *Colorimetry of total phenolic with phosphomolybdic-phosphotungstic acid agents.* Am. J. Enol. Vitic., 1965. **16**: p. 144-158.

13. Benzie, I.F. and J.J. Strain, *The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay.* Anal Biochem, 1996. **239**(1): p. 70-6.

14. Yilmaz, Y. and R.T. Toledo, *Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin, and gallic acid.* J Agric Food Chem, 2004. **52**(2): p. 255-60.

15. Wu, J.H., et al., *Phenolic antioxidants from the heartwood of Acacia confusa.* J Agric Food Chem, 2005. **53**(15): p. 5917-21.

16. Nagaraj, R.H., et al., *Effect of pyridoxamine on chemical modification of proteins by carbonyls in diabetic rats: characterization of a major product from the reaction of pyridoxamine and methylglyoxal.* Arch Biochem Biophys, 2002. **402**(1): p. 110-9.

17. Gomes, R.A., et al., *Yeast protein glycation in vivo by methylglyoxal.* Febs J, 2006.

18. Wu, X. and V.M. Monnier, *Enzymatic deglycation of proteins.* Arch Biochem Biophys, 2003. **419**(1): p. 16-24.

19. Wu, C.H. and G.C. Yen, *Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts.* J Agric Food Chem, 2005. **53**(8): p. 3167-73.

20. Ou, P., et al., *Activation of aldose reductase in rat lens and metal-ion chelation by aldose reductase inhibitors and lipoic acid.* Free Radic Res, 1996. **25**(4): p. 337-46.

21. Leopoldini, M., et al., *Iron chelation by the powerful antioxidant flavonoid quercetin.* J Agric Food Chem, 2006. **54**(17): p. 6343-51.

22. Babu, P.V., K.E. Sabitha, and C.S. Shyamaladevi, *Therapeutic effect of green tea extract on oxidative stress in aorta and heart of streptozotocin diabetic rats.* Chem Biol Interact, 2006. **162**(2): p. 114-20.

23. Lee, G.Y., et al., *Naphthopyrone glucosides from the seeds of Cassia tora with inhibitory activity on advanced glycation end products (AGEs) formation.* Arch Pharm Res, 2006. **29**(7): p. 587-90.

24. Scalbert, A., et al., *Absorption and metabolism of polyphenols in the gut and impact on health.* Biomed Pharmacother, 2002. **56**(6): p. 276-82.

25. Kiho, T., et al., *Tomato paste fraction inhibiting the formation of advanced glycation end-products.* Biosci Biotechnol Biochem, 2004. **68**(1): p. 200-5.

26. Hegde, P., G. Chandrakasan, and T. Chandra, *Inhibition of collagen glycation and crosslinking in vitro by methanolic extracts of Finger millet (Eleusine coracana) and Kodo millet (Paspalum scrobiculatum).* J Nutr Biochem, 2002. **13**(9): p. 517

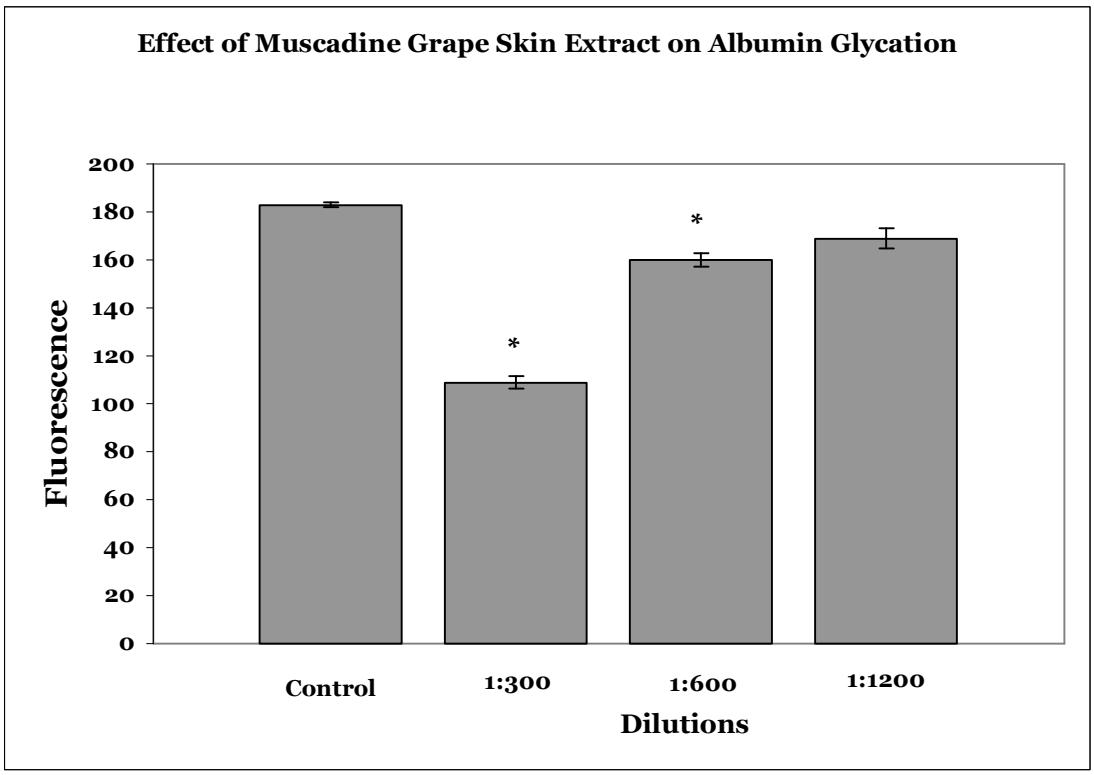

Figure 1. Effect of Muscadine Grape Skin Extract on Albumin Glycation. Briefly, bovine serum albumin (10 mg/mL) was incubated with fructose (250 mM) in potassium phosphate buffer (200 mM; pH 7.4; 0.02% sodium azide) and treated with varying concentrations of the extract, as shown, for 72 hours at 37°C. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Results represent the mean ± SEM of triplicate determinations. *$P < 0.05$ when compared to controls.

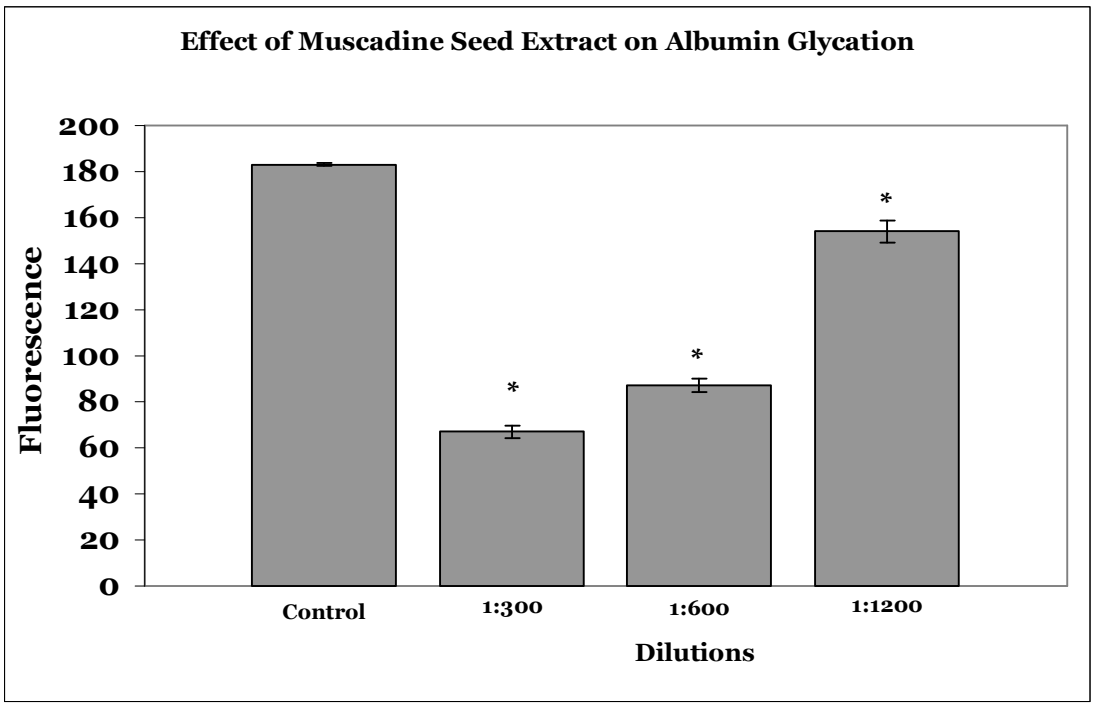

Figure 2. Effect of Muscadine Grape Seed Extract on Albumin Glycation. Bovine serum albumin (10 mg/mL) was incubated with fructose (250 mM) in potassium phosphate buffer (200 mM; pH 7.4; 0.02% sodium azide) and treated with varying concentrations of the extract, as shown, for 72 hours at 37°C. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Results represent the mean ± SEM of triplicate determinations. *$P < 0.05$ when compared to controls.

