

DEVELOPMENT OF NUTRACEUTICALS AND FUNCTIONAL FOODS:  
ELDERBERRY (*SAMBUCUS NIGRA*), MUSCADINE (*VITUS ROTUNDIFOLIA*), AND  
CRAFT BEER

by

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(Under the Direction of Phillip Greenspan)

ABSTRACT

Nutraceuticals and functional foods are both part of complementary and alternative medicine. Nutraceuticals are natural products that may manage disease symptoms, whereas functional foods provide benefit when included in the diet, due to antioxidants and phenolics. Study of craft beer showed porters and stouts to have higher phenolic and antioxidant content than other beers. When the antioxidant content was determined 12 and 24 months after time of purchase, that content decreased significantly in beers higher in hops, likely due to degradation of antioxidants in hop plants. These data indicate craft beer may represent a functional beverage in the American diet. Elderberry has been used for influenza treatment and prevention, effects attributed the phenolics myricetin and quercetin. Both myricetin and quercetin are contained in muscadine, a grape that has been shown to benefit human health. Our research shows elderberry and muscadine decrease cytotoxicity in cells infected with influenza strains, including a Tamiflu<sup>®</sup>-resistant strain. This activity occurred at various time points and

concentrations of elderberry and muscadine extracts. Craft beer, namely American pale ale and stout, also decreased cytotoxicity in Tamiflu<sup>®</sup>-resistant influenza at higher concentrations. Thus, elderberry and muscadine may represent nutraceutical agents for the treatment and prevention of influenza, whereas craft beer may prevent influenza infection as a functional beverage. Craft beer also had activity against protein glycation, a process responsible for the development of diabetic complications. All beers, particularly porter and stout, significantly inhibited protein glycation based on volume, and most beers inhibited glycation based on phenolic content. A commercial American domestic beer failed to inhibit glycation; all beers significantly inhibited protein glycation compared to the commercial American beer based on antioxidant content. A porter and Imperial India pale ale also inhibited dicarbonyl formation, a highly reactive compound formed in the process of protein glycation. Taken together, these results support the use of elderberry and introduce muscadine as a nutraceutical agent for the treatment and prevention of influenza, and suggest craft beer as a functional beverage for the delivery of phenolics to prevent influenza infection and diabetic complications.

**INDEX WORDS:** Nutraceuticals, Functional foods, Elderberry, Muscadine, Craft beer, Antioxidants, Phenolics, Influenza, Protein glycation

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## DEDICATION

This dissertation is dedicated to my family, particularly my father, Luther Elrod, for initiating my interest in this field, and to my husband, Erik Hofmeister, for his support in continuing it.

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## CHAPTER ONE

### LITERATURE REVIEW

Complementary and alternative medicine (commonly abbreviated CAM) involves the use of therapies not associated with pharmaceutical intervention. This may include chiropractic care, acupuncture, and other methods. Central to this research are nutraceuticals and functional foods, two related but distinct approaches to non-pharmaceutical treatments.

The term "nutraceutical" was defined in 1989 by Stephen DeFelice as " any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease."(1, 2) Functional foods are known as foods that confer some medical benefit beyond their inherent nutritional value.(3) Functional foods may generally be thought of as an extension of a healthful diet, whereas nutraceuticals tend to be more supplemental in nature.

Many modern pharmaceutical medications have origins in traditional uses of plants; aspirin, digoxin, and Taxol<sup>®</sup> are all well-known pharmaceuticals derived from willow bark, foxglove, and the Pacific yew tree, respectively. (4-6) Traditional use of plant therapies has provided rationale for development of medication.(7) As such, research into nutraceuticals may lead to development of a formulation of natural products as prevention or treatment of medical conditions, as well as compounds that may be exploited for pharmaceutical development. Compounds contained in plants, collectively referred to as phytochemicals, are thus the target of research into plant-based therapy,

either as part of complementary and alternative medicine or as the basis for pharmaceutical development.

Phytochemicals are defined as compounds with virtually no role in photosynthesis but that are still contained in high concentrations in plants. Many are associated with benefit to human health, but in contrast to vitamins, they have no effect on immediate or short-term function. Rather, the benefits from phytochemicals tend to be in preserving general good health and protecting against more chronic conditions.(8)

The rationale for the use of nutraceuticals and functional foods is rooted in the presence of bioactive compounds in plants, including antioxidant, anti-inflammatory, and antiviral compounds.(9-11) Plants have evolved these compounds as a means to defend against predation, disease, and to repel or attract insects. Humans have long used plants for their medicinal properties, exploiting these mechanisms for benefit against disease or microorganisms. It is generally accepted that some of these compounds will confer health benefits to patients consuming the foods or supplements.(12) Many nutraceutical supplements may be used in addition to or instead of pharmaceutical therapies, as suggested by the term "complementary and alternative medicine."

Research into nutraceuticals and functional foods often involves the use of whole foods, but may also include research into individual compounds within the food that may contribute to medical benefit. Understanding of the types of compounds contained within medicinal foods can lead to expansion of research into similar foods or nutraceuticals. Many phytochemicals contained within plants have antioxidant function, which is related to chemical structure.

Because of the wide-ranging implications of chronic inflammation in humans, many of the health benefits derived from consumption of plant-based compounds may be attributed to antioxidants and related compounds. Thus, the presence of antioxidants is an excellent starting point for assessing whether a plant or plant derivative may have use as a nutraceutical or functional food.

### **Antioxidants as a Rationale for Nutraceuticals and Functional Foods**

Any discussion of the utilization of antioxidants in human health must begin with an understanding of endogenous production of free radicals and the human body's mechanism for dealing with such. Because of the chemistry of the oxygen atom, the  $O_2$  molecule preferentially accepts single electrons sequentially in the process of reduction. These sequential reduction reactions yield  $\bullet O_2^-$  and hydrogen peroxide ( $H_2O_2$ ). Both these molecules are highly reactive individually, and may combine to generate the highly reactive  $\bullet OH$  hydroxy radical. Electron transport within the mitochondria is responsible for much of this generation of  $\bullet O_2^-$  and  $H_2O_2$ . Indeed, it was this generation of reactive oxygen species by mitochondria that furthered the theories associated with free radicals and cell damage and aging. The observation that species with higher metabolic rates show a faster aging process is linked to the generation of free radicals by mitochondrial respiration.(13)

Though mitochondria appear to be the major source of endogenous free radical production, other normal chemical reactions in humans also appear to produce free radicals. Peroxidation of fatty acids generates  $H_2O_2$ , and the P450 metabolism of xenobiotic compounds may cause chronic regeneration of  $\bullet O_2^-$ . Furthermore, inflammatory processes induce an immune response involving various free radicals. As

such, chronic inflammation may cause a high burden of free radicals in the body. Additionally, tissue-specific reactions may yield free radicals and part of normal physiology.(13)

It should be clear that, while the human body generates free radicals as part of normal functions, various means of coping with these compounds also exist. Superoxide dismutase is a highly conserved enzyme, implying that most animals have developed various means of generating free radicals and also preventing free radicals from doing an excessive amount of harm to the body. Other endogenous enzymes also exist to help remove these normally-generated free radicals. Thus it appears that free radical generation and scavenging are important parts of most metabolic systems, including that of humans.(13)

Phytochemicals acting as antioxidants contain a benzene ring and at least hydroxy group, allowing for reaction with the free radical and stabilization of the charge by the benzene ring. Thus, a higher number of hydroxy groups on the benzene ring allows for greater antioxidant power of the phytochemical. Compounds of this type are known as polyphenols, due to the presence of at least one phenol group.(14) Many of the benefits associated with the nutraceuticals and functional foods studied in this research may be attributed to the presence of antioxidant compounds in said medicinal foods.

### **Antioxidants in Diet**

Various studies have established the link between consumption for healthful foods and longevity, and the benefits of a diet high in fruits and vegetables are now accepted phenomena. While it is recommended that people consume 5 portions of fruits and vegetables daily, particular fruits or vegetables tend not to be recommended over others;

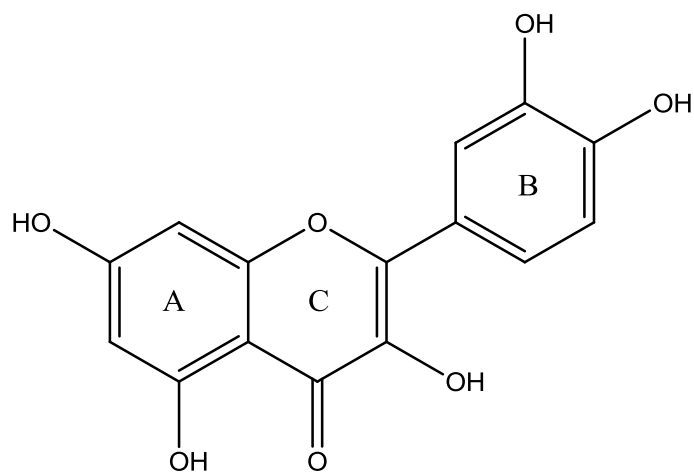
rather, the emphasis is on consuming a wide range of these foods in order to obtain a wide range of phytochemicals.(8) Given the implication of free radicals as detrimental to human health, and the high content of antioxidants in fruits and vegetables, it can be inferred that antioxidants may be largely responsible for these benefits. However, supplementation with antioxidants found in these foods does not confer the same benefits seen with consumption of the whole foods. Various theories have been put forth about this differential between isolated antioxidants and the whole foods, including synergy between various compounds in the whole food, and contribution of non-antioxidant phytochemicals. More recent data, however, suggests that the consumption of antioxidants in foods may confer benefits other than free radical scavenging, that these compounds are affecting cell pathways in order to prevent cancer, cardiovascular issues, etc.(8)

### **Benefits of Polyphenols**

As previously mentioned, a significant category of antioxidants are polyphenols. Polyphenols include phenolic acids, hydroxycinnamic acids, xanthenes, stilbenes, and flavonoids.(8) Flavonoids will feature largely in discussion of this research, but ferulic acid, a hydroxycinnamate, will also be discussed.

Polyphenols are available in many parts of the diet. While fruits and vegetables are well-known as having benefit to the human diet, they are a less significant source of polyphenols than beverages such as coffee, tea, and red wine, due not just to the amounts of polyphenols in these foods but also to the amounts in which these foods are consumed.(8)

Flavonoids are ubiquitous in plants, and as such, may contribute significantly to the antioxidant protection associated with fruit and vegetable consumption. Based on their structure, flavonoids may be subdivided further into flavanols, flavanones, flavonols, flavones, anthocyanidins, and isoflavanones. As mentioned, the structure of the phenol will determine the degree of its antioxidant power. Studies have shown that quercetin is a highly effective antioxidant due to two hydroxy groups in the 3 and 5 position of the A-ring, two ortho-hydroxy groups in its B-ring, and a 2-3 double bond and keto group in the 4 position of the C-ring, as shown below. Each of these structure characteristics has been shown to allow for effective free radical scavenging due to an increased capacity to stabilize the radical form after reaction with a free radical. Quercetin has been shown to prevent oxidation of low-density lipoproteins, giving it significant cardioprotective effects. While research into the bioavailability and metabolism of consumed flavonoids and other antioxidants is ongoing, some studies have found that metabolites of quercetin and other compounds are themselves antioxidants, thus creating the potential for sustained antioxidant activity even after the metabolism of the compound.(9)



**FIGURE 1.1 Quercetin**

The majority of flavonoids exist naturally as glycosides. Quercetin in particular exists primarily in an O-glycoside form, as do many other flavonoids. The content of flavonoid compounds will vary not only by food but by season and variety; that is, different flavonoid profiles may be seen in similar foods according to when and where they are harvested. Food processing may also increase or decrease flavonoids and other phenols.(8)

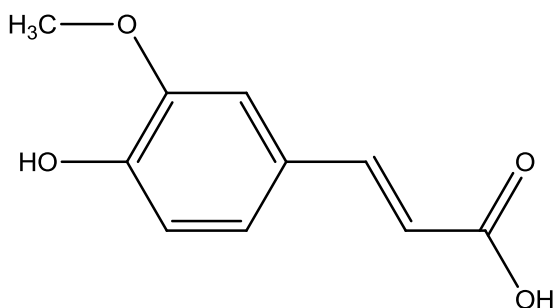
Flavonoids in particular have been studied for their beneficial effects against inflammation, cancer, and viruses.(8, 15) Most flavonoids are absorbed from the small intestine, where the glycoside moiety may be released by hydrolysis via lactase phloridizin hydrolase in the brush border of intestinal epithelial cells. The aglycone may then diffuse into epithelial cells and undergo sulfation, glucuronidation, or methylation by various enzymes. Upon being absorbed into the blood stream, the flavonoid metabolites may undergo phase II metabolism. Thus, one flavonoid may have a wide range of metabolites in the body following absorption, though two or three metabolites tend to dominate; quercetin has up to 20 metabolites.(8)

Quercetin and myricetin, in addition to being major flavonoids widely available from the diet, have been shown to have great benefit in anti-inflammatory, anti-proliferative and other health effects. Quercetin is contained in significant quantities in berries and other fruits, including elderberry and muscadine.(16, 17) Beyond its anti-inflammatory effects, quercetin has been shown to have benefits against platelet aggregation, hyperlipidemia, and diabetes.(18, 19) Clearly, quercetin may contribute significantly to the benefits associated with consuming berries and other fruits containing

this flavonoid. Additionally, it has been well-established that quercetin and related glycosides are well-absorbed into the body.(8)