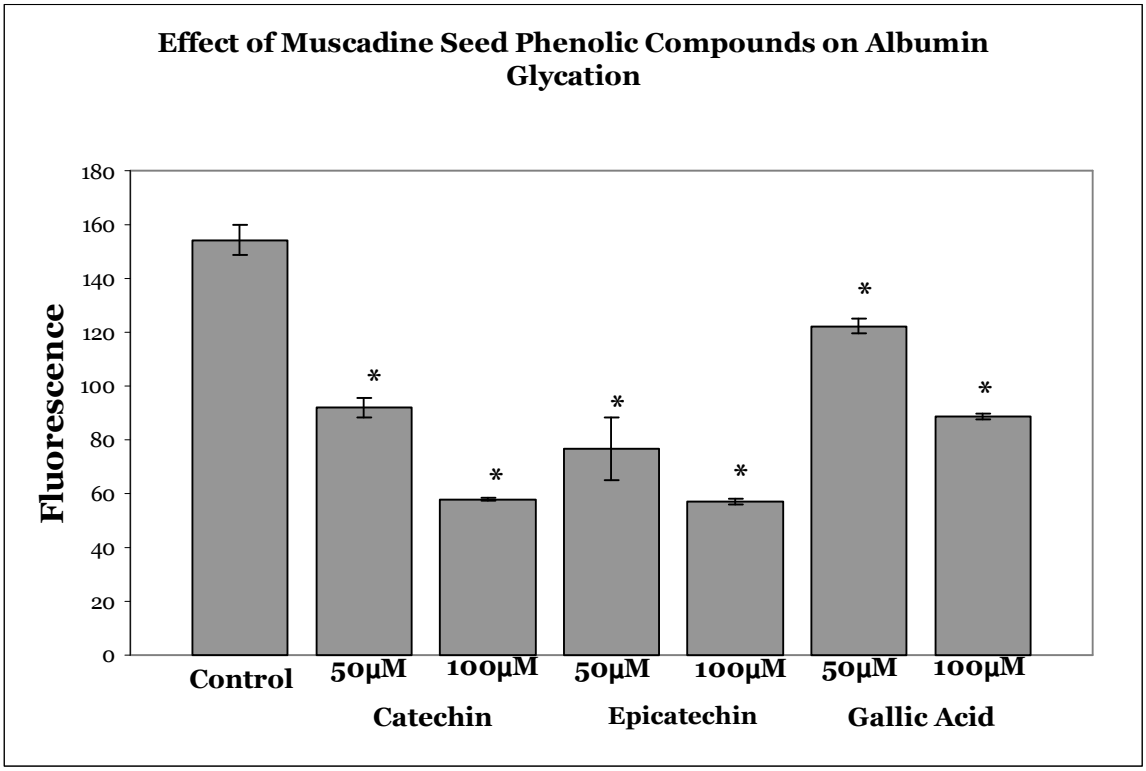

Figure 3. Effect of Muscadine Seed Phenolic Compounds on Albumin Glycation. Bovine serum albumin (10 mg/mL) was incubated with fructose (250 mM) in potassium phosphate buffer (200 mM; pH 7.4; 0.02% sodium azide) and treated with varying concentrations of phenolic compounds found in seed extracts, as shown, for 72 hours at 37°C. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Results represent the mean ± SEM of triplicate determinations. *$P < 0.05$ when compared to controls.

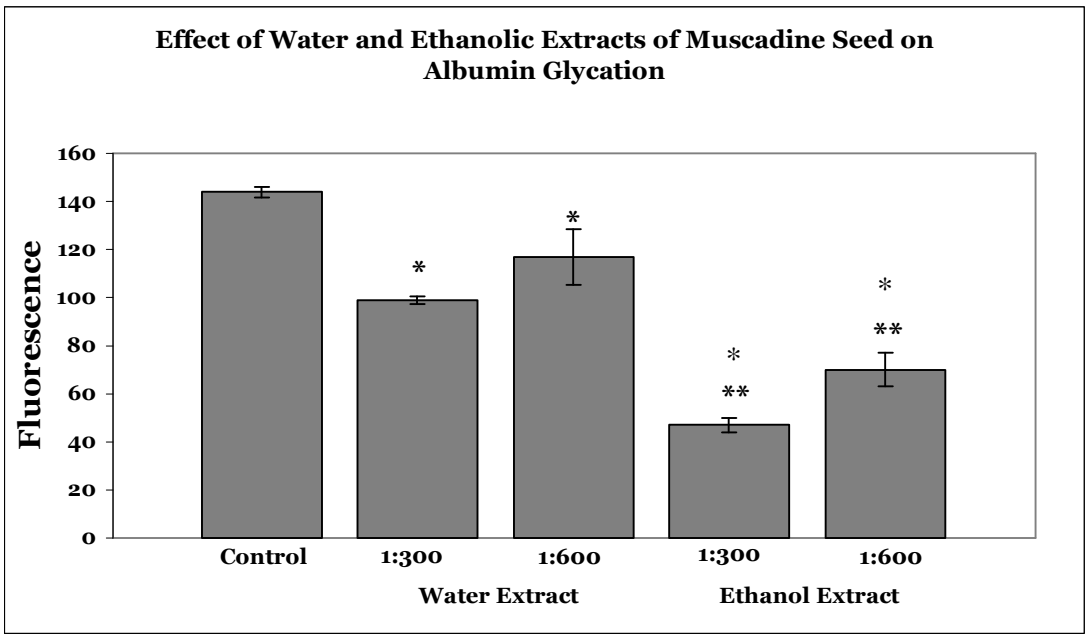

Figure 4. Effect of Water and Ethanolic Extracts of Muscadine Seed on Albumin Glycation. Bovine serum albumin (10 mg/mL) was incubated with fructose (250 mM) in potassium phosphate buffer (200 mM; pH 7.4; 0.02% sodium azide) and treated with varying concentrations of both water and ethanolic derived extracts of muscadine seeds, as shown, for 72 hours at 37°C. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Results represent the mean ± SEM of triplicate determinations. *$P < 0.05$ when compared to controls, **$P < 0.05$ when compared to the corresponding dilution of the water extract.

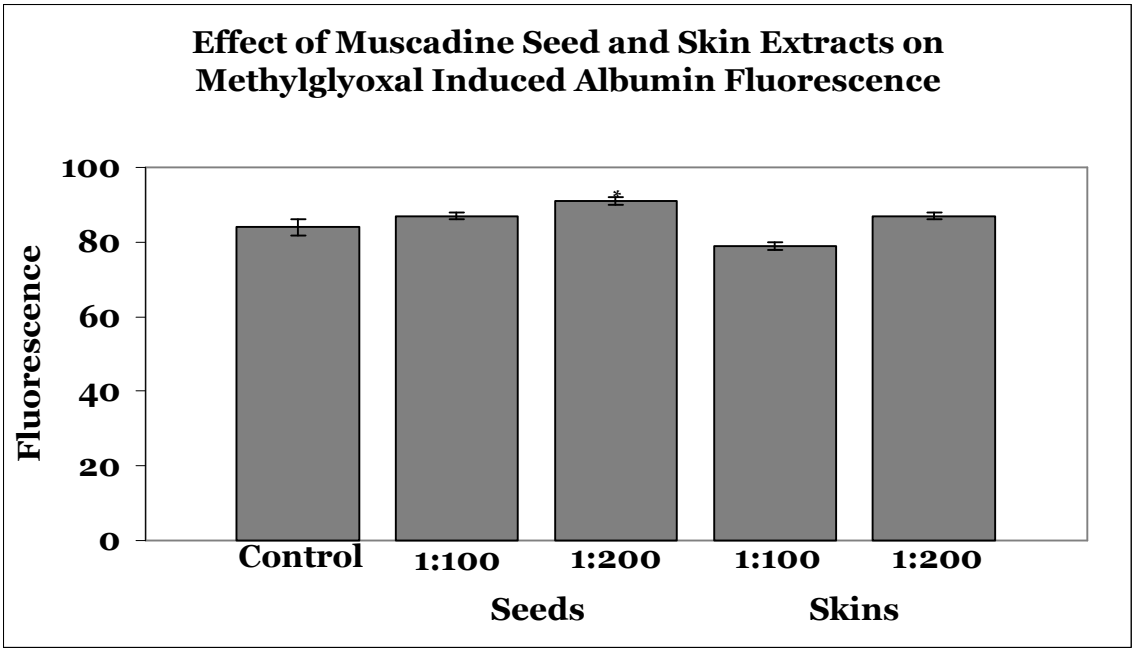

Figure 5. Effect of Muscadine Seed and Skin Extracts on Methylglyoxal Induced Albumin Fluorescence. Bovine serum albumin (100 μM) was incubated with 1 mM methylglyoxal in 0.1 M sodium phosphate, pH 7.0. After 96 hours, the fluorescence was measured using the wavelength pair of 350 and 409 nm. Results represent the mean ± SEM of triplicate determinations.

Chapter III - Novel Nutraceutical Property of Select Sorghum Brans: Inhibition of Protein Glycation

---

[1]Farrar, JL, Hartle, DK, Hargrove, JL, Greenspan, P*, to be submitted to the *Journal of Agricultural and Food Chemistry*

Abstract.

Despite the high levels of phytochemicals in grain sorghum and its position as a major food staple, there has been a lack of research on its effects on both animal and human health and disease prevention. These phenolic compounds, mainly located in the bran fraction, result in the plant having substantial antioxidant properties. In this study, we examined the effect of ethanolic extracts of several varieties of sorghum bran on albumin glycation, a non-enzymatic process thought to be important in the pathogenesis of many diabetic complications. Sorghum brans with high phenolic content and high antioxidant properties inhibited protein glycation; whereas sorghum brans that are low in these properties did not inhibit this process. Ethanolic extracts of wheat, rice or oat bran did not inhibit protein glycation. Although one high phenolic sorghum bran variety (sumac) inhibited protein glycation mediated by the auto-oxidation of fructose by approximately 60%, the inhibition of methyglyoxal mediated albumin glycation was minimized. These results suggest that certain varieties of sorghum bran may affect important biological processes that are important in diabetes and insulin resistance. These results distinguish select sorghum brans from the common food brans and suggest a nutraceutical rationale for its human consumption.

KEYWORDS: sorghum bran, diabetes mellitus, protein glycation, advanced glycation endproducts (AGEs)

Introduction.

Consistent consumption of foods that contain significant levels of phytochemicals and dietary fiber correlates with tangible disease prevention. For example, whole grain consumption is known to help in reducing cases of heart disease, diabetes, and other chronic diseases partly due to components in their brans, especially dietary fiber and phytochemicals [1,2,4]. This has led to the dietary guideline that increased consumption of whole grains. Cereals are the most widely and consistently consumed food staples of diets all over the world [2].

Sorghum grain has been a dietary staple for millenia in parts of India, Africa, and China [3]. Much of the growth in the world's population will be in the semiarid, developing countries where drought-tolerant sorghum and millet varieties are major crops [4] that contribute to the protein and energy requirements. Some sorghum varieties have extremely high contents of phenolic compounds that aid in the natural defense of plants against pests and diseases. These phenolic compounds, mainly located in the bran fraction, result in the plant having significant antioxidant properties [1].

Sorghum phenolic compounds fall into two major categories; phenolic acids and flavonoids. The phenolic acids are benzoic or cinnamic acid derivatives, whereas the flavonoids are largely tannins and anthocyanins. Limited data exists on the levels of anthocyanins found in cereals and grains; however, anthocyanins are a significant component of sorghum bran [5, 26].

Despite the high levels of phytochemicals in sorghum and its position as a major grain, there has been a paucity of medical research on its effects on human health. The effect of sorghum on pathological processes, such as inflammation, has not been

examined. Researchers have tested the hypothesis that ethanolic extracts of sorghum brans will inhibit protein glycation, and present the results in this communication. This non-enzymatic reaction between reducing sugars and proteins is inhibited by antioxidants such as flavonoids [6] and is thought to be extremely important in the pathogenesis of diabetic complications. The results presented suggest that ingestion of sorghum bran could have previously unrecognized health benefits especially important to metabolic syndrome and diabetes patients.

Materials and Methods.

Chemicals.

Bovine serum albumin (BSA) (Fraction V, Essentially Fatty Acid Free, D- (-) fructose, Chelex 100 (sodium form), Folin-Ciocalteu reagent, methylglyoxal solution, and TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) were purchased from Sigma Chemical Company, (St Louis, MO). Sorghum brans were gifts from Dr. Lloyd Rooney of Texas A& M University and Dr Scott Bean, USDA, Manhattan, KS. Rice and wheat brans were purchased from Bob's Red Mill Natural Foods, Inc (Milwaukee, OR). Oat bran was obtained from a health food store in Georgia.

Preparation of Sorghum Extracts.

To prepare the bran extracts, dried bran was made into a powder with a commercial coffee grinder. The bran was extracted with a 1:10 (w/v) with 50% ethanol for 2 hours at room temperature with periodic vortexing. The extract was then centrifuged to remove the precipitate and filter sterilized to obtain the final extract..

Total Phenolic Content.

Phenolic content of samples was determined with the Folin-Ciocalteu method as described by Singleton [7]. Gallic acid was employed as the standard. Absorbance was measured at 765 nm in a Beckman DU 600 series spectrophotometer. Results are expressed as milligrams of gallic acid per gram of bran.

Ferric Reducing Antioxidant Protein (FRAP).

FRAP values were determined with a modified version of the Benzie and Strain method [8], with ferrous sulfate as the reference standards. Absorption was measured at 593 nm in a Beckman DU 600 series spectrophotometer. The FRAP assay is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex to the ferrous form. The results are expressed as millimoles of ferrous sulfate formed per 100 grams of dry weight of bran.

Albumin Glycation.

The fluorescence assay to determine the glycation of albumin was performed as essentially described by McPherson, et. al. [9] Bovine serum albumin (BSA; 10 mg/mL) was incubated with D- (-) fructose (250 mM) in potassium phosphate buffer (200 mM; pH 7.4; 0.02% sodium azide) in a 5% carbon dioxide incubator at 37°C for 72 hours. The buffer was treated with Chelex 100 prior to use. Various concentrations of the extracts were added to the 3-ml incubation mixture. To control for the ethanol present in the extract, control incubations were incubated in the presence of the appropriate concentration of ethanol. The fluorescence intensity was measured at an

excitation/emission wavelength pair of 370/440 nm using a Perkin-Elmer LS 55 Luminescence Spectrometer. Slit widths were set a 3 nm. Values were corrected for the intrinsic fluorescence of bran extracts.

Modification of Albumin by Methylglyoxal.

Bovine serum albumin (100μM) was incubated with 1mM methylglyoxal in 0.1 M sodium phosphate, pH 7.0 according to the method of Packer and colleagues[10]. The buffer was treated with Chelex 100 prior to use. After 96 hours, the fluorescence was measured using the wavelength pair of 350 and 409 nm. Values were corrected for the intrinisic fluorescence of bran extracts.

Statistical Analysis.

Experiments were performed in triplicate. Values are expressed as mean ± SEM.. Data within groups were analyzed using one-way analysis of variance (ANOVA) and multiple comparisons were performed employing the Duncan's Multiple Range test. $P < 0.05$ was considered statistically significant.

Results.

Effect of Sumac Sorghum Bran Extract on Albumin Glycation.

Sorghum bran (sumac variety) was extracted with 50% ethanol (10% w/v) and the phenolic content of the extract was determined. Sumac bran had a phenolic content of 52.8 mg/g and a FRAP value of 47.2 mmoles/100g, in agreement with high antioxidant capacity of this tannin-rich sorghum [15]. The effect of sumac sorghum bran extract on

the glycation of albumin was initially examined at four different dilutions of the extract (Figure 1). Control incubations of fructose and albumin resulted in significant albumin glycation; the relative fluorescence intensity was found to be approximately 135 units. When fructose and albumin were incubated in the presence 1:300 and 1:600 dilutions of the sorghum extract, a significant concentration dependent decrease in fluorescence intensity was observed. When the 1:1200 and 1:2400 dilutions were examined, a significant difference from the control incubation was not observed.

Since sumac sorghum bran significantly inhibited protein glycation, five other sorghum brans were examined to determine their effect on this non-enzymatic reaction. Four sorghum brans, sumac, shanqui, black and mycogen varieties at dilutions of 1:300 significantly inhibited the glycation of albumin. Fontanelle and white sorghum bran varieties, however, did not significantly produce a decreased fluorescence intensity at this dilution (Figure 2). The phenolic content and the FRAP values for the extracts of these sorghum brans were also determined (Table 1). Fontanelle and white were found to have a very low content of phenolic compounds and low FRAP values, while the sorghum brans that inhibited glycation (sumac, shanqui, black and mycogen) had much greater phenolic contents and antioxidant FRAP values. From these data, it is apparent that the degree of glycation inhibition correlates with the phenolic content/FRAP values.

Effect of Various Bran Extracts on Albumin Glycation.

Since sorghum bran was found to significantly inhibit albumin glycation, the effect of other cereal brans was also examined. As seen in Figure 3, neither rice, wheat nor oat bran significantly inhibited the glycation of albumin. The phenolic content and

FRAP values of the bran extracts were examined (Table 2). In contrast to the high phenolic content and FRAP values in certain sorghum varieties, these non-sorghum brans possessed a very low content of phenolic compounds and were found to have a negligible antioxidant capacity.

Effect of Sumac Sorghum Bran and Muscadine Grape Seed Extracts on Albumin Glycation.

In our laboratory, the muscadine grape seed has previously been shown to significantly inhibit protein glycation [12]. This grape, native to the southeastern United States has one of the highest antioxidant capacities of any fruit. In this experiment, sumac sorghum bran was and muscadine grape seeds were extracted with 50% ethanol and their inhibitory properties on albumin glycation determined. As shown in figure 4, both concentrations (1:300 and 1:600 dilutions) of both the sumac sorghum and muscadine seeds extracts significantly inhibited protein glycation These results demonstrate that both sumac sorghum and muscadine seed extracts are efficacious inhibitors of protein glycation..

Comparison of Water and Ethanolic Extracts of Sumac Sorghum Bran on Protein Glycation.

In this experiment, water and ethanolic extracts of sumac sorghum bran were prepared with distilled water or 50% ethanol. After the 2 hours of incubation with periodic vortexing, the phenolic content of the extracts was determined. The ethanolic extract had a phenolic content of 5.6 mg/ml while the content of the aqueous extract was 2.1 mg/ml. The antioxidant property of the extracts was determined employing the FRAP assay. In agreement with phenolic content, the ethanolic extract has a greater

FRAP values (48.6 mmoles/100 grams vs 20.6 mmoles/100 grams, respectively). The fluorescence intensity of the glycation of albumin was measured in the presence of two dilutions (1:300 and 1:600) of the sorghum bran extracts. As seen in figure 5, both ethanolic and aqueous extracts at both dilutions inhibited the glycation of albumin as evidenced by a significant decrease in fluorescence. The ethanolic extract inhibited glycation to a greater extent than the corresponding aqueous extract, in agreement with the greater phenolic concentration found in the ethanolic extracts.

Effect of Sumac Sorghum Bran on Methylglyoxal Induced Albumin Fluorescence.

Methylglyoxal has been shown to be an important intermediate in the autooxidation of reducing sugars and can readily glycate proteins [13]. Protein glycation by methylglyoxal is a nonenzymatic modification whereby arginine and lysine side chains of proteins participate in forming a heterogeneous group of advanced glycation end-products [14]. As seen in Figure 6, methylglyoxal, when incubated with albumin produced significance fluorescence at the wavelength pair of 350 and 409 nm, indicative of albumin glycation. When two dilutions (1:100 and 1:200) of ethanolic extracts of sumac sorghum bran were incubated with methylglyoxal, a significant decrease in fluorescence intensity was observed. However, a dilution of 1:400 did not produce a significant decrease in glycation

Discussion.