Ferulic acid is a phenolic acid (not a flavonoid) contained in a variety of foods, including roasted coffee, barley and other grains, artichokes, grapefruit, oranges, and bananas.(20) Ferulic acid was discovered in the resin of *Ferula foetida*, a plant similar to fennel.(21) Studies of the Mediterranean diet have estimated daily consumption of ferulic acid to be approximately 150 to 250 mg/day. As with many phenolic compounds, ferulic acid is available in foods largely as covalent conjugates. Ferulic acid and its major conjugate 5-O-feruloyl-L-arabinofuranose are not degraded by stomach acid, instead being absorbed in the colon. The conjugates are cleaved by cinnamoyl esterase, xylanase and FA esterase, with ferulic acid then being absorbed primarily by passive diffusion and to a much lesser extent by the monocarboxylic acid transporter. Absorption occurs quickly, with the  $T_{max}$  (i.e. time to maximum concentration) less than 30 minutes. The  $C_{max}$  (maximum concentration) is decreased and the  $T_{max}$  and half-life increased with ferulic acid mono- and disaccharides. Metabolism is carried out by UDP-glucuronosyltransferases 1A1 and 2B7 (though this does not appear to significantly affect metabolism of co-administered drugs). Ferulic acid metabolites are thus glucuronide and sulfoglucuronide. Ferulic acid and its metabolites are renally excreted; again, conjugation affects this parameter, slowing excretion by 15-fold. As with other phenolics, the antioxidant effects of ferulic acid are due to its structure: ferulic acid contains a 3-methoxy group and a 4-hydroxyl group, both of which help to stabilize the radical intermediate generated by interaction with free radicals. These groups may also terminate the free radical pathway. A carboxylic acid group adjacent to a carbon-carbon

double bond also helps stabilize the radical intermediate and provides an additional point of interaction with free radicals.(20)



**FIGURE 1.2 Ferulic Acid**

Despite its potent antioxidant effects, various studies have shown ferulic acid to have significant non-antioxidant effects that also act to protect cells. Many of its beneficial effects have been associated with brain health; it has been shown to inhibit lipid peroxidation, cell death due to peroxy compounds, and decrease amyloid-beta protein induced epithelial and intrinsic nitric oxide synthase in hippocampal cells. It has also been shown to prevent intrinsic nitric oxide synthase induction associated with cerebral ischemia. In other areas of the body, it has been shown to inhibit liver toxicity due to carbon tetrachloride and increase superoxide dismutase and catalase (endogenous antioxidants) in the myocardium. Other cardiovascular effects include hypolipidemic and anti-atherogenic effects and anti-hypertensive effects comparable to the ACE inhibitor captopril. It has also been shown to improve cardiac symptoms in patients unable to take pharmaceutical agents. It has a wide range of protective benefits in cancer, including decreasing toxicity associated with cancer treatments.(20) Ferulic acid has been shown to inhibit some viruses, including Epstein-Barr and HIV. (22-24) Limited studies have also shown it to inhibit influenza.(25)

In diabetes, ferulic acid shows various mechanisms of protections, including increasing superoxide dismutase and catalase in the pancreas, decreasing blood glucose and increasing insulin levels and inhibiting nephropathy. It also shows synergy with metformin and thiazolidinediones; it also decreases adverse effects associated with these drugs.(20)

### **Methods of Phenolic/Antioxidant Analysis**

Clearly, any research into the amount of antioxidants and phenols contained in foods and beverages can establish a potential role for said foods and beverages as a part of a disease-preventative diet. Our laboratory uses two major assays for establishing the amount of phenols and antioxidants food products: the Folin-Ciocalteu method(26) for determining total phenolic content and the Ferric-Reducing Antioxidant Power (FRAP) method(27) for determining antioxidant content. These assays have been widely used in nutraceutical research and have been generally accepted as viable methods for determining these parameters regarding food products. It has also been established that there is good correlation between the TPC and FRAP content of foods.(28)

### **Craft Beer as a Functional Food**

A beverage with major potential to be included in a functional diet is craft beer, due to popularity of beer in general and the quantity in which it tends to be consumed. Craft beer is defined as beer from any brewery producing less than 6,000,000 barrels a year.(29) Craft beer has been gaining in popularity in recent years, with consumption of craft beer increasing even as overall beer consumption decreases.(30) Beer contains hops and barley, plants which have been shown to contain

phytochemicals beneficial to health.(31, 32) The roasting of barley to create malt increases the ferulic acid content.(33) Additionally, craft beer also contains added ingredients, including coffee, fruit, and spices, all of which can increase phenolic/antioxidant content.(34-36) Craft beers contain a wider range of beer styles than commercial American beer, and also have a smaller consumer base due to the size of their production. As such, there is greater potential for significant phytochemical content than commercial beer. In recent years, beer has come to be recognized for its health benefits; one study found beer to have similar benefits to that of red wine.(37) In the Mediterranean diet, beer was one of the greatest beverage sources of antioxidants.(38)

A few studies have been done to establish craft beer as a source of phenolics and antioxidants, but very limited studies exist on the variation of phenolic and antioxidant content according to beer style.(39-42) All these studies involved analysis within a few days of purchase. A niche therefore exists for research into which major craft beer styles may afford a higher phenolic/antioxidant delivery to the diet, thus allowing consumers to select beer with a greater potential to contribute to a functional diet.

### **Nutraceuticals/Functional Foods and Influenza A**

As mentioned above, phytochemicals have been shown to have antiviral effects. While some compounds may act more to preserve cell health than to directly inhibit the virus, there has been research conducted showing that phytochemicals may have the capacity to directly affect viral infection. This section will elucidate the need for nutraceutical intervention in influenza, the current state of research, and the results of our recent work into this area.

Every year, strains of the influenza A virus account for millions of infections and thousands of deaths throughout the world.(43) In an attempt to prevent such infection, vaccines for the virus are administered in many populations. However, these vaccines impart immunity to certain strains of the virus, but not necessarily to the strains that will be of concern during a given season. The strains are updated each year, and certain vaccines have not adequately predicted the strains that will be of concern that season. Furthermore, issues with availability of the influenza vaccine have arisen.(44) Therefore, through a lack of immunization or lack of vaccine effectiveness, numerous patients contract the influenza A virus and must receive treatment.

The influenza A virus is an RNA virus with three surface proteins of pharmacologic relevance: hemagglutinin, neuraminidase, and matrix protein.

Hemagglutinin is responsible for the binding and entry of the influenza virus to host cells, whereas neuraminidase is responsible for the release of the virus from the cell to allow for further infection of host cells.(45) The matrix protein is involved in the transport of hydrogen ions, allowing for the change in pH, which is necessary for the uncoating of the virus and the invasion of host cells.(46) Currently, the treatment options for influenza involve inhibition of neuraminidase and the M2 matrix protein. No hemagglutinin inhibitors are available as pharmaceutical agents, though such agents are under investigation.(47)

The M2 inhibitors currently available are amantadine (Symmetrel<sup>®</sup>) and rimantadine (Flumadine<sup>®</sup>), in the drug class known as adamantanes. These medications were developed for the prophylaxis and the treatment of influenza viruses. Although available in the more affordable generic form, the use of these medications may be

limited due to issues of resistance with many influenza strains, including the recent H1N1 pandemic virus, and significant adverse effects. Adamantanes are associated with adverse effects in the central nervous system, especially amantadine, in which such extreme adverse effects as suicides have been reported.(48, 49) Furthermore, the resistance of influenza virus strains to adamantanes has increased since 2003, to the point that these medications are no longer recommended for influenza prophylaxis or treatment.(50)

### **Anti-Influenza Medications: Neuraminidase Inhibitors**

Neuraminidase inhibitors are oseltamivir (Tamiflu<sup>®</sup>) and zanamivir (Relenza<sup>®</sup>). (51) Neither of these medications have a generic form available, making them cost-prohibitive for certain patient populations. Furthermore, both medications are limited in their use by side effects, the most alarming of which are neurologic issues in Tamiflu<sup>®</sup> and brochospasm in Relenza<sup>®</sup>. (52, 53) This issue with Relenza<sup>®</sup> is of concern due to the fact that brochospasm occurs most frequently in patients with asthma, who are at higher risk of developing the influenza virus.(54) This effectively limits patients with asthma to Tamiflu<sup>®</sup> or amantadine for treatment of influenza. This was a special concern with the recent H1N1 pandemic, in which amantadine was found to be ineffective against the virus and reports of resistance to Tamiflu<sup>®</sup> increased as the pandemic progressed.(55)

A review of the currently available pharmaceutical agents for influenza treatment clearly demonstrates a need for more treatment options. The mere fact that only four drugs are available would demonstrate this need, yet issues exist with each individual medication that might theoretically prohibit certain patients from being treated with any antiviral agent for a specific case. These issues, combined with the fact that vaccines are

less than fully reliable for influenza prophylaxis, indicate a need for more treatment options for the influenza virus.

### **Elderberry as an Influenza Nutraceutical**

*Sambucus nigra*, or black elder, is a tree found in the floodplains or rich soil areas of North America, Europe, and parts of the Middle East.(56) Studies have suggested that elder products, usually elderberry, might have health benefits in constipation, diabetes, cancer, inflammation, and in limiting the pathogenicity of methicillin-resistant *Staphylococcus aureus*.(57-61) More research focuses on the potential antiviral, particularly anti-influenzal, effects of *Sambucus nigra* than for any one of these other benefits, although the results of such studies are far from conclusive.(16, 62-64)

Although the mechanism of these effects is currently unknown, hemagglutinin and/or neuraminidase inhibition is supported by certain studies.(16, 62) Furthermore, use of elderberry products in humans suggests such products might be a safe and effective alternative to current pharmaceutical agents.(63, 64) *Sambucus nigra* contains significant amounts of flavonoids beneficial to humans, including cyanidin, quercetin, myricetin, and kaempferol, of which quercetin- and myricetin-based compounds have been implicated in influenza inhibition.(16, 65-67)

In a 1995 study of the effects of Sambucol<sup>®</sup> elderberry extract in human patients with influenza B (see *Sambucus nigra: Human Studies*), the authors also conducted *in vitro* studies of different strains of influenzas A and B.(64) In this study, Manin-Darby canine kidney cells were inoculated with two separate isolates each of H3N2 and H1N1 influenza A viruses as well as three separate isolates of influenza B. The viruses were titrated on the cell cultures to determine the virus dilution necessary to induce complete

cytopathic effect (CPE). Virus concentrations used for the *in vitro* studies were at the concentration required to induce CPE or higher. In order to determine the effect of elderberry on virus inhibition, the authors incubated various concentrations of Sambucol D<sup>®</sup> with the influenza strains. The strains incubated with Sambucol D<sup>®</sup> were then used to infect the MDCK cells in triplicate, with each test being performed four separate times. The plates were stained in order to determine cytopathic effect. The authors report a dose-dependent inhibition of two strains of H3N2, three strains of influenza B, and several strains of H1N1. However, the authors neglect to present statistical analysis of their *in vitro* data, making interpretation of these results difficult. Viruses were also mixed with 1% sheep red blood cells in order to test the hemagglutination properties of elderberry. The authors found a 1:4 dilution of Sambucol D<sup>®</sup> inhibited hemagglutination of one strain each of H3N2 and H1N1 influenza A subtypes and two influenza B strains after one hour. No specific data of the hemagglutination studies were presented by the authors, again impeding interpretation of the authors' results.(64)

A more recent study examined the *in vitro* effect of elderberry extract on the H1N1 virus exclusively. This 2009 study makes use of a technique known as Direct Analysis in Real Time Mass Spectrometry (DART TOF-MS) in order to identify the specific compounds that account for the purported antiviral activity of elderberry. To determine this antiviral activity, the authors infected MDCK cells with the H1N1 virus and dilutions of elderberry. This study found the extract to have an IC<sub>50</sub> of 252 ± 34 µg/mL and an IC<sub>100</sub> of 1000 µg/mL. Notably, the authors did not present the specific concentrations of elderberry extract used, although a dose-dependent curve is included.

A later assay, in which elderberry concentrations of 252 and 1108 µg/mL were incubated with H1N1 virions, yielded 58% and 95% inhibition of viral activity.(16)

Because natural products are not regulated by the Food and Drug Administration, they may be administered to humans without FDA approval. Because of this, despite the paucity of reliable *in vitro* and *in vivo* data, two studies have been conducted into the effects of elderberry in humans who contracted seasonal influenza. Both studies used Sambucol<sup>®</sup>, the commercial elderberry extract. In each case, the total amount of flavonoids (although not the amount of each flavonoid) was standardized prior to administration. In 1995, Sambucol<sup>®</sup> was studied in patients with influenza B, finding both improvement and complete cure to be achieved in about two to three days with Sambucol<sup>®</sup>, versus about six days with placebo. This double blind study was performed in a limited capacity, among only 27 patients (thirteen of the original 40 patients were excluded for various reasons). Interestingly, however, unlike many early human studies of antiviral medication, this study includes children: the patients ranged in age from five to fifty-six years of age. The patient population also included twice as many female patients as male (eighteen females to nine males), which is somewhat unusual for human studies. The study involved the administration of four tablespoons of Sambucol<sup>®</sup> daily to adults and two tablespoons daily for children for three days. The spacing/frequency of the doses was not specified (i.e. whether the adult dosage was all four tablespoons at once or one tablespoon every 6 hours, etc.); furthermore, the ages of children versus adults were not defined. This is an interesting omission, given the age range of the patients. That is, if a “child” was defined as a patient younger than twelve, and the patient population included patients clustered around that age, it would be interesting to see how

a patient of twelve or thirteen compared to a patient of ten or eleven, being administered twice the dose of Sambucol<sup>®</sup>. As no adverse effects were reported, one may assume no complications might have resulted in young adults receiving the higher dose, but it would be useful to know if older children showed a lower response to the lower (i.e. children's) dose. Other drawbacks to this study include ambiguous definitions of endpoints: although serological studies were performed on each patient before and after treatment, the terms "improvement" and "complete cure" are used without defining these terms. The authors state "[f]eelings of improvement and complete cure were...noted" by the patients, suggesting that the conclusions that Sambucol<sup>®</sup> improves symptoms and induces an earlier cure compared to placebo is dependent upon the patient's perception rather than achievement of objective, previously established, endpoints.(64)