The data presented here support the hypothesis that phenolic compounds in certain sorghum bran varieties significantly inhibit protein glycation. The inhibitory

sorghum brans were those with high phenolic content and high antioxidant FRAP values. The high levels of antioxidants present in sumac bran and black brans and the relatively low levels present in white bran are in agreement with previous reports [1, 11]. Numerous specialty sorghum varieties are now being cultivated. Experimental manipulation has led to the development of sorghum brans that are either high in tannins (sumac, black) or very low in these compounds (white, fonatelle). These high tannin sorghum bran varieties contain anthocyanins such as luteolinidin and apigeninidin [15] and are the varieties that strongly inhibit the glycation reaction. These results are in agreement with previous findings that demonstrated that antioxidants [16] and plant extracts with high contents of flavonoids [17] can significantly inhibit protein glycation

In comparison to sorghum, other cereal brans, such as oat, rice and wheat had low phenolic contents and low antioxidant FRAP values. The FRAP values of these brans were approximately 1-2% of that found for the sumac sorghum bran. These findings on the antioxidant properties of the brans are in agreement with the findings of Awika *et. al.*[11]. Sumac sorghum bran was found to have twenty-five times higher antioxidant capacity than red wheat bran as measured by the antioxidant ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) assay [1]. Using another antioxidant assay, the TEAC (Trolox Equivalent Antioxidant Capacity) assay, wheat bran was shown to have three times the antioxidant capacity than oat bran [18]. Finally, rice flour was found to have a similar antioxidant capacity to that of wheat flour [19]. Therefore, the antioxidant properties observed here are consistent with those measured by other investigators.

Although sumac sorghum bran was a potent inhibitor of fructose mediated protein glycation , its affect on methylglyoxal-induced glycation was not as great. For instance at 1:200, an extract of sorghum bran sumac produced an 8% inhibition in methylglyoxal mediated albumin glycation whereas a 1:300 dilution produced a 60% reduction in fructose mediated glycation. The failure to block the methylglyoxal reaction suggests that oxidative processes may not participate in the glycation of albumin mediated by methylglyoxal to same extent as it does in fructose-induced glycation. A similar profile of strong inhibition on glucose mediated glycation and weak, but significant, inhibition on methylglyoxal inhibition was observed for epicatechin and epigallocatechin [20].

The inhibition of protein glycation by high tannin sorghum bran varieties was found to be quite similar to that produced by muscadine grape seeds. These two crops have several commonalities. Both have extremely high phenolic content and antioxidant capacities. Both the muscadine grapes and high tannin sorghum brans can be grown in arid environments; the high content of antioxidants found in these plant parts protects them from pests and diseases and allows them to flourish in such environments [11, 21, 22].

This study illustrates that select sorghum brans have potential to modulate processes involved in inflammatory diseases. The present results show that phenolic-rich sorghum brans inhibit a non-enzymatic process thought to be important in the pathogenesis of diabetic complications, such as neuropathy and cardiovascular disease. To date, most of the substances known to inhibit protein glycation are either antioxidants [16], spices [23] or extracts of plant substances [20]. One study has documented that an extract of tomato paste can inhibit protein glycation [24]. Clearly, effects of food staples on protein glycation deserves study. Millets, from the Kodo and Finger varieties, have

been shown to inhibit protein glycation [25]. The results of this study confirm that specialty sorghum brans are far superior to impact human health when compared with other brans commonly used in the diet [26]. The high antioxidant and anti-glycation properties provide a rationale for nutraceutical and food ingredient use of select sorghum brans and their extracts.

Literature Cited

1. Awika, J.M., C.M. McDonough, and L.W. Rooney, *Decorticating sorghum to concentrate healthy phytochemicals.* J Agric Food Chem, 2005. **53**(16): p. 6230-4.
2. Kushi, L.H., K.A. Meyer, and D.R. Jacobs, Jr., *Cereals, legumes, and chronic disease risk reduction: evidence from epidemiologic studies.* Am J Clin Nutr, 1999. **70**(3 Suppl): p. 451S-458S.
3. Zhao, Z.Y., *Sorghum (Sorghum bicolor L.).* Methods Mol Biol, 2006. **343**: p. 233-44.
4. Klopfenstein, C.H., RC, *Nutritional Properties of Sorghum and the Millets*, in *Sorghum and Millets, Chemistry and Technology*, D. Dendy, Editor. 1995, American Association of Cereal Chemists, Inc.: Upton.
5. Lietti, A., A. Cristoni, and M. Picci, *Studies on Vaccinium myrtillus anthocyanosides. I. Vasoprotective and antiinflammatory activity.* Arzneimittelforschung, 1976. **26**(5): p. 829-32.
6. Cervantes-Laurean, D., et al., *Inhibition of advanced glycation end product formation on collagen by rutin and its metabolites.* J Nutr Biochem, 2006. **17**(8): p. 531-40.
7. Singleton, V.L.R. *Colorimetry of total phenolic with phosphomolybdic-phosphotungstic acid agents.* Am. J. Enol. Vitic., 1965. **16**: p. p. 144-158.

8. Benzie, I.F.a.J.J.S., *The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay.* Anal Biochem, 1996.

239(1): p. p. 70-6.

9. McPherson, J.D., B.H. Shilton, and D.J. Walton, *Role of fructose in glycation and cross-linking of proteins.* Biochemistry, 1988. **27**(6): p. p. 1901-7.

10. Packer, L., E.H. Witt, and H.J. Tritschler, *alpha-Lipoic acid as a biological antioxidant.* Free Radic Biol Med, 1995. **19**(2): p. p. 227-50.

11. Awika, J.M., et al., *Screening methods to measure antioxidant activity of sorghum (sorghum bicolor) and sorghum products.* J Agric Food Chem, 2003. **51**(23): p. 6657-62.

12. Farrar, J., Hartle, DK, Hargrove, JL, Greenspan, P (2006) *Inhibition of protein glycation by skins and seeds of the muscadine grape.* . FASEB J. **Volume**, A1002

13. Lee, C., et al. , *Oxidation-reduction properties of methylglyoxal-modified protein in relation to free radical generation.* J Biol Chem, 1998. **273**(39): p. p. 25272-8.

14. Wu, X.a.V.M.M., *Enzymatic deglycation of proteins.* Arch Biochem Biophys, 2003. **419**(1): p. p. 16-24.

15. Dykes, L., et al., *Phenolic compounds and antioxidant activity of sorghum grains of varying genotypes.* J Agric Food Chem, 2005. **53**(17): p. 6813-8.

16. Selvaraj, N., Z. Bobby, and V. Sathiyapriya, *Effect of lipid peroxides and antioxidants on glycation of hemoglobin: an in vitro study on human erythrocytes.* Clin Chim Acta, 2006. **366**(1-2): p. 190-5.

17. Hsieh, C.L., et al., *Inhibitory effect of some selected nutraceutic herbs on LDL glycation induced by glucose and glyoxal.* J Ethnopharmacol, 2005. **102**(3): p. 357-63.

18. Martinez-Tome, M., et al., *Evaluation of antioxidant capacity of cereal brans.* J Agric Food Chem, 2004. **52**(15): p. 4690-9.

19. Perez-Jimenez, J. and F. Saura-Calixto, *Literature data may underestimate the actual antioxidant capacity of cereals.* J Agric Food Chem, 2005. **53**(12): p. 5036-40.

20. Wu, C.H. and G.C. Yen, *Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts.* J Agric Food Chem, 2005. **53**(8): p. 3167-73.

21. Greenspan, P., et al., *Antiinflammatory properties of the muscadine grape (Vitis rotundifolia).* J Agric Food Chem, 2005. **53**(22): p. 8481-4.

22. Hartle DK, Greenspan, P., Hargrove JL, *Muscadine Medicine*. 1st ed. ed. 2005, St George's Island: Lulu.

23. Ahmad, M.S. and N. Ahmed, *Antiglycation properties of aged garlic extract: possible role in prevention of diabetic complications.* J Nutr, 2006. **136**(3 Suppl): p. 796S-799S.

24. Kiho, T., et al., *Tomato paste fraction inhibiting the formation of advanced glycation end-products.* Biosci Biotechnol Biochem, 2004. **68**(1): p. 200-5.

25. Hegde, P., G. Chandrakasan, and T. Chandra, *Inhibition of collagen glycation and crosslinking in vitro by methanolic extracts of Finger millet (Eleusine coracana) and Kodo millet (Paspalum scrobiculatum).* J Nutr Biochem, 2002. **13**(9): p. 517.

26. Awika, J.M. and L.W. Rooney, *Sorghum phytochemicals and their potential impact on human health.* Phytochemistry, 2004. **65**(9): p. 1199-221.

Table 1. Phenolic content and FRAP values of various sorghum bran extracts.

Extract	Phenolic Value (mg/g)	FRAP Value (mmoles/100g)
Sumac Sorghum Bran	62.4 ± 0.9	28.4 ± 0.1
Shanqui Sorghum Bran	47.7 ± 0.7	15.3 ± 0.1
Black Sorghum Bran	22.7 ± 0.5	7.9 ± 0.2
Mycogen Sorghum Bran	5.6 ± 0.1	2.2 ± 0.1
Fontanelle Sorghum Bran	2.5 ± 0.1	1.0 ± 0.1
White Sorghum Bran	3.9 ± 0.1	1.2 ± 0.1

Data represent mean ± SEM of triplicate determinations.

Table 2. Phenolic content and FRAP values of various bran extracts.

Extract	Phenolic Value (mg/g)	FRAP Value (mmoles/100 g)
Sorghum Bran	52.8 ± 0.5	47.3 ± 0.8
Wheat Bran	2.0 ± 0.1	0.7 ± 0.1
Oat Bran	0.6 ± 0.1	0.4 ± 0.1
Rice bran	6.0 ± 0.3	5.3 ± 0.6

Data represent mean ± SEM of triplicate determinations.

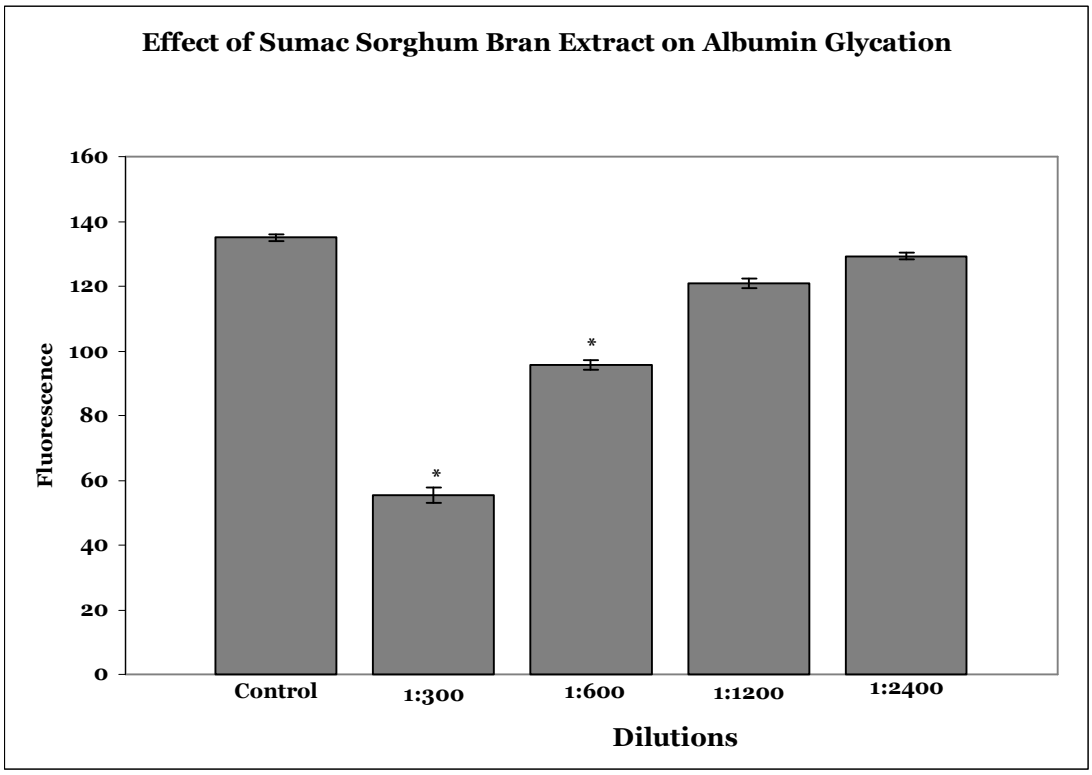

Figure 1. Effect of Sumac Sorghum Bran Extract on Albumin Glycation. Briefly, bovine serum albumin (10mg/mL) was incubated with fructose (250 mM) in potassium phosphate buffer (200mM; pH 7.4) and treated with varying concentrations of the extract for 72 hours at 37°C. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Results represent the mean ± SEM of triplicate determination. *$P < 0.05$ when compared to controls.

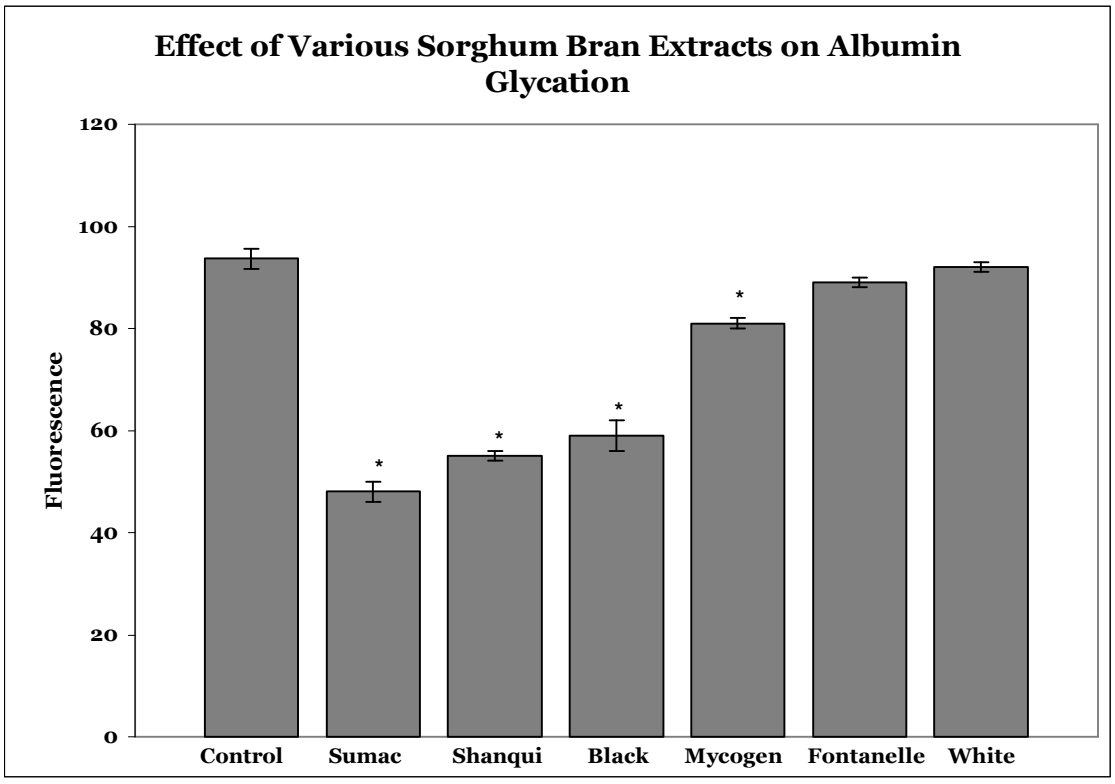

Figure 2. Effect of Various Sorghum Bran Extracts on Albumin Glycation. Bovine serum albumin 10 mg/mL) was incubated with fructose (250 mM) in potassium phosphate buffer (200 mM; pH 7.4) and incubated with a 1:300 dilution of various extracts for 72 hours at 37°C. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Results represent the mean ± SEM of triplicate determination. *$P < 0.05$ when compared to controls.

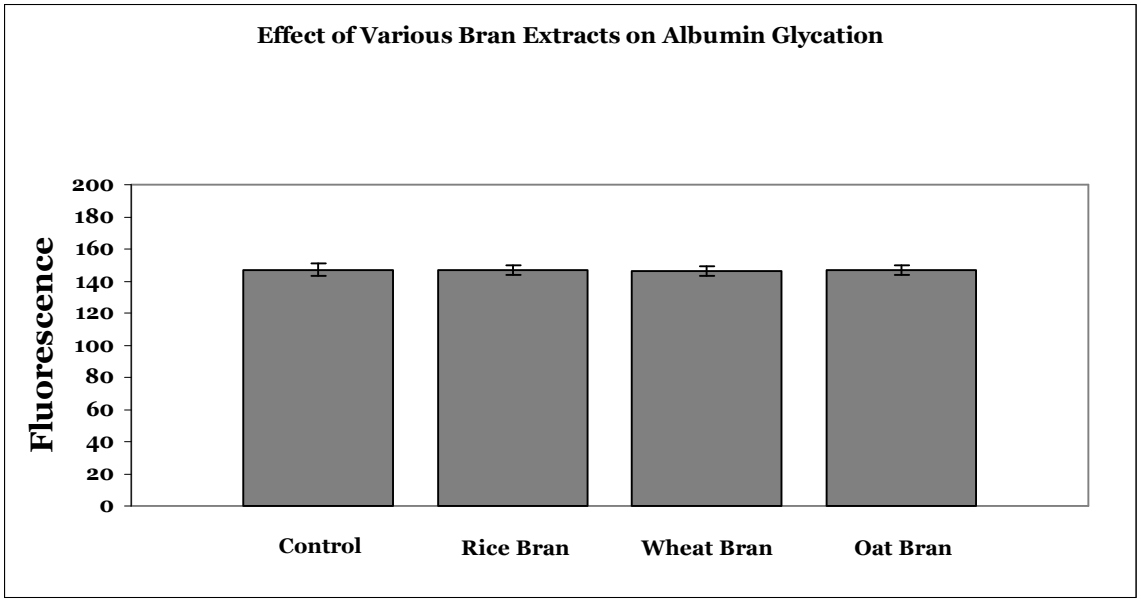

Figure 3. Effect of Various Bran Extracts on Albumin Glycation. Bovine serum albumin (10 mg/mL) was incubated with fructose (250 mM) in potassium phosphate buffer (200 mM; pH 7.4) and treated at a dilution of 1:300 with the various bran extracts for 72 hours at 37°C. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Results represent the mean ± SEM of triplicate determination. *P < 0.05 when compared to controls.

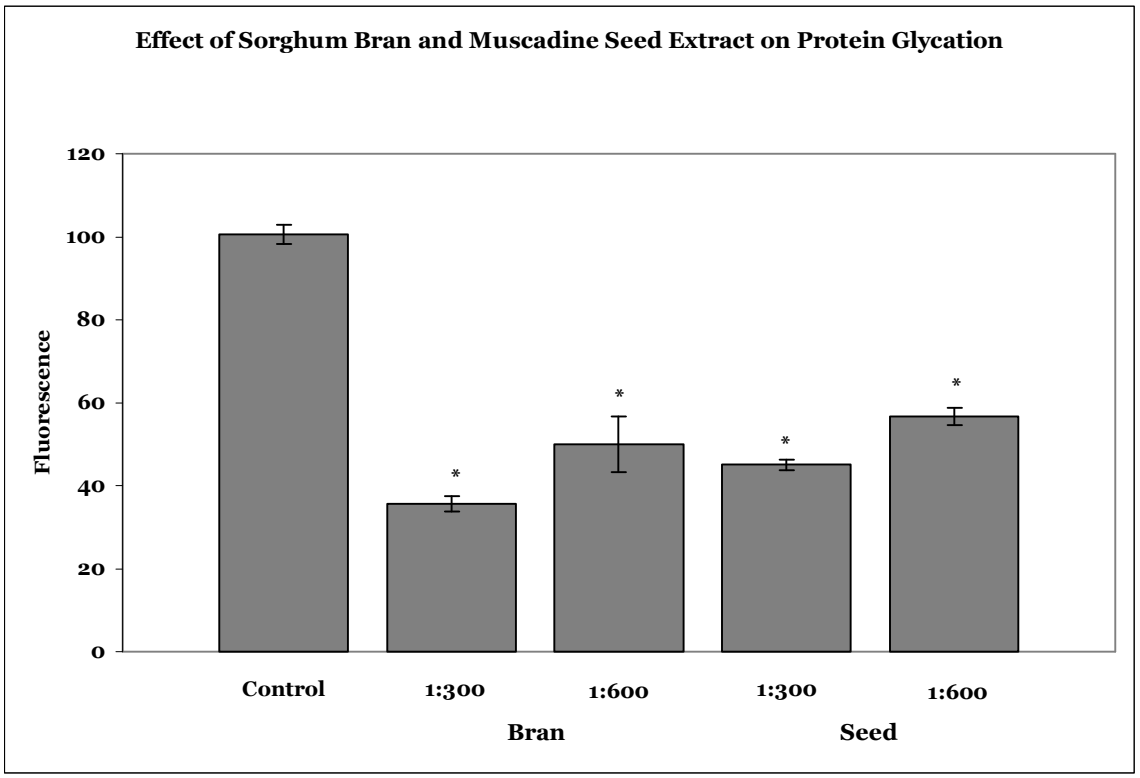

Figure 4. Effect of Sorghum Bran and Muscadine Grape Seed Extracts on Albumin Glycation. Bovine serum albumin (10 mg/mL) was incubated with fructose (250 mM) in potassium phosphate buffer (200 mM; pH 7.4) and treated with varying concentrations of different sorghum bran and muscadine seed extracts for 72 hours at 37°C. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Results represent the mean ± SEM of triplicate determination. *$P < 0.05$ when compared to controls.