In a 2004 Norwegian study, researchers found Sambucol<sup>®</sup> to relieve influenza A and B symptoms four days earlier than placebo. This study of 60 patients found those treated with one tablespoon of Sambucol<sup>®</sup> four times a day for five days (compared to three days in the 1995 study) reported an improvement in symptoms significantly earlier than those treated with placebo, as well as a significantly lower use of medications for symptomatic treatment (i.e. nasal spray and antipyretic/analgesic medication). Although no patients reported any adverse effects associated with Sambucol<sup>®</sup>, patients who had been vaccinated against influenza and those with chronic diseases (a target demographic for influenza medication) were excluded from the study. However, this study did use a number scale for improvement of symptoms, thereby indicating more objective endpoints than the 1995 study. The mechanism of symptomatic improvement is also unknown; as

such, it may either be inhibition of viral infection or improvement in immune system response that accounts for these benefits.(68)

### **Elderberry Phytochemicals and Influenza**

As stated previously, a study has identified two elderberry flavonoids to be potentially responsible for the anti-influenza effects of elderberry. Roschek et al.(16) utilized DART TOF-MS to determine the elderberry compounds binding to H1N1 virions. Upon incubation of H1N1 virions with elderberry extract, the bound and unbound components were both subjected to DART TOF-MS. Using this technique, it was determined that the quercetin derivative 5,7,3',4'-tetra-*O*-methylquercetin and the myricetin derivative 5,7-dihydroxy-4-oxo-2-(3,4,5-trihydroxyphenyl)chroman-3-yl-3,4,5-trihydroxycyclohexanecarboxylate were the flavonoids bound to H1N1 virions. Upon determining these flavonoids to be potentially responsible for antiviral activity, the authors synthetically created the quercetin derivative and used the racemic mixture of dihydromyricetin. Dihydromyricetin was used due to free energy studies indicating the ester group of 5, 7-dihydroxy-4-oxo-2-(3, 4, 5-trihydroxyphenyl) chroman-3-yl-3, 4, 5-trihydroxycyclohexanecarboxylate was likely not necessary for antiviral activity. Subsequent to identification of the flavonoids binding to H1N1 virions, the authors used similar techniques to explore the potential mechanism of these flavonoids and the extract. H1N1 virions were incubated with elderberry extract or synthesized flavonoids, with the unbound portion removed; bound and unbound portions were subjected to DART TOF-MS. The flavonoid-bound virions were also used to infect MCDK cells. Using these techniques, the authors found that, upon incubation with the extract, the quercetin derivative bound to the virion at approximately twice the ratio to the myricetin derivative

as was found in the extract (2.9:1 ratio binding the virion; 1.5:1 ratio in extract), thus indicating binding might be specific in nature. DART TOF-MS analysis of synthesized flavonoids in the H1N1 virus indicated these flavonoids do indeed bind to the virions, supporting the proposed mechanism of inhibition of viral entry into cells via hemagglutinin effects. Cell assays using the synthesized flavonoids yielded  $IC_{50}$  values of 0.13  $\mu\text{g}/\text{mL}$  for the quercetin derivative and 2.8  $\mu\text{g}/\text{mL}$  for dihydromyricetin, about 1000 and 100 times lower (i.e. more potent) than that of the extract, respectively. The quercetin derivative therefore shows an  $IC_{50}$  similar to that of Tamiflu<sup>®</sup>, one of the current treatments recommended for H1N1 infection. (Recall that Tamiflu<sup>®</sup> has been associated with resistance issues in H1N1.) The authors also report that the  $IC_{50}$  of amantadine falls between the quercetin and myricetin derivatives. Free energy analysis of the quercetin and myricetin derivatives indicates that these flavonoids fit the spatial constraints for hemagglutinin binding, potentially binding to the mannose-rich domain of this protein.(16)

The aglycone form of quercetin (i.e. without carbohydrate groups) has not been studied as extensively as its glycone forms. Indeed, *in vitro* data is limited on the aglycone, confined to a study on the effect of 10 nM and 100 nM quercetin in peripheral blood mononuclear cells. In combination with shikimic acid (a compound used in the synthesis of Tamiflu<sup>®</sup>), these concentrations of quercetin induced significant increases in IL-8 and IL-6, indicating a potential immunomodulatory mechanism in influenza inhibition, as well as a possible role as an adjunct to Tamiflu<sup>®</sup> therapy.(69)

There are several conditions that might increase the likelihood of viral infection, including exercise. Davis, et al. found that mice subjected to three consecutive days of

treadmill running before virus inoculation were significantly more likely to develop infection than control, as well as significantly more likely to die from the infection. The exercise group also showed significantly shorter time to infection and time to mortality compared to the control. Administration of 12.5 mg/kg quercetin not only increased the time to infection and time to mortality of the exercise group, it decreased the percent of mice infected, percent mortality, and symptom severity compared to placebo.

Quercetin's effects were higher in the exercise group than the placebo group, bringing the data for the exercise group close to that of the placebo-fed mice who were not subjected to exercise. Quercetin also lessened the disease severity for mice in the non-exercise group.(70)

This flavonoid has also been studied for protective effects against oxidative damage associated with the virus, thereby potentially providing an additional mechanism for benefits in influenza. An *in vivo* study of mice infected with the H3N2 virus showed 1 mg/day quercetin for five days to increase catalase and decrease glutathione and superoxide dismutase concentrations in the lung.(71)

Another study examining the potential benefits of quercetin in influenza therapy beyond viral inhibition found quercetin at 20 mg/kg body weight prevented an increase in concentrations of lipid peroxidation products and hepatic oxidative damage associated with influenza infection. This same concentration also prevented radical oxygen species and neutrophil activation in the lungs. The authors also found that this dose of quercetin suppressed cytochrome P450 in healthy animals, though it maintained P450 levels in animals infected with influenza. Quercetin also suppressed other drug-metabolizing enzymes, raising concerns regarding drug interactions with these flavonoids.(72)

In early 2009, a study published in the European Journal of Pharmaceutical Science all but predicted the H1N1 pandemic that would occur later that year. This study cited the fact that H1N1 isolates had shown resistance to oseltamivir in the 2007-2008 influenza season as an impetus for further exploring flavonoids, including quercetin 3-rhamnoside, as anti-influenzals. Quercetin 3-rhamnoside showed activity against H1N1 strain A/WS/33 at 100 µg/mL (86% viral activity reduction) and 10 µg/mL (66% viral activity reduction). These results compared favorably to the oseltamivir control, which showed moderate antiviral activity against 58% of the virus at 100 µg/mL and 49% of the virus at 10 µg/mL. Furthermore, in studying the cytopathic effect associated with the virus, 10 µg/mL of the flavonoid inhibited virus-related cytotoxicity to a greater extent than an equal concentration of oseltamivir.(15)

Another study looked at the effects of quercetin-3-glucoside, also known as isoquercetin, along with several other polyphenols both *in vitro* and *in vivo*. In *in vitro* studies using MCDK cells, isoquercetin showed the lowest effective dose and highest toxic dose of all phenolic compounds studied (including other glycosylated forms of quercetin), thus indicating a therapeutic index wider than the other compounds.

Isoquercetin was also effective at preventing the development of drug resistance both alone and in combination with amantadine and oseltamivir at a concentration of 2 µM.

Two and 10 mg/kg/day isoquercetin were also associated with improvement in virus titers and lung pathology in a mouse model.(73)

Fan, et al. studied the effects of 3 and 6 mg/kg quercetin-3-O-β-D-glucuronide on a mouse model of influenza A. Administered daily for 4 days, this therapy resulted in decreased lung edema, with inhibitory effects comparable to that of ribavirin (an antiviral

indicated for Hepatitis C, not currently approved for influenza therapy in the United States).(74, 75)

An *in vivo* study of the effects of quercetin-3-rhamnoside found 6.25 mg/kg daily in influenza A-infected mice decreased weight loss and mortality to an extent similar to that seen with oseltamivir. Virus titers in the lung however, were about two times lower than those seen in the oseltamivir group.(15)

Myricetin, like quercetin, is a type of flavonoid known as a flavonol. Indeed, myricetin and quercetin are structurally quite similar, indicating potential similarity in the biological effects of these flavonols. As would be expected, myricetin does indeed share many beneficial effects with quercetin, including benefits against cancer, hyperlipidemia, platelet aggregation, diabetes, and bacterial infection.(76) Oddly, however, no studies have been conducted on the anti-influenza effects of myricetin, and very few have been conducted on its antiviral effects. This discrepancy is perhaps due to fewer studies conducted with myricetin than quercetin overall.

Anthocyanidins are another class of flavonoids associated with numerous benefits in fruits. These compounds are responsible for the blue and red colors of many fruits and as such are especially high in fruits such as berries. The glycosylated forms of these flavonoids are known as anthocyanins. Limited data into this class of flavonoids suggests that they may act to prevent influenza infection *in vitro*, perhaps via inhibition of viral adhesion and release from cells.(77, 78)

Although the data is much more limited than quercetin, studies on the stilbene resveratrol in influenza have shown some significant advances in the elucidation of the mechanism of influenza inhibition. Palamara, et al. found dose-dependent inhibition of

an H1N1 virus at concentrations of 10 to 40  $\mu\text{g}/\text{mL}$  in MDCK cells, which seemed to be due to inhibition of protein kinase C, a signaling molecule activated during viral infection. This inhibition ultimately resulted in the decreased expression of viral proteins. In mice, one week treatment of 1 mg/kg/day of resveratrol significantly decreased mortality and viral titers over placebo.(79)

Like quercetin, unmodified resveratrol may not be the most potent form of this polyphenol. Huang, et al. studied a tetramer of resveratrol known as (+)-vitisin A, finding it to be effective at inhibiting virus-mediated Akt and STAT phosphorylation. This phosphorylation ultimately causes the release of regulated on activation, normal T cell expressed and secreted (RANTES) chemokines from infected lung epithelial cells. The  $\text{EC}_{50}$  associated with RANTES secretion was much lower for (+)-vitisin A than for resveratrol (0.27  $\mu\text{M}$  vs. 28.37  $\mu\text{M}$ ). (80) These data present two different potential mechanisms for resveratrol's benefits in influenza: direct effects of the unmodified stilbene on viral protein expression and effect of the tetramer on virus-associated lung inflammation processes.

### **Muscadine and Influenza**

Given the evidence presented above regarding the flavonoids contained in elderberry, examination of the composition of other fruits allow for speculation that those fruits may also exhibit anti-influenza effects. Some fruits, such as cranberry, have been studied for this indication and show some promise.(81) For other fruits, such as muscadine, research of this type would be novel contributions to the field of influenza therapy.

Muscadine is a grape indigenous to the southeastern United States that has been studied extensively in our laboratory for medicinal benefits.(82-86) Like *Sambucus nigra*, muscadine (*Vitis rotundifolia*) has been studied for beneficial effects in inflammation(84, 86-88), diabetes(82, 89), and cancer(85, 90-93); additionally, muscadine has been suggested to help alleviate Alzheimer's disease(94) and infection due to several different microbes(95-100). Notably, however, the antiviral effects of muscadine have not been studied. Given the significant content of quercetin and myricetin in muscadine, as well as similar potential benefits, it is reasonable to suspect that muscadine might have anti-influenza properties similar to those of elderberry.(101, 102)

### **Craft Beer and Influenza**

Hops, one of the major ingredients in beer, contains chalcones, flavonoids that have been shown to have various health benefits.(103-105) Chalcones have been shown to have anti-influenza activity, not just via neuraminidase inhibition, but due to cytoprotection by inhibition of caspases and interleukins.(106-108) While none of these studies involve chalcones derived from hops, it is reasonable to suppose that hop-containing foods, including craft beer, may show anti-influenza activity. As mentioned above, very limited work has been done on ferulic acid, a component of barley, in influenza(25), further supporting the possibility of craft beer showing benefit with influenza.

### **Protein Glycation**

Complications associated with Type 2 diabetes include retinopathy, nephropathy, and peripheral neuropathy. These complications are associated with the chronic

hyperglycemia seen with Type 2 diabetes. Hyperglycemia allows for the reaction of blood glucose with endogenous proteins to form advanced glycation end products (AGE), which cross link with proteins and result in the above complications. Hyperglycemia is also associated with oxidative stress; as such, polyphenols have been shown to inhibit protein glycation and to preserve cell viability *in vitro* and *in vivo*.(109, 110)

Glycation can be defined as non-enzymatic glycosylation. The early stage of protein glycation involves the reaction of glucose with the N-terminal of a protein. This forms a compound known as a Schiff base, which undergoes rearrangement to an Amadori product. These Amadori products degrade to dicarbonyl compounds. This entire process occurs non-enzymatically. The ultimately formed dicarbonyls are more reactive than the parent sugars, and may not only cross link proteins themselves, they may also form additional reactive end-products, thus increasing the potential for complications associated with hyperglycemia. This process occurs in hemoglobin and is the basis for the A1c test in Type 2 diabetes.(109, 111)

Proteins exposed to high levels of glucose cause complications in the body for various reasons, including conformational changes and decreased ligand binding.(109) Clearly, such changes impact proteins' usual activity and impair function.

One of the major proteins glycated is collagen, a protein responsible for elasticity in the body, especially in the joints, arteries, and lungs. Protein glycation has been shown to be responsible for changes in the body as a normal part of aging, but some age-related conditions are increased in diabetic patients.

In addition to its effects on protein, glucose itself acts as oxidant and decreases superoxide dismutase *in vitro*. It also may lead to LDL accumulation and atherosclerosis.

Furthermore, studies on glucose-associated protein changes have shown that oxidation is essential to these processes. Amadori products and dicarbonyls also act as oxidants, further supporting the use of polyphenols to inhibit protein glycation and other adverse diabetic effects.

Muscadine has already been shown to significantly inhibit protein glycation.(82) In this case, gallic acid and catechins were shown to be largely responsible for this inhibition. Commercial beers have been shown to contain these compounds; it is reasonable to believe they may be contained in craft beer as well.(40) Furthermore, compounds related to chalcones (flavonoids in hops) have been shown to inhibit formation of advanced glycation end products, as has ferulic acid.(112-118) Thus, craft beer has the potential to be a functional beverage to inhibit protein glycation and perhaps prevent diabetic complications.