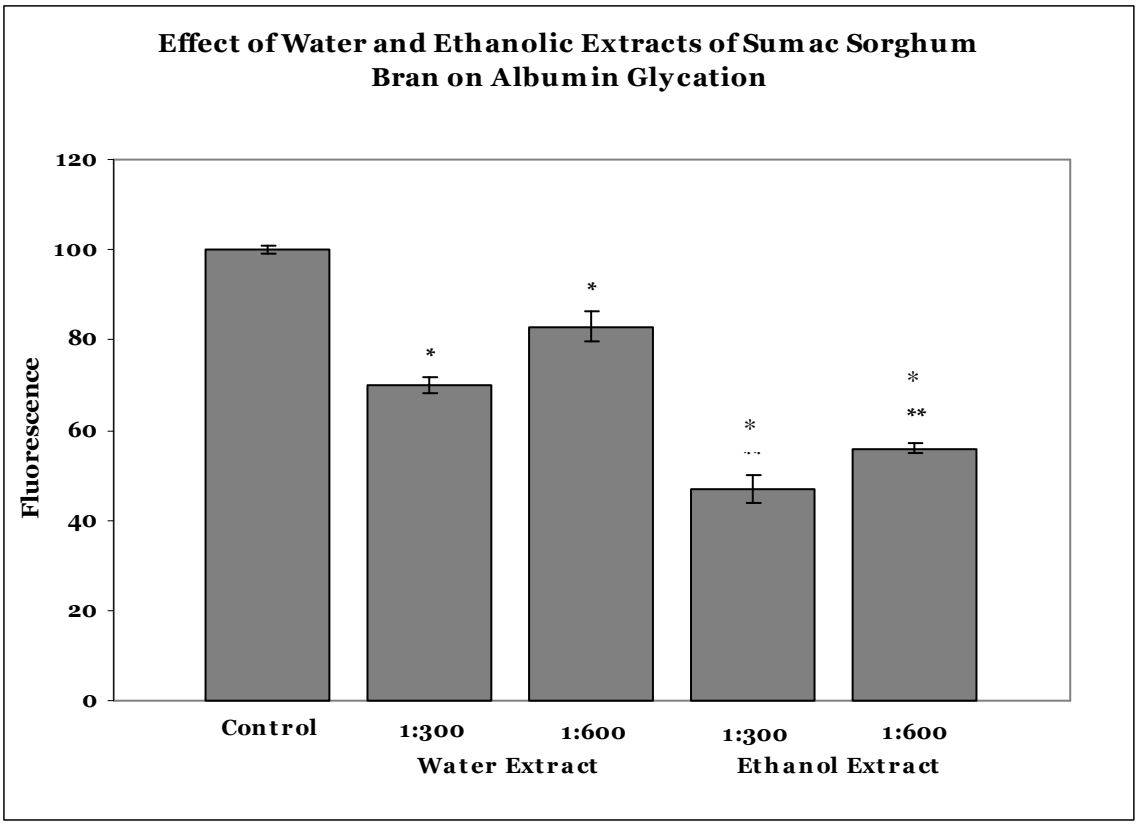

Figure 5. Effect of Water and Ethanolic Extracts of Sumac Sorghum Bran on Albumin Glycation. Bovine serum albumin (10 mg/mL) was incubated with fructose (250 mM) in potassium phosphate buffer (200mM; pH 7.4) and treated with varying concentrations of both water and ethanolic extracts of sumac sorghum bran for 72 hours at 37°C. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Results represent the mean ± SEM of triplicate determinations. *$P < 0.05$ when compared to controls. **$P< 0.05$ when compared to the same dilution of the water extract.

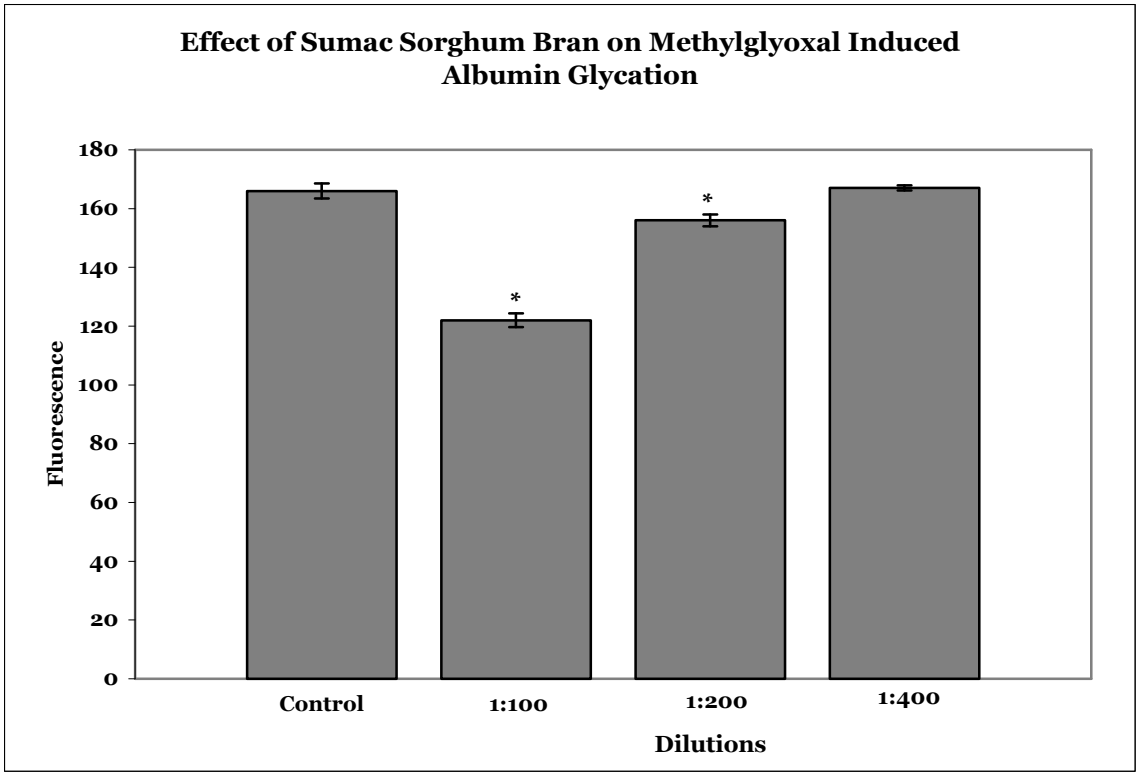

Figure 6. Effect of Sumac Sorghum Bran on Methylglyoxal Induced Albumin Fluorescence. Bovine serum albumin (100µM) was incubated with 1mM methylglyoxal in 0.1 M sodium phosphate, pH 7.0. At 37ºC, after 96 hours, the fluorescence was measured using the wavelength pair of 350 and 409 nm Results represent the mean ± SEM of triplicate determinations.

Chapter IV - Resveratrol, a Major Constituent of Polygonum cuspidatum, is an Inhibitor of Protein Glycation

---

[1]Farrar, JL, Hartle, DK, Hargrove, JL, Greenspan, P*, to be submitted to

Phytochemistry

Abstract.

Polygonum cuspidatum (Japanese knotweed, Mexican bamboo, *P. cuspidatum*, etc.) is a traditional herb used in China and Japan to treat skin burns, gallstones, hepatitis, and inflammation. Resveratrol, a phenolic compound present in this herb, has been shown to have significant pharmacological action in numerous biological pathways. This communication, tested the hypothesis that ethanolic extracts of *Polygonum cuspidatum* and resveratrol would inhibit protein glycation. Both the ethanolic extracts of *Polygonum cuspidatum* and resveratrol were found to significantly inhibit this non-enzymatic process. The results show that resveratrol may inhibit protein glycation, a biological process thought to be important in aging and diabetes.

KEYWORDS: *polygonum cuspidatum*; Japanese knotweed; Mexican bamboo; advanced glycation end products (AGEs); diabetic complications; antioxidant activity, oxidative processe

Introduction.

Polygonum cuspidatum is a perennial plant with spreading rhizomes and numerous reddish-brown stems that are traditionally used in East Asia to treat skin burns, gallstones, hepatitis and inflammation. It has also been used in Korea to maintain oral hygiene [1]. This plant contains a large number of flavonoids, which are frequently found as glucosides, and other constituents including phenyl alcohols, sterols, essential oils and amino acids [2]. Yang, *et. al*. identified resveratrol as a major phenolic constituent of *P. cuspidatum* [3]. Resveratrol, a polyphenolic constituent of grapes, berries, juices and wines, has gained significant attention for its apparent therapeutic value [4]. Resveratrol has been shown to exert multifaceted antioxidant and anti-inflammatory effects in various disease models [5]. Experimentally, resveratrol has been shown to possess both anti-atherosclerotic and anti-cancer activities [6]. The biological activity of resveratrol has been studied in aging; this compound has been shown to increase longevity in mice fed a high fat diet [6].