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## CHAPTER TWO

### EFFECT OF STYLE AND TIME ON PHENOLIC AND ANTIOXIDANT CONTENT OF CRAFT BEER

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## **Abstract**

Craft beer typically contains ingredients likely to impart phenolic compounds and other antioxidants to the consumer. At least five different beers from five different styles (American Pale Ale, India Pale Ale, Imperial IPA, Porter, and Stout) were selected and subjected to the Folin-Ciocalteu and ferric-reducing antioxidant power (FRAP) assays in order to determine the total phenolic content (TPC) and antioxidant capacity, respectively. There was a significant relationship between TPC and alcohol content and FRAP and alcohol content, but no relationship between TPC or FRAP concentration and International Bitterness Units. The alcohol-normalized TPC concentration in Stouts was significantly higher than in American Pale Ale, IPA or Imperial IPA, alcohol-normalized FRAP concentration in Porters was significantly higher than in IPA or Imperial IPA. These findings may allow consumers to select beer more likely to impart beneficial compounds to their diets.

## **Introduction**

Craft beer can be defined as beer produced by a brewery that produces less than 6,000,000 barrels a year.(1) Overall beer consumption in the United States is in decline, decreasing 1% in 2011, while craft beer has increased 13% in that same period of time.(2) Beer is generally divided into lager beers, produced by fermentation at lower temperatures with the yeast *Saccharomyces uvarum*, and ale beers, produced by fermentation at higher temperatures with the yeast *Saccharomyces cerevisiae*. The Beer Judge Certification Program recognizes 23 major categories and 80 distinct styles.(3) Styles are defined by their aroma, appearance, flavor, mouthfeel, bitterness, and alcohol content. Generally, beer of a certain style from different brewers will taste more similar than beers of different styles from the same brewer, although some differences may be too subtle for some to detect.

In recent years, beer has come to be recognized for its health benefits; one study found beer to have similar benefits to that of red wine.(4) In the Mediterranean diet, beer was one of the greatest beverage sources of antioxidants.(5) Antioxidants scavenge free radicals in the body, thus preventing oxidative damage to cells, proteins, lipids, and DNA. This is hypothesized to prevent numerous health complications, including cardiovascular disease, diabetes, and cancer.(6-8)

Beer has significant concentrations of polyphenols beneficial to human health. Xanthohumol and related compounds are found exclusively in hops; therefore, beer is virtually the sole source of these chemoprotective flavonoids in the human diet.(9) In addition to hops, beer contains barley, which may be malted into order to generate

enzymes to partially break down sugars. Malted barley or raw barley may be roasted or kilned to create different flavors, such as caramel, chocolate, or coffee.(10)

The effect of beer style on antioxidant properties has been investigated previously. In one study, lager beers were reported to have a lower antioxidant content than ale beers, although this difference may have been due to other variables, such as kilning of the barley.(11) Seven styles of beer have been evaluated for their antioxidant properties.(12) This study was conducted with beers produced in Europe, which may differ from American beers. Moreover, the craft beer phenomenon is most prominent in the United States, while in Europe the movement is only just gaining momentum. The study did not supply the names of the beers analyzed, so it is impossible to say whether they were produced by macrobreweries or craft breweries. That study also did not define how beers were classified according to style, and at least one style was erroneously labeled as “ale”.(12) A third study evaluated the antioxidant properties for four different styles.(13) Again, the exact source of the beer was not described, and the specific style was not defined.

Another limitation of the studies above is that all beers were analyzed within a few days of purchase, often within 24 hours. However, beer purchasers may not consume beer within such a time frame; indeed, some craft beer drinkers may “cellar” beer for a period of time in order to appreciate changes in the beer with time.(119) Thus, if beer consumers are choosing beer styles in order to increase their dietary antioxidants, it is essential that they be aware of whether purchase-to-consumption time might affect the beer’s phenolic/antioxidant content. Furthermore, researchers must be aware of the effect storage has on antioxidant levels, to inform study design.

To study the phenolic and antioxidant content of craft beer and to determine the effects of time on these parameters, we performed the Folin-Ciocalteu and ferric-reducing antioxidant power (FRAP) assays(15, 16) on at least five different beers from five different style both within 24 hours of purchase and 12 and 24 months after purchase.

## **Materials and Methods**

### **Materials**

Folin-Ciocalteu reagent, TPTZ (2, 4, 6-tri[2-pyridyl]-s-triazine, ferrous sulfate heptahydrate, and anhydrous ferric chloride were purchased from Sigma Chemical Company (St. Louis, MO). Beers were purchased from Five Points Bottle Shop in Athens, GA.

### **Phenolic Content and Antioxidant Analysis**

The selected beers were subjected to the Folin-Ciocalteu assay to determine the total phenolic content (TPC) and the ferric-reducing antioxidant power (FRAP) assay to determine the antioxidant capacity. Briefly, for the Folin-Ciocalteu method of determining total phenolic content, 20  $\mu\text{L}$  of beer was combined with 1.58 mL of deionized water, 100  $\mu\text{L}$  of Folin-Ciocalteu reagent, and 300 of  $\mu\text{L}$  sodium carbonate and incubated at room temperature for 45 minutes. Absorbance was measured using spectrophotometer set at 765 nm and results were expressed in gallic acid equivalents per milliliter based on a standard curve.

For the FRAP assay, the reagent was made fresh daily by combining 2.5 mL of 20 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 2.5 mL of 10 mM TPTZ (in 40 mM HCl), and 25 mL of 300 mM acetate buffer. Thirty microliters of beer samples were added to 300  $\mu\text{L}$  reagent, incubated for 5 minutes, then 340  $\mu\text{L}$  deionized water added. Absorbance was measured

at 593 nm and results expressed as mmol Fe(II) equivalents per liter based on FeSO<sub>4</sub>•7H<sub>2</sub>O standard curve. The beers were purchased and analyzed within 24 hours to determine baseline TPC and FRAP values, and again at 12 and 24 months.

### **Statistical Methods**

Normality was determined using the D'Agostino-Pearson method. FRAP and TPC were normalized to percent alcohol, available from each brewery. The results were then compared among groups with a one-way ANOVA with post-hoc testing by Tukey's test for normally distributed data or with a Kruskal-Wallis with post-hoc testing by Dunn's test for non-normally distributed data. The relationship between TPC and FRAP and between TPC, FRAP, percent alcohol, and IBU was determined using linear regression. For the 12 and 24 month assays, values were compared with values obtained at baseline using a paired t-test. Post-hoc testing was performed by Tukey's test for normally distributed data and a Kruskal-Wallis with post-hoc testing by Dunn's test for non-normally distributed data. Significance was set at alpha <0.05.

### **Results**

Based on previous publications of phenol content in beer styles, to detect a 25% difference among beer styles, a minimum number of 5 different beers within each style were required based on a  $\beta$  of 0.20 and an  $\alpha$  of 0.05. We increased the sample size to 9 to increase power.

A range of beers across styles and color range was selected on the basis of perceived popularity of style within the American craft beer culture. This perception was based on trade magazines and the numbers of beers of a style available at local specialty beer stores. Five styles were chosen: American pale ale (APA), India pale ale (IPA),

Imperial IPA (Imp), porter, and stout. Pumpkin beers were included in the 12 and 24 month analysis, but not in the comparison according to style, due to the small sample size available. One white wine and two red wines were also selected for comparison purposes, due to the current perception of wine as an antioxidant-rich beverage.

American pale ale has a low to medium malt flavor (often described as caramel-like) and a medium to medium-high hop character (i.e. bitterness, citrus-like notes, etc.). India pale ale has medium-high to very high hop bitterness, and medium maltiness. Imperial IPA has higher alcohol content than IPA and very high hop bitterness. According to the Brewers Association 2013 Beer Style Guidelines, “The intention of this style of beer is to exhibit the fresh and bright character of hops”.(10) Porter is dark brown to very dark in color, with acceptable roasted malt flavor, but not roasted barley. Stout, a black-colored beer, should contain the flavor of roasted barley. Pumpkin beers must contain pumpkin or winter squash, which should be obvious in the flavor. They may contain the spices often associated with pumpkin flavor, but this is not necessary.(10)

For each chosen style, a list of craft beers available from local specialty beer stores was obtained. *Beer Advocate*(18), a craft beer oriented magazine, was consulted to determine the quality of each potential beer. Beers that scored a B+ or higher were considered for inclusion. The beers with the greatest number of ratings on *Beer Advocate* were included for analysis.

International Bitterness Units (IBU), a measure of hop bitterness in a beer, was available for 7 APAs, 8 IPAs, 7 Imperial IPAs, 6 porters, and 9 stouts. There was a significant relationship between TPC and percent alcohol ( $r^2=0.23$ ,  $p<0.001$ ) and FRAP

and percent alcohol ( $R^2=0.1$ ,  $P<0.03$ ). There was no relationship between TPC or FRAP concentration and IBU. Normalized TPC and FRAP concentrations are presented in Table 2.1. The alcohol-normalized TPC concentration in stouts was significantly higher than in APA, IPA or Imperial IPA ( $P<0.05$ ). The alcohol-normalized FRAP concentration in porters was significantly higher than in IPA or Imperial IPA ( $P<0.05$ ). There was a strong and significant relationship between TPC and FRAP concentrations ( $P<0.0001$ ;  $R^2=0.89$ ).

A white wine (Pinot Grigio) and two red wines (Pinot Noir and Cabernet Sauvignon) were analyzed within 24 hours of purchase for comparison purposes. The non-normalized TPC values showed that all wines were lower in phenolic content than beers; the order of TPC of craft beer was APA, porter, IPA, stout, and Imperial IPA. (Figure 2.1) Non-normalized FRAP values showed that Pinot Grigio was lowest in antioxidant content, followed by APA, IPA, stout, Imperial IPA, porter, Pinot Noir, and Cabernet Sauvignon. (Figure 2.2)

Alcohol-normalized TPC increased significantly for APA between 12 and 24 months, Imperial IPA between baseline and 24 months, and stout between baseline and 24 months and between 12 months and 24 months ( $P<0.05$ ). (Table 2.2) FRAP values decreased significantly for IPA between baseline and 12 months and Imp between baseline and 24 months ( $P<0.05$ ). (Table 2.3)

## **Discussion**

These data support the results from other studies that there is a correlation between the phenolic content and antioxidant capacity of beers and a difference in the phenolic/antioxidant content according to beer type. Barley and hops may both

contribute to the antioxidant content of beer, with hops containing a high level of antioxidants according to studies.(9, 19) In this study, the phenolic/antioxidant content of the porters and stouts were higher than the beer styles with a high concentration of hops (i.e. APA, IPA, Imperial IPA). This supports other findings that barley contributes more significantly to the antioxidant capacity than hops.(11, 12) Beers which used more roasted grains had higher antioxidant content. The other beer styles, higher in hop content, show lower phenolic/antioxidant contents. Because hops are high in prenylated flavonoids, this is a somewhat surprising finding.(6) However, barley processing yields an increase in ferulic acid, which may contribute to an overall increase in phenolic acids and antioxidants.(20) Furthermore, the bitterness from hops is approximated by the IBU value of beer, which is an imprecise measurement of hop content, mostly dictated by isomerized alpha acids. The isomerization of alpha acids changes a six-membered ring to a five-membered ring, which would not be detected by the Folin-Ciocalteu or FRAP methods. As alpha acid isomerization is a function of hop type and duration of boiling during beer production, it is possible that hop processing may alter the antioxidant content of hops by destroying the six-membered ring which is partially responsible for antioxidant activity.(21)

The relationship of phenolic compounds and antioxidants to alcohol content is an interesting finding. It is possible the ingredients that increase alcohol content, specifically the fermentable sugars produced during the mashing stage of brewing, may increase the TPC and FRAP results. Furthermore, a higher alcohol content likely increases the solubility of phenols. A higher alcohol content is obtained by using a larger

volume of malt barley, which has a relatively high phenolic acid and antioxidant content.(20)

For comparison purposes, a major commercial American brand of beer was subjected to the same analysis described above. The TPC and FRAP values of this beer were approximately half or less that of any of the craft beer analyzed when adjusted for alcohol content (data not shown). Our TPC values are higher than those found by Zhao, et al. in commercial beers or by Granato, et al. in lagers and brown ales, but similar to those found in ales, abbey beers, and bock beers by Piazzon, et al.(11, 12, 22) This is consistent with our findings that craft beer tends to have higher phenolic values, and darker beers tend to have more phenols than lighter beers. Craft beer in general, then, may have health benefits not associated with more popular American beers. However, the styles of beer are dramatically different between the major brand and the craft beers analyzed in this study. The major brand beer is a light lager, with less barley and hops as compared with the other styles analyzed in this study.

Also interesting were the results when non-normalized TPC and FRAP values were compared to white and red wine. All three wines were lower in TPC values than craft beer, but craft beer fell between white and red wine in FRAP values. This suggests that craft beer at least compares favorably to red and white wine in phenolic and antioxidant content. Indeed, craft beer appears to contain higher amounts of phenolics. It also indicates that red wine may contain non-phenolic antioxidants. It is important to remember that both TPC and FRAP values are expressed based on volume. Because a standard drink is considered 12 ounces for beer and 5 ounces for wine, normalizing these results to amount per drink shows that wine delivers 30 to 70  $\mu\text{g}$  phenolics per standard

drink, whereas craft beer delivers 200 to 300  $\mu\text{g}$  phenolics per drink. For antioxidant content, each glass of the white wine analyzed delivers approximately 0.2 mmol Fe(II) equivalents per standard drink, craft beer delivers 1.3 to 2 mmol, and red wine delivers 2 to 2.2 mmol. Clearly, the larger volume of craft beer ingested increases the amount of phenolics and antioxidants delivered, exceeding or approximating the amount delivered even from red wine. Studies have estimated that the typical American may consume approximately 1 gram phenolics per day, most of that coming from beverages such as beer and wine.(23) Another study estimated that typical flavonoid consumption by Americans is approximately 190 mg per day, with about 4 mg per day coming from wine consumption.(24) Clearly, this study found much lower values for phenolic content of these beverages, likely due to variation by laboratory and methodology. Regardless, the trends reported here indicate that craft beer can contribute more significantly to the typical American consumption of phenolics and other antioxidants than wine, due to the difference in volume consumed.