The effect of resveratrol on protein glycation, a spontaneous non-enzymatic aminocarbonyl reaction between reducing sugars and proteins [7, 8], has not been previously examined. In this communication, the inhibitory effects of ethanolic extracts of *Polygonum cuspidatum* and resveratrol on the glycation of albumin are reported.

Materials and Methods.

Chemicals.

Bovine serum albumin (BSA) (Fraction V, Essentially Fatty Acid Free, D- (-) fructose, Chelex 100 (sodium form), and resveratrol were purchased from Sigma Chemical Company, (St Louis, MO). *Polygonum cuspidatum* was obtained from

Supplemental Health Formulations, LLC, Mayer, AZ. This commercial product is a proprietary extract of the plant and is sold in powder form.

Preparation of the P. cuspidatum Ethanolic Extracts.

The P. cuspidatum product obtained from Supplemental Health Formulations was extracted with a 1:10 (w/v) with 50% ethanol for 2 hours at room temperature with periodic vortexing. The extract was then centrifuged to remove the precipitate and filter sterilized to obtain the final extract.

Analysis of the Concentration of Resveratrol in the Polygonum cuspidatum commercial product.

The ethanolic extract was diluted 400-fold and subjected to HPLC analysis using an ESA (Chelmsford, MA) system consisting of a Model 582 Solvent Delivery Module, a Model 542 autosampler maintained at 6ºC and a Model 5600A CoulArray detector at 250 mV. The column was an MCM C18 (4.6 X 150 mm, 5-120A) from MC Medical, Japan. Mobile phase A was 75 mM citric acid, 25 mM ammonium acetate and 10% acetonitrile; Mobile phase B was similar to A but with 50% acetonitrile. The gradient was linear from 0-17 minutes from 10%A to 80%B. Flow rate 1.0 ml/min and 20 µl of sample was injected. Resveratrol eluted between 16.2 and 16.8 minutes.

Albumin Glycation.

The fluorescence assay to determine the glycation of albumin was performed as essentially described by McPherson, et. al. [9] Bovine serum albumin (BSA; 10 mg/mL) was incubated with D- (-) fructose (250 mM) in potassium phosphate buffer (200mM; pH

7.4; 0.02% sodium azide) in a 5% carbon dioxide incubator at 37°C for 72 hours. The buffer was treated with Chelex 100 prior to use. Various concentrations of the extracts or concentrations of resveratrol were added to the 3-ml incubation mixture. To control for the ethanol present in the extract, control incubations were incubated in the presence of the appropriate concentration of ethanol. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Slit widths were set at 3 nm. Values were corrected for the intrinsic fluorescence of *P. cuspidatum* extracts.

Statistical Analysis.

Experiments were performed in triplicate. Values are expressed as mean ± SEM. Data within groups were analyzed using one-way analysis of variance (ANOVA) and multiple comparisons were performed employing the Duncan's Multiple Range test. $P < 0.05$ was considered statistically significant.

Results.

Analysis of the Resveratrol Content of Polygonum cuspidatum Extract.

The ethanolic extract of *Polygonum cuspidatum* was analyzed by HPLC to determine the concentration of resveratrol in the commercial product. As seen in Figure 1, resveratrol was the major phenolic compound detected in the ethanolic extract of *Polygonum cuspidatum*. Other peaks were eluted from the column however their peak height was significantly smaller than that of resveratrol. Analysis revealed that the concentration of resveratrol in the commercial product, based on extraction with 50%

ethanol, was 3.8% (w/w); the 10% (w/v) ethanolic extract prepared from this product represents a concentration of resveratrol of 3.8 mg/mL.

Effect of P. cuspidatum Extract on Albumin Glycation.

The effect of *Polygonum cuspidatum* on the glycation of albumin was examined at four different dilutions of the extract. Control incubations of fructose and albumin resulted in significant albumin glycation; the relative fluorescence intensity was found to be approximately 115 units. When fructose and albumin were incubated with 1:300, 1:600, 1:1200 and 1:2400 dilutions of the *P. cuspidatum* extract, a significant concentration dependent decrease in fluorescence intensity was observed at all four dilutions. At a 1:2400.dilution, the extent of albumin glycation, as determined by fluorescence was inhibited by approximately 35%.

Effect of Resveratrol on Albumin Glycation.

The effect of four different concentrations of resveratrol, ranging from 50 to 500 μM, on the glycation of albumin was examined. As seen in Figure 2, a dose dependent inhibition of albumin glycation was observed; 50 μM resveratrol inhibited protein glycation by approximately 30% while 500 μM resveratrol produced a 50% reduction in this process.

Discussion.

The results presented here show that an ethanolic extract of *Polygonum cuspidatum* and resveratrol both inhibit the glycation of albumin by fructose.

Based on the concentration of resveratrol that was extracted with 50% ethanol, the concentration in the 1:300 dilution of the extract is approximately 50 μM resveratrol. Resveratrol, at this concentration, inhibited protein glycation by 27% while the 1:300 dilution of the extract was a stronger inhibitor of glycation, producing a 58% inhibition in this process. These results suggest that the phenolic compounds present in *Polygonum cuspidatum*, which also include catechin, contribute to inhibition observed with the extract. The degree of inhibition observed with the extract of *Polygonum cuspidatum* was greater than that observed with extracts of sorghum brans or muscadine skins and seeds. A 1:2400 dilution of the *Polygonum cuspidatum* extract significantly inhibited protein glycation while this concentration of sumac sorghum bran or muscadine seeds did not produce a significant effect. The probable reason for this difference is not that the extract of *Polygonum cuspidatum* is a more powerful inhibitor, but rather, the *Polygonum cuspidatum* material employed in these experiments was purchased as an already concentrated product.

Resveratrol, in other studies, has been shown to increase the uptake of glucose by tissues and increase the synthesis of glycogen in the streptozotocin diabetic rat [2]. In a recent study, resveratrol, when given to mice fed a high caloric diet, decreased fasting blood glucose concentrations and also decreased insulin levels in both the fed and fasted state. When a glucose bolus was given to these animals, an increase in insulin sensitivity was observed, thought to be a result of an increase in AMPK kinase [6]. Resveratrol has also been shown to attenuate diabetic nephropathy in in steptozotocin-treated rats; it decreased both the excretion of albumin in the urine and the blood urea nitrogen concentration. While resveratrol has not been shown to inhibit the formation of age-

related glycation products (AGE), the prototype inhibitor of protein glycation, aminoguanidine, has been shown to decrease fluorescence in the streptozotocin diabetic rat and attenuate the diabetic nephropathy [10]. In this manner, the effect of resveratrol in improving renal function in the streptozotocin-treated rat [11] may well be a result of inhibition of protein glycation.

Literature Cited

1. Song, J.H., et al., *In vitro inhibitory effects of Polygonum cuspidatum on bacterial viability and virulence factors of Streptococcus mutans and Streptococcus sobrinus.* Arch Oral Biol, 2006.
2. Su, H.C., L.M. Hung, and J.K. Chen, *Resveratrol, a red wine antioxidant, possesses an insulin-like effect in streptozotocin-induced diabetic rats.* Am J Physiol Endocrinol Metab, 2006. **290**(6): p. E1339-46.
3. Yang, F., T. Zhang, and Y. Ito, *Large-scale separation of resveratrol, anthraglycoside A and anthraglycoside B from Polygonum cuspidatum Sieb. et Zucc by high-speed counter-current chromatography.* J Chromatogr A, 2001. **919**(2): p. 443-8.
4. Bakhtiarova, A., et al., *Resveratrol inhibits firefly luciferase.* Biochem Biophys Res Commun, 2006.
5. Labinskyy, N., et al., *Vascular dysfunction in aging: potential effects of resveratrol, an anti-inflammatory phytoestrogen.* Curr Med Chem, 2006. **13**(9): p. 989-96.
6. Baur, J.A., et al., *Resveratrol improves health and survival of mice on a high-calorie diet.* Nature, 2006.
7. Ahmed, N., *Advanced glycation endproducts--role in pathology of diabetic complications.* Diabetes Res Clin Pract, 2005. **67**(1): p. 3-21.

8. Iwashima, Y., et al., *Advanced glycation end product-induced peroxisome proliferator-activated receptor gamma gene expression in the cultured mesangial cells.* Biochem Biophys Res Commun, 1999. **264**(2): p. 441-8.

9. McPherson, J.D., B.H. Shilton, and D.J. Walton, *Role of fructose in glycation and cross-linking of proteins.* Biochemistry, 1988. **27**(6): p. 1901-7.

10. Soulis-Liparota, T., et al., *The relative roles of advanced glycation, oxidation and aldose reductase inhibition in the development of experimental diabetic nephropathy in the Sprague-Dawley rat.* Diabetologia, 1995. **38**(4): p. 387-94.

11. Sharma, S., et al., *Resveratrol, a polyphenolic phytoalexin, attenuates diabetic nephropathy in rats.* Pharmacology, 2006. **76**(2): p. 69-75.