The results of the 12 and 24 month analyses were initially confounding: TPC values increased significantly for APA between the 12 and 24 month timepoints, for Imperial IPA between baseline and 24 months, and for stout between baseline and 12 and 24 months. FRAP content, however, decreased significantly for IPA between baseline and 12 months and for Imperial IPA between baseline and 24 months. The TPC values can be understood based on other studies showing increases in phenolic content with storage time.(25, 26) The decrease in FRAP values is unusual in conjunction with an increase in TPC, however, especially in Imperial IPA, in which an increase in TPC is combined with a decrease in FRAP content. Degradation of non-phenolic antioxidants

may account for the decreasing FRAP values, but would not explain the increase in TPC. Because Imperial IPA is a very highly hopped beer, it is possible that xanthohumol and other phenols in hops may undergo molecular rearrangement during storage time.

### **Conclusions**

In summary, these results place craft beer alongside wine as a functional beverage for the delivery of phenolics and antioxidants. They also show that, while antioxidant content may decrease over time, phenolic content may actually increase; thus, it is unlikely that time will have a significant effect on phenolic/antioxidant content. However, darker beers such as porter and stout have higher phenolic/antioxidant content than lighter, or hoppier beer. Darker beers also retain their phenolic/antioxidant content over time. This information may allow beer drinkers to make purchasing and consumption decisions based on potential benefits of craft beer in general and craft beer styles in particular.

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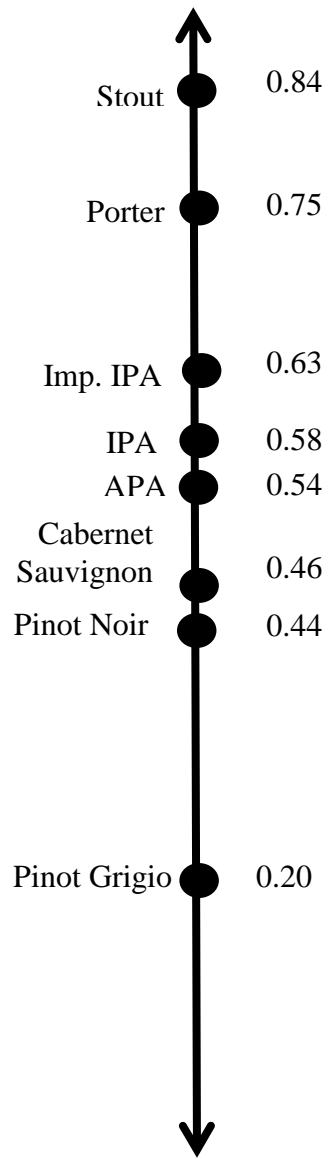
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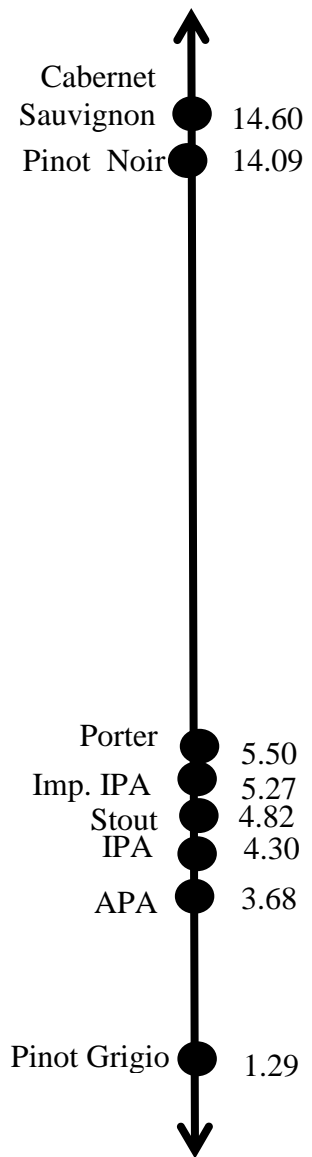
**Table 2.1: The total phenolic content and ferric reducing antioxidant potential of popular craft beer styles**

<b>Beer Style</b>	<b>Normalized TPC</b>	<b>Normalized FRAP</b>	<b>TPC</b>	<b>FRAP</b>	<b>% Alcohol</b>
<b>APA</b>	0.10 ± 0.02	0.67 ± 0.09	0.54 ± 0.11	3.68 ± 0.5	<b>5.5 ± 0.4</b>
<b>IPA</b>	0.09 ± 0.03	0.65 ± 0.08	0.63 ± 0.15	4.30 ± 0.57	<b>6.7 ± 0.6</b>
<b>Imp. IPA</b>	0.10 ± 0.03	0.62 ± 0.14	0.84 ± 0.17	5.27 ± 1.0	<b>8.7 ± 1.3</b>
<b>Porter</b>	0.10 ± 0.02	0.95 ± 0.24#	0.58 ± 0.10	5.50 ± 1.4	<b>5.8 ± 0.3</b>
<b>Stout</b>	<b>0.13 ± 0.01*</b>	<b>0.82 ± 0.12</b>	<b>0.75 ± 0.12</b>	<b>4.82 ± 0.86</b>	<b>5.9 ± 0.9</b>

**TPC = Total Phenol Concentration; FRAP = Ferric-Reducing Antioxidant Power. Normalized TPC and FRAP calculated by dividing results by alcohol content. Results expressed as mean ± standard deviation µg gallic acid equivalents/mL for TPC and mmol Fe(II) equivalents/L for triplicate determinations. \* = values are significantly different from APA, IPA, and Imp; # = values are significantly different from IPA and Imp.**



**Figure 2.1: Comparison of TPC values of craft beer and wine. Values expressed as  $\mu\text{g}$  gallic acid equivalents per milliliter.**



**Figure 2.2: Comparison of FRAP values of craft beer and wine. Values expressed as mmol Fe (II)/L.**

**Table 2.2: The effect of time on alcohol-normalized total phenolic content of craft beer styles.**

<b>Beer Style</b>	<b>Baseline</b>	<b>12 Months</b>	<b>24 Months</b>
<b>APA</b>	0.54 ± 0.11	0.49 ± 0.10 <sup>a</sup>	0.57 ± 0.07 <sup>a</sup>
<b>IPA</b>	0.56 ± 0.13	0.61 ± 0.07	0.50 ± 0.10
<b>Imperial IPA</b>	0.53 ± 0.13 <sup>a</sup>	0.79 ± 0.11	0.97 ± 0.17 <sup>a</sup>
<b>Porter</b>	0.75 ± 0.17	0.82 ± 0.23	0.83 ± 0.18
<b>Stout</b>	0.74 ± 0.12 <sup>a</sup>	0.76 ± 0.14 <sup>b</sup>	0.96 ± 0.18 <sup>ab</sup>
<b>Pumpkin</b>	0.56 ± 0.26	0.68 ± 0.27	0.71 ± 0.37

**Results expressed as mean ± standard deviation, µg gallic acid equivalents/mL for triplicate determinations. A value with the same superscript denotes significant difference among years within a style.**

**Table 2.3: The effect of time on the alcohol-normalized ferric-reducing antioxidant potential of craft beer styles.**

<b>Beer Style</b>	<b>Baseline</b>	<b>12 Months</b>	<b>24 Months</b>
<b>APA</b>	3.7 ± 0.5	3.0 ± 0.6	3.2 ± 1.3
<b>IPA</b>	4.5 ± 0.7 <sup>a</sup>	2.8 ± 1.1 <sup>a</sup>	3.6 ± 1.2
<b>Imperial IPA</b>	5.3 ± 0.9 <sup>a</sup>	4.1 ± 0.5	3.8 ± 1.1 <sup>a</sup>
<b>Porter</b>	5.2 ± 1.2	6.0 ± 1.6	5.6 ± 1.4
<b>Stout</b>	5.0 ± 0.9	5.1 ± 1.8	5.1 ± 1.3
<b>Pumpkin</b>	4.7 ± 1.7	3.0 ± 1.4	3.2 ± 2.2

**Results expressed as mean ± standard deviation mmol Fe (II) equivalents/L for triplicate determinations. A value with the same superscript denotes significant difference among years within a style.**

## CHAPTER THREE

### EFFECT OF MUSCADINE JUICE CONCENTRATE AND COMMERCIAL ELDERBERRY PREPARATIONS ON OSELTAMIVIR-RESISTANT H1N1 VIRUS

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## **Abstract**

Recent evidence indicates Tamiflu<sup>®</sup> (oseltamivir) may not be as effective against influenza as once believed. During the H1N1 pandemic, the limited treatment options available made Tamiflu<sup>®</sup> first-line therapy, but oseltamivir-resistant H1N1 strains have been emerging. The mechanism of action of Tamiflu<sup>®</sup> involves inhibition of neuraminidase, one of the two enzymes responsible for viral infection. Certain plant-based compounds have been shown to inhibit neuraminidase, and as such it is theoretically possible to develop a plant-based treatment alternative to Tamiflu<sup>®</sup>. Additionally, compounds found in elderberries that are related to quercetin and myricetin have been shown to inhibit the influenza virus hemagglutinin. As such, elderberry and muscadine, fruits that are high in quercetin and myricetin, may inhibit H1N1 via a different mechanism than Tamiflu<sup>®</sup>. To study these effects, we incubated oseltamivir-resistant H1N1 directly with various concentrations of Sambucol<sup>®</sup> and Sambucus<sup>®</sup> (two commercial elderberry products) and muscadine juice concentrate and added them to Manin-Darby canine kidney (MDCK) cells under a variety of experimental conditions. In other experiments, the extracts were incubated with cells either prior to or after inoculation with the virus. Both elderberry and muscadine showed inhibition of virus-mediated cytotoxicity. The therapeutic index of all extracts was shown to be greater than 20, indicating a wide difference between the concentration at which the extract is effective and the concentration at which it is toxic. The therapeutic index of muscadine was widest of the three extracts. It is therefore possible that these extracts may be used for influenza infection, even in viruses that have become resistant to the drug.

## **Introduction**

Every year, strains of the influenza A virus account for millions of infections and thousands of deaths throughout the world.(1) After the 2009 pandemic, H1N1 has become endemic in the population and is included in the annual influenza vaccine.(2) The influenza vaccine helps prevent many of these viral infections, although the live influenza virus is not recommended for several patient populations, including patients younger than two or older than 50 years of age, pregnant women, and patients with chronic diseases such as asthma or cardiovascular disease. The inactivated virus is considered safe for most patients, although those with severe allergies to vaccine components or with a history of Guillain-Barré Syndrome may not be able to receive this vaccine.(3) Furthermore, vaccine shortages will occasionally necessitate the limitation of vaccine administration to patients with significant need for influenza protection, as occurred during the 2004-2005 season.(4) Thus, despite the preventative capability of the vaccine, it is essential that other influenza treatment options become available.

The currently available treatment options for influenza infection are Tamiflu<sup>®</sup> (oseltamivir) and Relenza<sup>®</sup> (zanamivir). Both these medications are neuraminidase inhibitors; that is, they inhibit the influenza virus enzyme responsible for cleaving the sialic acid component of the virion and releasing the virus from the host cell; recent research has revealed that this enzyme is also involved with viral entry (along with hemagglutinin).(5, 6) Neither neuraminidase inhibitor has a generic form available, making them cost-prohibitive for certain patient populations. Furthermore, both medications are limited in their use by side effects, the most alarming of which are neurologic issues in Tamiflu<sup>®</sup> and bronchospasm in Relenza<sup>®</sup>. Zanamivir must be

administered via an inhaled dosage form due to low bioavailability and this inhaled dosage occasionally induces bronchospasm, often in patients with asthma, who are at higher risk of developing the influenza virus.(7) For these reasons, Relenza<sup>®</sup> has been declining in use.(8) Thus, many patients are limited to the use of Tamiflu<sup>®</sup> for treatment of influenza infection. Because H1N1 has been associated with instances of Tamiflu<sup>®</sup> resistance, it is theoretically possible that patients with H1N1 may have no treatment options available.(9) As such, research is needed for the development of new influenza treatments.

Recently, research has been performed on the use of elderberry for treatment of influenza, including the H1N1 strain.(10-13) It appears that quercetin and myricetin-associated flavonoids in elderberry are responsible for H1N1 inhibition, due to their ability to bind the influenza enzyme hemagglutinin, which is responsible for the binding of influenza virions to the host cells.(12) Quercetin and myricetin flavonoids are also present in high concentrations in the muscadine fruit, a fruit associated with numerous health benefits.(14-17) Furthermore, quercetin has been shown to have benefit in preventing drug resistance in influenza viruses.(18) Thus, we elected to study an oseltamivir-resistant H1N1 virus and hypothesize that elderberry and muscadine will show benefit in inhibiting this strain in vitro.

## **Materials and Methods**

### **Materials**

Manin-Darby canine kidney cells were obtained from ATCC (Manassas, VA).

Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM),

and phosphate-buffered saline solution (PBS) were obtained from Thermo Scientific (Suwanee, GA). Gentamicin was obtained from MP Biomedicals (Santa Ana, CA); TPCK-trypsin was obtained from Worthington (Lakewood, NJ). The CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay kit was obtained from Promega (Madison, WI). The viral strain, A/Mississippi/3/2001, was propagated using chicken eggs. Sambucol<sup>®</sup> is manufactured by Pharmicare US Inc.; Sambucus<sup>®</sup> is manufactured by Nature's Way Products, Inc.; both were obtained from a local health food store. Muscadine juice concentrate was generously provided by Muscadine Products Corporation, Wray, GA.

### **Folin-Ciocalteu Method for Determining Total Phenolic Concentration**

Because phenolic compounds have been implicated in fruits' ability to affect influenza, the fruit extracts were subjected to the Folin-Ciocalteu assay in order to determine the total phenolic concentration of each extract.(19) Briefly, this involves combining 20  $\mu$ L of sample dilutions with 1.58 mL deionized water, 100  $\mu$ L Folin-Ciocalteu reagent and 300  $\mu$ L sodium carbonate and incubating at room temperature, protected from light, for 45 minutes. The absorbance was determined using a Beckman DU 650 spectrophotometer at a wavelength of 765 nm. The total phenolic concentration of each extract was quantified using average absorbance from triplicate readings of each dilution based on a gallic acid standard curve.