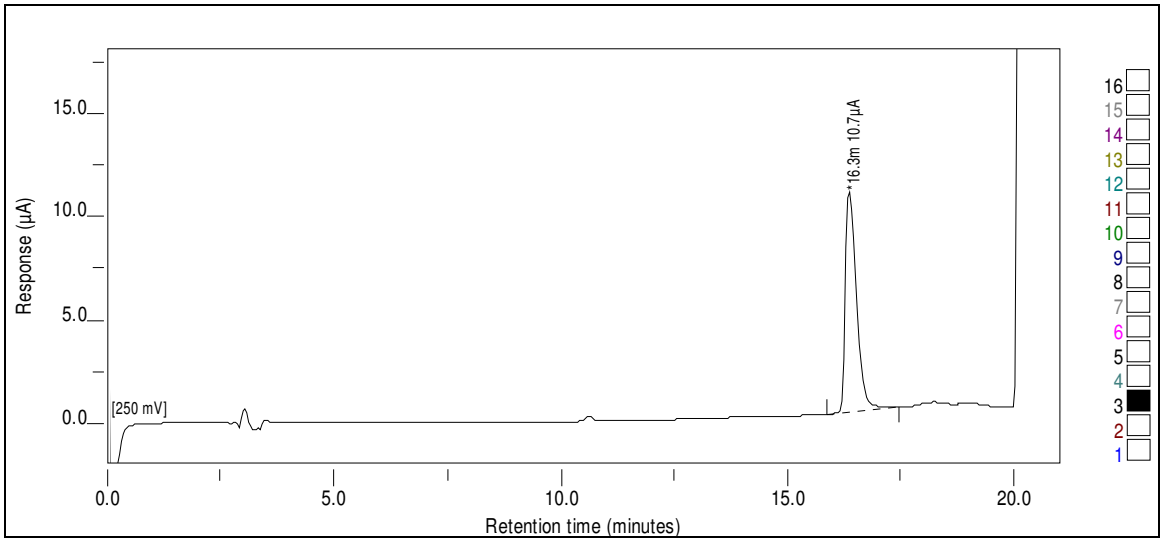

Figure 1. Chromatogram of the Ethanolic Extract of the Commerical *Polygonum cuspidatum* Extract.

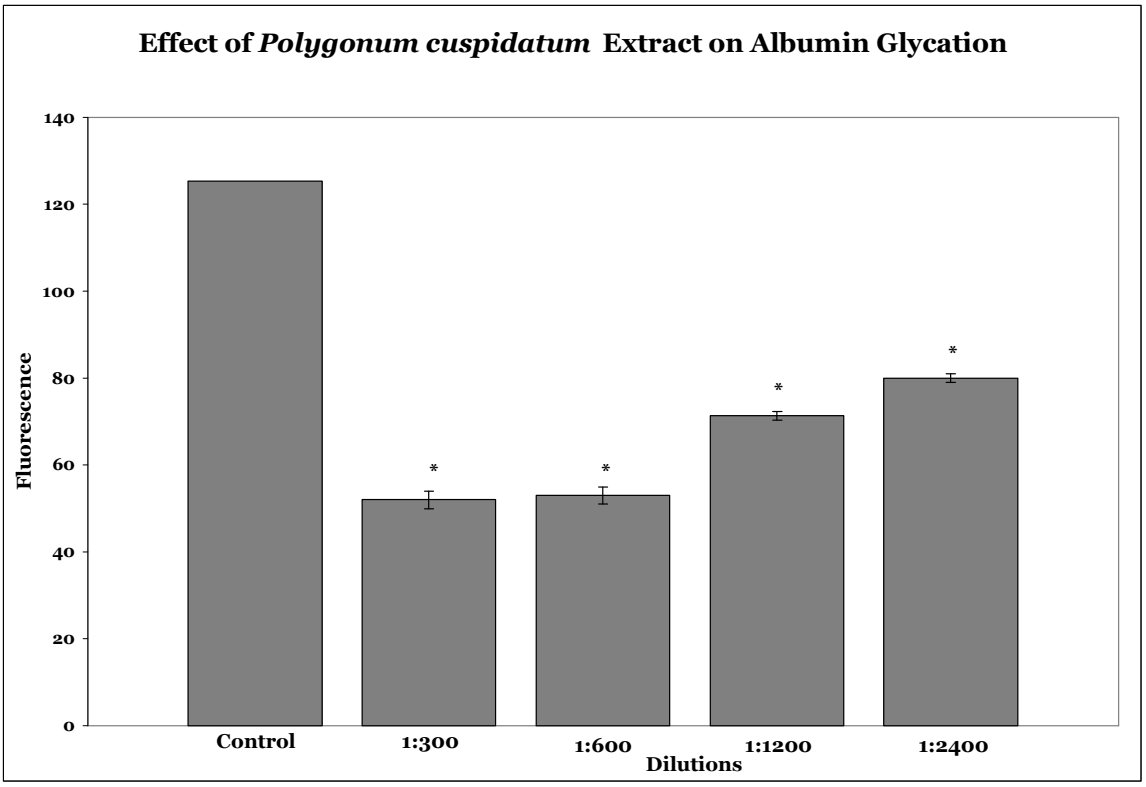

Figure 2. Effect of *Polygonum cuspidatum* Extract on Albumin Glycation. Bovine serum albumin (BSA) was incubated with fructose (250 mM) in potassium phosphate buffer (200 mM; pH 7.4) and treated with four concentrations of the extracts for 72 hours at 37°C. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Results represent the mean ± SEM of triplicate determination. *P < 0.05 when compared to controls.

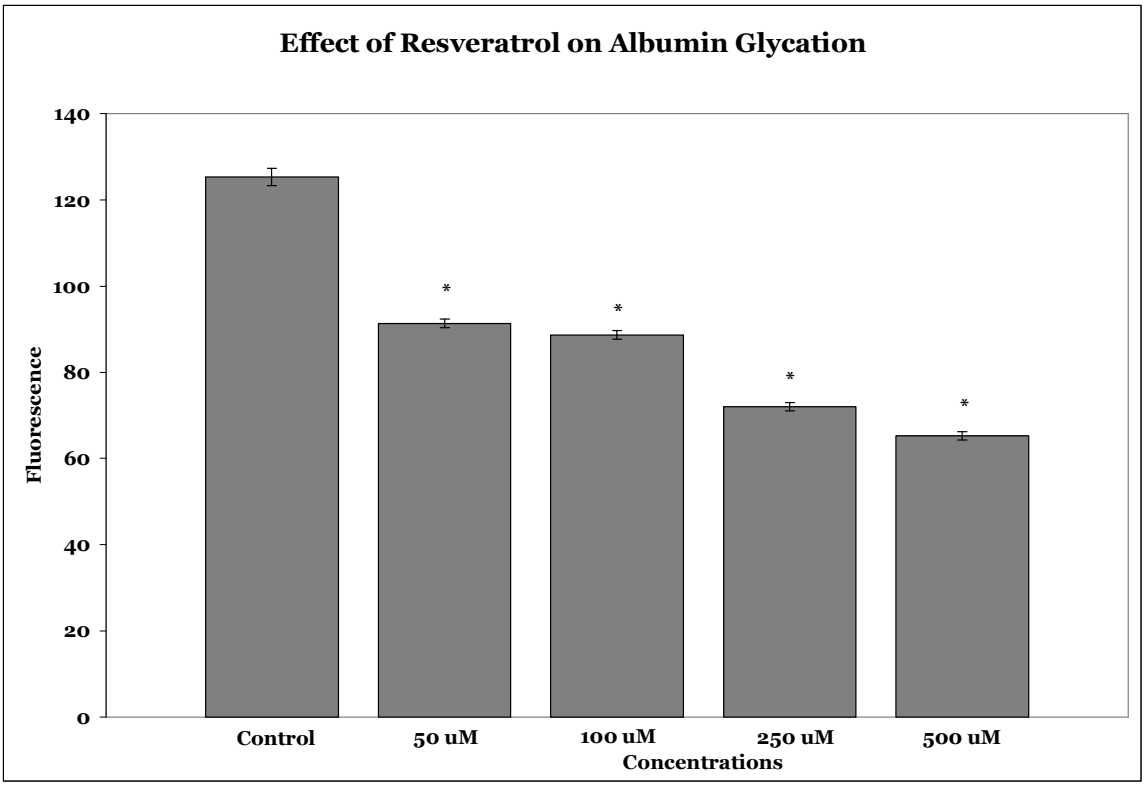

Figure 3. Effect of Resveratrol on Albumin Glycation. Bovine serum albumin (10 mg/mL) was incubated with fructose (250 mM) in potassium phosphate buffer (200 mM; pH 7.4) and treated with four concentrations of resveratrol for 72 hours at 37°C. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm. Results represent the mean ± SEM of triplicate determination. *$P < 0.05$ when compared to controls.

Chapter V - Conclusions.

The results presented in this dissertation demonstrate that certain food products, high in antioxidant capacity, can inhibit protein glycation. Ethanolic extracts of muscadine skins and seeds and special varieties of sorghum bran were found to contain a high concentration of phenolic compounds, high antioxidant capacity and strongly inhibited the fructose mediated non-enzymatic glycation of albumin. This inhibitory activity was also seen with *Polygonum cuspidatum* and its major phenolic constituent, resveratrol. The mechanism of action of these products is probably a result of their antioxidant properties; inhibition of protein glycation has been observed by antioxidants such as vitamins A, C and E.

The glycation of proteins and subsequently cross-linking of proteins in the diabetic state is thought to participate in pathogenesis of diabetic complicatons, such as diabetic nephropathy. In this manner, resveratrol has been shown to attenuate the progression of this nephropathy in a diabetic rat model. The results of these studies provide a rationale to study the effect of high tannin sorghum brans and muscadine seeds and skins on the progression of diabetic nephropathy in experimental animals. The outcome of this research approach is to incorporate high antioxidant foods, such as specialty sorghum brans and muscadine products into functional foods and nutraceuticals in an attempt to prevent diabetic complications.

According to the American Diabetes Association, this disease and its complications affected approximately 21 million Americans – or 7% of the population. It is the third

leading cause of death in the United States, only after heart disease and cancer. Economically speaking, the disease accounts for nearly $132 billion spent in both direct and indirect expenditures. Globally, the statistics are staggering. Diabetes and diabetic complication inflict a significant cost burden to humanity. Eliminating or reducing the health problems caused by diabetes through nutritional intervention could significantly improve the quality of life for people with diabetes while simultaneously potentially reducing expenditures for health care services and increasing productivity in the global economies.