### **Plaque Assay**

In order to calculate the plaque-forming units for the H1N1 strains to be used, 5 x 10<sup>5</sup> Manin-Darby canine kidney (MDCK) cells were incubated with DMEM + 5% FBS + 1% L-glutamine + 0.1% gentamicin growth medium in 12 well plates for 24 hours and

allowed to grow to confluency. The H1N1 was subjected to 10-fold serial dilution in MEM infection media with 1µg/mL TPCK-treated trypsin. The growth media was aspirated from each well of the 12-well plate and rinsed with PBS. Dilutions of the H1N1 virus were then added to each well of the 12-well plate at 100 µL volumes. The plate was then incubated at 37°C with 5% CO<sub>2</sub> for 2 to 3 hours, after which the infection medium was aspirated and 2 mL 1.2% Avicel overlay treated with 1:1000 TPCK-trypsin was added to each well and incubated again for 72 hours. After 72 hours, the overlay was aspirated and the cells rinsed with PBS and fixed with cold acetone: methanol (60:40) for 10 minutes. After fixation, the acetone: methanol solution was removed and the cells allowed to air dry. At this point, 1 to 2 mL Crystal Violet Solution was added to each well and incubated for 10 minutes at room temperature. Upon removal of the Crystal Violet and rinsing with deionized water, plaques visible in the violet stain were counted. The calculation of the virus titer was then performed as follows with a correction factor of 10:

$$(\text{Number of plaques})(\text{Dilution log})(\text{Correction factor}) = \text{Plaque forming units (pfu) per mL}$$

As muscadine represents a novel product for influenza inhibition, we attempted to visualize the effects of muscadine on cells incubated with the oseltamivir-resistant H1N1 virus. The plaque assay was repeated as described in the presence of 40 µg phenolics/mL muscadine. The cells were photographed using an EVOS<sup>®</sup> FL Cell Imaging System at a 40 X magnification.

## Cell Conditions and Time Points

MDCK cells grown to confluency in a 96-well plate were used for the viral assays, with serial dilutions of the elderberry and muscadine extracts added to the cells at various time points relative to addition of 100 pfu virus. The cell plates were incubated for 48 hours after virus addition, and then subjected to the CytoTox96<sup>®</sup> and hemagglutination assays to determine the effect of the extract on the cells and the virus.

Various timepoints were selected for the experiments described. Pre-incubation studies were performed by incubating three volumetric dilutions (1:40, 1:160, and 1:640) of extracts with the virus for one hour before adding them to the cells, and by adding extract to the cells one hour before addition of the virus. In other experiments, extracts and virus were also added simultaneously to the cells. Post-virus addition assays were also performed in which the middle dilution (i.e. 1:160) of extract was added 1, 6, and 24 hours after the virus was added. Analysis of the total phenolics content of the extracts revealed that the 1:160 dilution was approximately equal to 40 µg phenolics/mL for muscadine. As such, 40 µg/mL phenolics concentration was selected for studies in which the extracts were added at 0 hours, removed at 1, 6, and 24 hours, then the virus added, and in which the virus was added at 0 hours, removed at 1, 6, and 24 hours, then the extract added.

In a separate experiment, the therapeutic index was calculated for each extract. Various concentrations of extracts were tested against cells alone to determine the TD50, the concentration at which the extract is 50% cytotoxic. Additionally, an EC50, the concentration at which the extract induces 50% maximal response, was determined for

each extract. The therapeutic index was calculated for each extract by calculating the ratio of the TD50 to the EC50.

### **CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay**

The pathogenesis of influenza involves virus-mediated cell death.(20) Death of infected cells not only directly damages tissues, but appears to be responsible for an inflammatory response that results in respiratory symptoms and other effects associated with influenza infection.(21) Thus, measuring inhibition of cell death indicates the ability of a treatment to prevent tissue damage and other viral effects. The CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay is commonly used to determine the effect of treatment on influenza-induced cytotoxicity.(22-24) This assay involves the reaction of a substrate with lactate dehydrogenase released into the supernatant due to cell lysis.(25) To perform this assay, 50 microliters supernatant are transferred from the cell plate to a 96-well round-bottom plate and combined with 50 microliters assay buffer. After incubation at room temperature for 30 minutes, 50 microliters stop solution are added to the wells and the absorbance of the plates are read at 490 nm. The absorbance values reflect the amount of lactate dehydrogenase released from lysed cells and are a marker for cytotoxicity. Maximum cell lysis was induced in control wells and maximum absorbance associated with these wells was found to be approximately 4. Higher absorbance indicates greater release of lactate dehydrogenase from lysed cells, with lower absorbance reflecting greater cellular viability and integrity.

## **Hemagglutination Assay**

In order to determine whether elderberry and muscadine affect the influenza virus via hemagglutinin effects, the supernatant from the viral assays were subjected to the hemagglutination assay. For these experiments, 50  $\mu\text{L}$  supernatant were transferred to 96-well round-bottomed plates and 50  $\mu\text{L}$  0.5% red blood cells added to each well. The plates were incubated for 30 minutes to 1 hour at room temperature, at which point the plates were tilted to observe whether the red blood cells were agglutinated, i.e. formed a lattice due to hemagglutinin activity. Cloudy wells indicated hemagglutinin activity, whereas dots forming at the bottom of the well indicated no hemagglutinin activity.

## **Statistical Analysis**

All numerical data were subjected to the D'Agostino-Pearson method to determine normality, and outliers were revealed using Grubbs' test. The data were then subjected to one-way ANOVA with post-hoc testing by Tukey's test.

## **Results**

### **Pre-Incubation of Extracts with Virus Prior to Addition to Cells**

In order to determine whether the extracts may interact with the virus in such a way as to prevent cell death, the extracts were incubated with the virus for one hour prior to addition to the cells. The lactate dehydrogenase assay revealed Sambucol<sup>®</sup> and Sambucus<sup>®</sup> at a dilution of 1:40 resulted in significantly lower absorbance (i.e. cytotoxicity) compared to the virus control (i.e. virus without extract). These products also showed significantly lower cytotoxicity compared to muscadine at this same

concentration. At 1:160, all fruit extracts provided cytoprotection when compared to virus alone, and Sambucol<sup>®</sup> was cytoprotective at 1:640. (Figure 3.1) The hemagglutinin assay results show that hemagglutinin activity is inhibited by muscadine at 1:40, Sambucol<sup>®</sup> (in two out of three wells) and muscadine at 1:160, and all extracts at 1:640, with Sambucus<sup>®</sup> and muscadine showing inhibition in two out of three wells. (Table 3.1) It appears, then, that muscadine may induce cytotoxicity in this experiment at 1:40 and prevent cytotoxicity at lower concentrations. The inhibition of cytotoxicity by muscadine at 1:160 and 1:640 may be at least partially hemagglutinin-mediated; hemagglutinin activity was observed at 1:40 but did not result in inhibition of cytotoxicity, likely due to slight cytotoxic effect of muscadine at this concentration. Both elderberry products inhibited cytotoxicity at 1:40, but this effect was likely due to a mechanism other than hemagglutinin inhibition. The fact that all extracts inhibited cytotoxicity at 1:160, but only muscadine inhibits hemagglutination at this concentration suggests that mechanisms other than hemagglutinin effects may account for these extracts' activities.

### **Addition of Extract Prior to Virus**

In order to determine whether extracts might interact with cells in such a way as to induce cytoprotection prior to viral infection, we added the virus 1 hour after the addition of the extracts to the cells (without extracts being removed). This assay showed that all extracts inhibited cytotoxicity at the 1:160 and 1:640 dilutions, and the cytoprotection of Sambucol<sup>®</sup> was significant when compared to that of Sambucus<sup>®</sup> at 1:640, due perhaps the different formulations of these products. (Figure 3.2) All concentrations of extracts showed inhibition of hemagglutination. (Table 3.2) Thus, it

appears that these effects may be due to hemagglutinin inhibition, but again, higher concentrations of the extract may have induced some cytotoxicity.

### **Simultaneous Addition of Extracts and Virus to Cells**

The effect of extracts on cytotoxicity when added at the same time as the virus were studied in order to determine the effect when extracts and virus were competing directly for access to cells. All extracts significantly inhibited cytotoxicity compared the influenza control at 1:40. Muscadine significantly inhibited cytotoxicity at 1:160 and 1:640. Muscadine also significantly inhibited cytotoxicity compared to the elderberry products at 1:160. (Figure 3.3) The hemagglutinin assay revealed inhibition of the enzyme at Sambucus<sup>®</sup> 1:640 (in two out of three wells) and all concentrations of muscadine. (Table 3.3) It is likely, then, that hemagglutinin inhibition accounts for part of muscadine's protective effects in this assay, but other mechanisms account for elderberry's effects at 1:40.

### **Addition of Extracts Up To 24 Hours After Addition of Virus**

Extracts were added 1, 6, and 24 hours after addition of virus without washing away the virus in order to determine whether the extracts may have the capacity to protect the cells even after the initial inoculation of the virus. The 1:160 concentration was selected for this set of experiments due to its effects in the previous assays. Sambucus<sup>®</sup> 1:160 inhibited cytotoxicity at 1 hour after viral addition; at this timepoint, Sambucus<sup>®</sup> was also significantly different from Sambucol<sup>®</sup> and muscadine. All extracts inhibited cytotoxicity at 6 hours; Sambucus<sup>®</sup> and muscadine were significantly different from Sambucol<sup>®</sup> at this timepoint, and Sambucus<sup>®</sup> was significantly different from

muscadine. No significant differences were observed when these fruit extracts were added 24 hours after the addition of the virus. (Figure 3.4) The HA assay reveals that the elderberry products show no hemagglutinin inhibition at any time after viral addition. Muscadine inhibited hemagglutinin activity at 1 and 6 hours after viral addition, but not at 24 hours. (Table 3.4) Again, it appears that muscadine's effects may be due to hemagglutinin inhibition up to 6 hours after viral addition. While new viruses are synthesized 6 hours after viral entry(26), these data show that the addition of the fruit extracts after the viral inoculation can only produce modest cytoprotection compared to previous experiments. (Figures 3.1 and 3.2)

### **Removal of Extract and Addition of Virus; Removal of Virus and Addition of Extract**

These extracts contain tannins, compounds which may precipitate proteins, including viral enzymes. In order to control for this, a set of experiments were designed in which the virus was added to the cells, removed and cells washed and then the extracts were added, or extracts were added to cells, removed and cells washed and then the virus was added. Thus, the cells were never exposed to both the virus and the extracts simultaneously. In order to directly compare the effect of the extracts based on phenolic content, these experiments were performed with extracts at 40 µg phenolics per mL. In the experiment involving extract added to the cells, extract removed and cells washed after one hour, then virus added for the remaining incubation time, no significant difference was observed with the extracts. (Figure 3.5) However, in the converse experiment when the virus was added to the cells for one hour, removed and then extracts added, all extracts showed significant inhibition of cytotoxicity compared to virus alone

at a concentration of 40 µg phenolics per mL. (Figure 3.5) When extracts were added at 0 hour and removed after a longer incubation period of 6 hours, then virus added, Sambucol<sup>®</sup> and muscadine both inhibited cytotoxicity when compared to virus, whereas muscadine inhibited cytotoxicity when the virus was added at 0 hour, then removed and extracts added at 6 hours. (Figure 3.6) These results demonstrate that cytoprotection by fruit extracts can be observed when they are presented to the cells either before or after the virus. In addition, these results demonstrate that the phenolics present in muscadine are as active and in some cases more efficacious than those found in elderberry in their cytoprotective effects against the influenza virus.

### **Therapeutic Index**

The TD50 reflects the concentration that results in 50% extract-induced cytotoxicity, whereas the EC50 reflects the concentration that results in 50% of the maximal inhibition of virus-induced cytotoxicity produced by each extract. The therapeutic index is the ratio of the TD50 to the EC50, with higher values indicating a wider range at which the treatment is effective but not toxic. The TD50, EC50, and therapeutic indices are shown in Table 3.5. Muscadine was shown to have the widest therapeutic index at approximately 166, and Sambucol<sup>®</sup> has the narrowest, at approximately 23.

### **Plaque Assay with Virus, Muscadine**

When a plaque assay was performed on MDCK cells incubated with 40 µg phenolics/mL muscadine and oseltamivir-resistant H1N1, clear differences could be seen

between cells exposed to virus alone and cells exposed to the virus with muscadine.  
(Figure 3.7)

## **Discussion**

There is a great need for additional treatment options for influenza. This is especially true of virus strains that have become resistant to current treatment options, such as oseltamivir-resistant H1N1. This resistance may be due to mutations of the neuraminidase enzyme allowing the enzyme to elude neuraminidase inhibition by oseltamivir (Tamiflu<sup>®</sup>). A phenomenon known as antigenic drift, in which the hemagglutinin or neuraminidase enzymes mutate, may create new strains of influenza which may evade a population's immunity, leading to an epidemic.(27, 28) This phenomenon is responsible for development of the influenza strain studied here, A/Mississippi/3/2001, an H1N1 virus in which the mutation of the Histidine 274 residue to Tyrosine conferred resistance to oseltamivir. This mutation is well-established as a mechanism of N1neuraminidase resistance to oseltamivir, but not to zanamivir.(7) This is due to differences in the structure of oseltamivir and zanamivir; the mutation at 274 induces a shift in the enzyme's side chain at the 276 residue. This shift induces change in orientation of the diethxoy moiety of oseltamivir, which decreases the ability of oseltamivir to bind strongly to neuraminidase. Zanamivir, however, binds the 276 side chain more strongly due to the shift, so the H274Y mutation is actually beneficial to zanamivir binding.(29) However, because of issues mentioned above, certain patients may not be able to use zanamivir or may prefer oseltamivir due to ease of dosage. Because these drugs must be started within 48 hours of symptom development, it may not be possible to establish whether a patient's viral infection is oseltamivir-sensitive prior to

treatment. The H274Y mutation has been increasing lately, even in areas where oseltamivir is not in wide use.(30, 31) During the 2008 influenza season, the incidence of oseltamivir-resistant viruses with this mutation increased from less than 1% of H1N1 infections to nearly 100% in Oceania, Southeast Asia, and South Africa.(32) This same mutation has been shown to cause resistance of the H5N1 virus to oseltamivir.(33, 34)

Due to the need for an alternative to current pharmaceutical agents in influenza, various studies have examined the effects of phenolics against influenza, particularly against oseltamivir-resistant H1N1.(35-39) These studies have found phenolics to be effective against the mutated virus due to the fact that they inhibit the enzyme via noncompetitive inhibition, a mechanism other than that of oseltamivir or zanamivir, both of which exhibit competitive inhibition.(40) Because these compounds likely bind an enzyme site other than the catalytic center bound by neuraminidase inhibitors, they may inhibit neuraminidase enzymes that exhibit the H274Y mutation in their catalytic sites(38). Phenolics have also been shown to inhibit the hemagglutinin enzyme (involved in viral entry of host cells) and the PI3K/AKT pathway (necessary for viral replication).(41-43) Because phenolics may affect numerous mechanisms of viral replication and infection, they offer an ideal alternative to neuraminidase inhibitors, which exhibit a single mechanism of action.

Other studies have found that phenolics and high-phenolic plants exhibit cytoprotection against influenza via inhibition of viral entry.(39, 41, 42) Additionally, elderberry phenolics have been shown to inhibit hemagglutinin, one of the enzymes responsible for viral entry.(12) To study whether elderberry and muscadine might inhibit viral entry, we added two elderberry products and a muscadine juice concentrate to

oseltamivir-resistant H1N1 and incubated the combination for one hour prior to addition to cells. The results of this study revealed that elderberry extracts inhibited cytotoxicity at 1:40; all three extracts inhibited cytotoxicity at 1:160, and Sambucol<sup>®</sup> and muscadine inhibited cytotoxicity at 1:640. (Figure 3.1) The hemagglutination assay supported the possibility that this might be due to hemagglutinin effects: muscadine inhibited hemagglutinin activity at all concentrations, Sambucus<sup>®</sup> inhibited hemagglutinin at 1:40, and Sambucol<sup>®</sup> inhibited hemagglutinin at 1:160, while both elderberry products inhibited hemagglutinin at 1:640. (Table 3.1) This indication that low concentrations of elderberry and muscadine may inhibit hemagglutinin, and thus, inhibit cytotoxicity due to influenza, supports their use both as treatment and prevention. This is the first study showing elderberry or muscadine to have efficacy when pre-incubated with the virus, suggesting a significant role in prevention of influenza infection. While elderberry products have been marketed as preventative measures for viral infection, these results support that concept and suggest muscadine as another preventative option.

The results presented here indicate that elderberry and muscadine inhibit cytotoxicity due to oseltamivir-resistant H1N1, often due to inhibition of hemagglutinin. Lower concentrations of extracts allow for cytoprotection even before the virus was added, supporting the use of these products for prevention of influenza infection. (Figure 3.2) This conclusion is supported by similar studies involving other medicinal plants in influenza, including oseltamivir-resistant mutants.(42, 44) These are the first findings involving pre-incubation of cells with elderberry, supporting its use for prevention of influenza infection, and the first findings involving muscadine and influenza.

While hemagglutinin inhibition appeared to account for some of the extracts' cytoprotection, the fact that this was not true in all cases indicates that these products may induce cytoprotection via mechanisms other than hemagglutinin activity. This is supported by other studies showing multiple mechanisms of phenolic-mediated cytoprotection against oseltamivir-resistant H1N1(35, 38, 39, 41, 42). No other studies have shown efficacy of elderberry or muscadine in an oseltamivir-resistant H1N1 viral strain, supporting this study as evidence of these products as an alternative to oseltamivir in resistant strains. It is possible that the extracts may not require access to the virus in order to show benefits to cells. Extracts inhibited cytotoxicity when the virus was removed and cells washed before extracts added. This indicates that the mechanism of antiviral activity of elderberry and muscadine occurs independent of a direct interaction between virus and fruit extract.

These studies, along with various others involving similar assays and other medicinal plants, indicate that elderberry, muscadine, and other natural products may inhibit influenza activity by various mechanisms. As previously mentioned, it has been shown that hemagglutinin effects account for elderberry's anti-influenza activity, a finding that our research supports. Additionally, unpublished data from our lab has shown that muscadine inhibits neuraminidase activity; particularly the neuraminidase 1 isozyme of H1N1. Because of the various compounds contained in natural extracts, and the complex synergy between them, it is quite possible that these extracts may inhibit influenza activity by many mechanisms of action, giving them an advantage over many pharmaceutical agents for influenza inhibition, which typically exhibit a single mechanism. That is, neuraminidase inhibitors are not effective in influenza strains that

may develop a neuraminidase mutant that prevents binding by the inhibitor. However, natural products that may inhibit both neuraminidase and hemagglutinin, along with various other mechanisms of providing cytoprotection against influenza, may act as an alternative to these pharmaceutical agents. The improvement of cell viability when cells are exposed to muscadine along with oseltamivir-resistant H1N1 was supported by visualization of these cells via plaque assay.

The therapeutic index of all extracts was shown to be greater than 20, indicating a wide difference between the concentration at which the extract is effective and the concentration at which it is toxic. The therapeutic index of muscadine was widest of the three extract, indicating the greatest range of safe and effective doses.

### **Conclusions**

This study supports other findings that elderberry products may have efficacy in treating the H1N1 virus and introduces the possibility of muscadine as a beneficial product in this regard. Many of these studies are the first of their kind in elderberry, and this is the first study to show any benefit of muscadine against influenza. Along with other studies into medicinal plants, these studies indicate that elderberry and muscadine may have various mechanisms to benefit cells infected with influenza. Because of this, these extracts may be used as complementary or alternative treatment or prevention for influenza, with muscadine being especially safe and efficacious.

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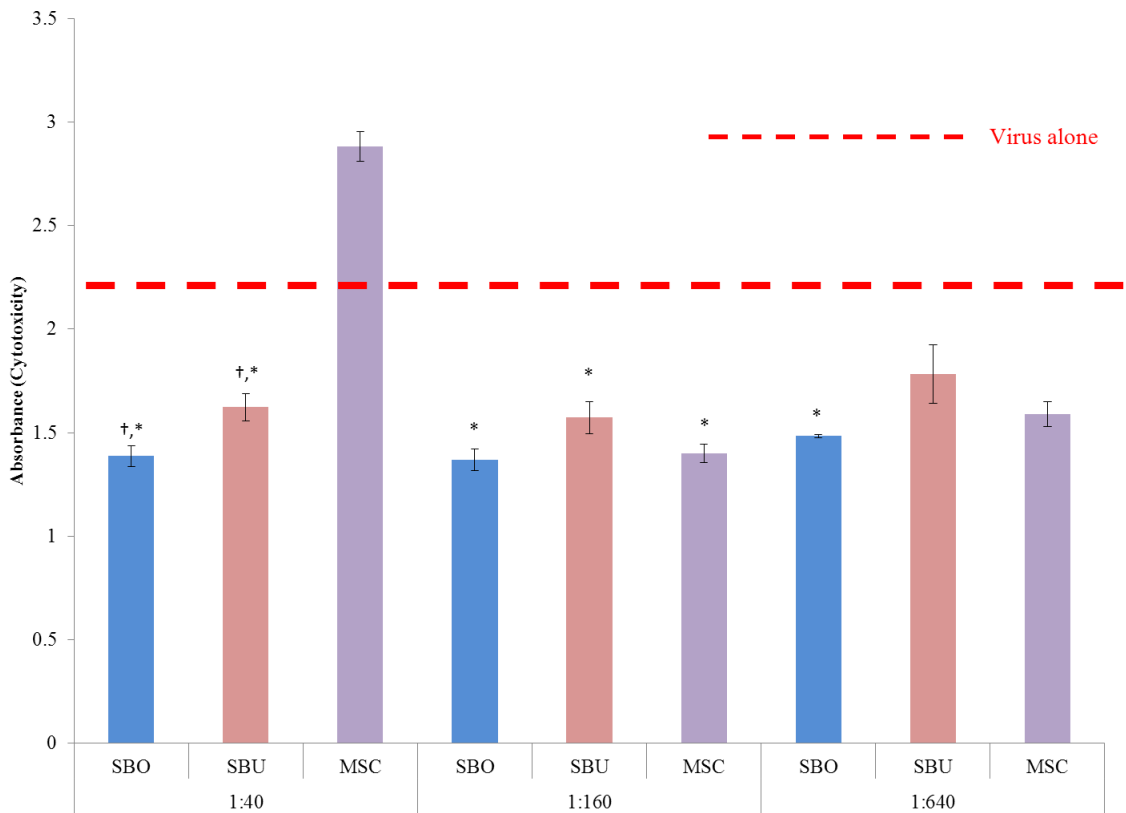
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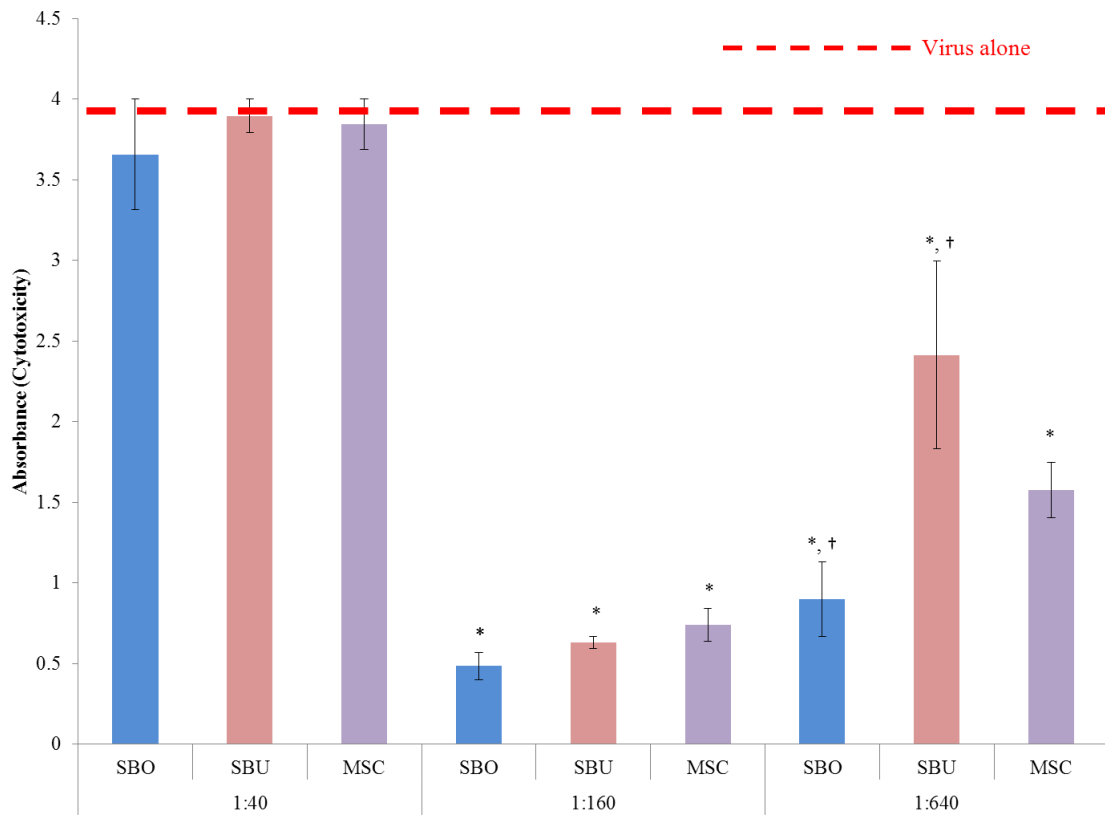
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**Figure 3.1: Results from the Pre-Incubation Assay. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine. Extracts were incubated with oseltamivir-resistant H1N1 virus for one hour prior to addition to MDCK cells; after 48 hours, cytotoxicity was determined. Results represent mean  $\pm$  standard absorbance of data in triplicate. \* = significant difference from virus alone, † = significant difference from muscadine 1:40,  $p < 0.05$ .**

**Table 3.1: Results from Pre-Incubation Hemagglutinin Assay. HA results presented in triplicate. SBO = Sambucol<sup>®</sup>; SBU = Sambucus<sup>®</sup>, MSC = muscadine. ND = no dot observed (i.e. hemagglutinin activity) ● = dot formed (i.e. inhibition of hemagglutinin activity).**

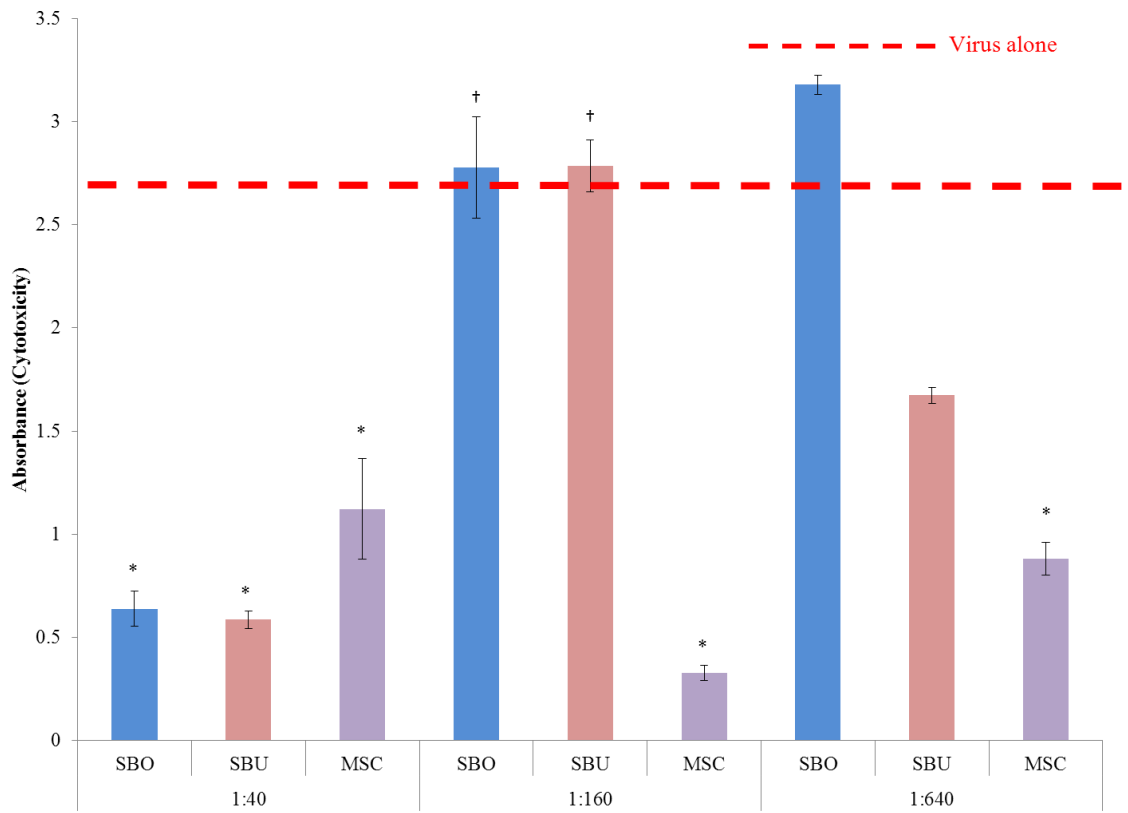
1:40			1:160			1:640		
SBO	SBU	MSC	SBO	SBU	MSC	SBO	SBU	MSC
ND	●	●	●	ND	●	●	ND	ND
ND	ND	●	●	ND	●	●	●	●
ND	ND	●	ND	ND	●	●	●	●



**Figure 3.2: Results from Extracts Added at 0 Hour, Virus Added at 1 Hour Assay.** SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine. Extracts added 1 hour prior to addition of oseltamivir-resistant H1N1 to MDCK cells; after 48 hours, cytotoxicity was determined. Results represent mean  $\pm$  standard error of data in triplicate. \* = significant difference from virus alone; † = data points significantly different from each other,  $p < 0.05$ .

**Table 3.2: Results from Hemagglutinin Assay of Extracts Added at 0 Hour, Virus Added at 1 Hour. HA results presented in triplicate. SBO = Sambucol<sup>®</sup>; SBU = Sambucus<sup>®</sup>, MSC = muscadine. ● = dot formed (i.e. inhibition of hemagglutinin activity).**

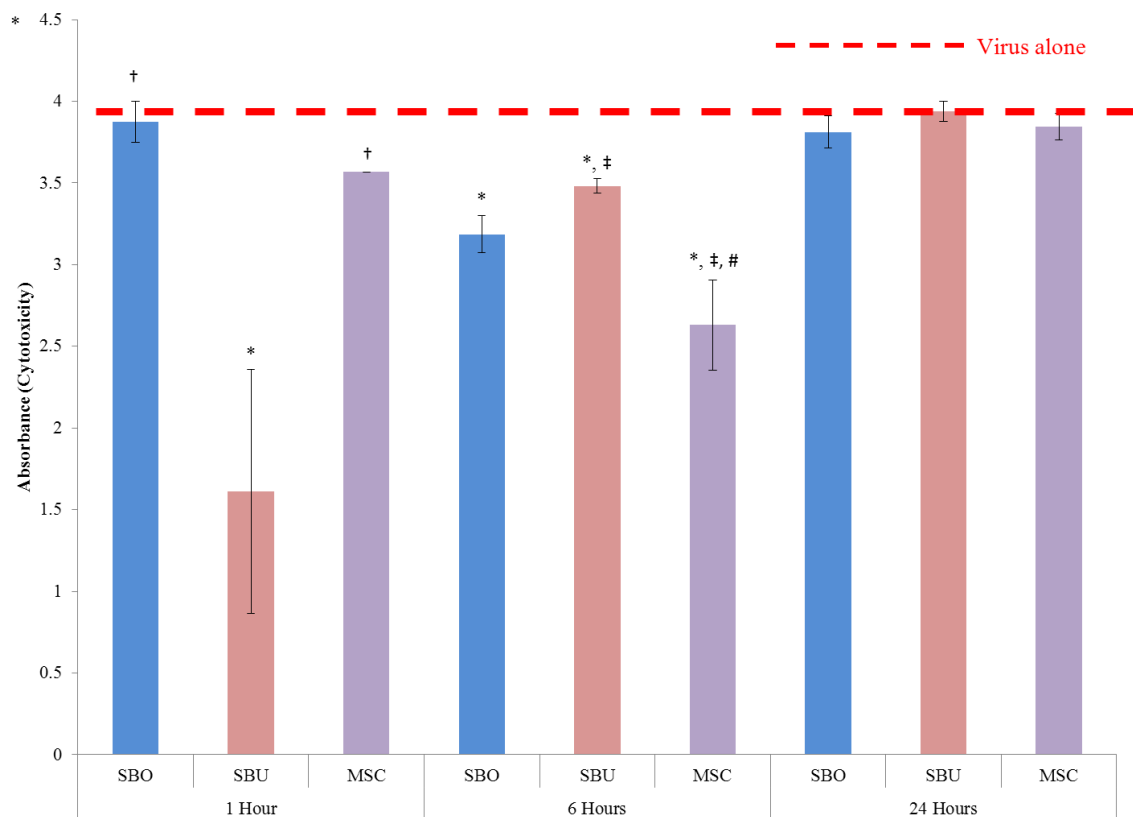
1:40			1:160			1:640		
SBO	SBU	MSC	SBO	SBU	MSC	SBO	SBU	MSC
●	●	●	●	●	●	●	●	●
●	●	●	●	●	●	●	●	●
●	●	●	●	●	●	●	●	●



**Figure 3.3: Results from Simultaneous Addition Assay. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine. Extracts and oseltamivir-resistant H1N1 virus added at same time to MDCK cells; after 48 hours, cytotoxicity was determined. Results represent mean  $\pm$  standard error absorbance of data in triplicate. \* = significant difference from virus alone; † = significant difference from muscadine at 1:160,  $p < 0.05$ .**

**Table 3.3: Results from Simultaneous Addition Hemagglutinin Assay. HA results presented in triplicate. SBO = Sambucol<sup>®</sup>; SBU = Sambucus<sup>®</sup>, MSC = muscadine. ND = no dot observed (i.e. hemagglutinin activity) ● = dot formed (i.e. inhibition of hemagglutinin activity).**

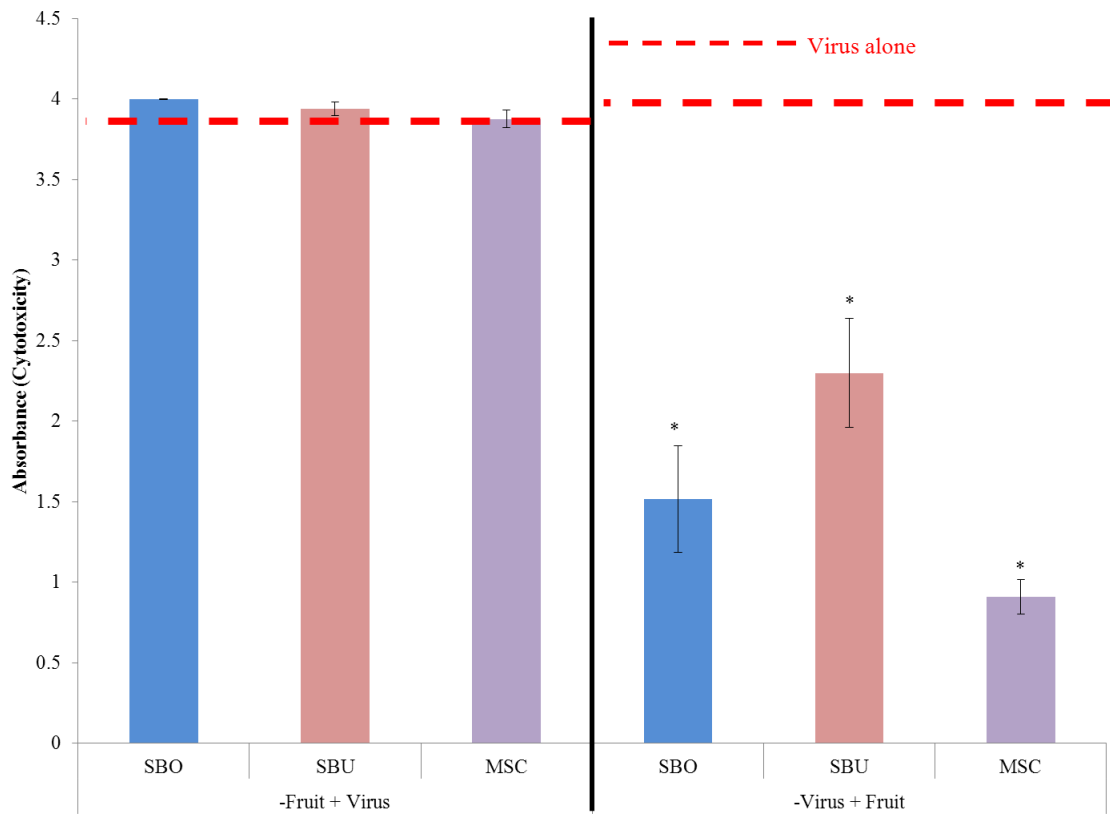
1:40			1:160			1:640		
SBO	SBU	MSC	SBO	SBU	MSC	SBO	SBU	MSC
ND	ND	●	ND	ND	●	ND	●	●
ND	ND	●	ND	ND	●	ND	●	●
●	ND	●	ND	ND	●	ND	ND	●



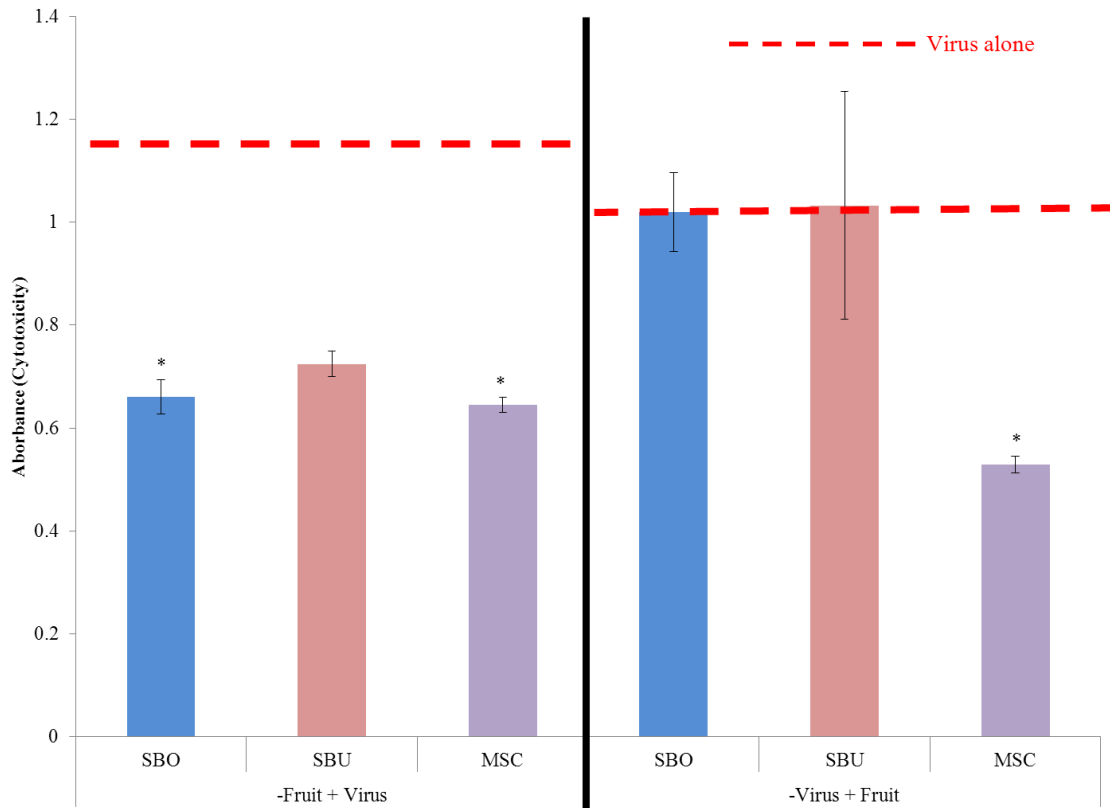
**Figure 3.4: Results from Post-Virus Addition Assay. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine. Extracts added at 1, 6, and 24 hours after addition of oseltamivir-resistant H1N1 virus to MDCK cells; after 48 hours, cytotoxicity was determined. Results represent mean  $\pm$  standard error of data in triplicate. \* = significant difference from virus alone; † = significant difference from Sambucus<sup>®</sup> 1 hour; ‡ = significant difference from Sambucol<sup>®</sup> 6 hour; # = significant difference from Sambucus<sup>®</sup> 6 hour,  $p < 0.05$ .**

**Table 3.4: Results from Post-Virus Addition Hemagglutinin Assay. HA results presented in triplicate. SBO = Sambucol<sup>®</sup>; SBU = Sambucus<sup>®</sup>, MSC = muscadine. ND = no dot observed (i.e. hemagglutinin activity) ● = dot formed (i.e. inhibition of hemagglutinin activity).**

1 Hour			6 Hours			24 Hours		
SBO	SBU	MSC	SBO	SBU	MSC	SBO	SBU	MSC
ND	ND	●	ND	ND	●	ND	ND	ND
ND	ND	●	ND	ND	●	ND	ND	ND
ND	ND	●	ND	ND	●	ND	ND	ND



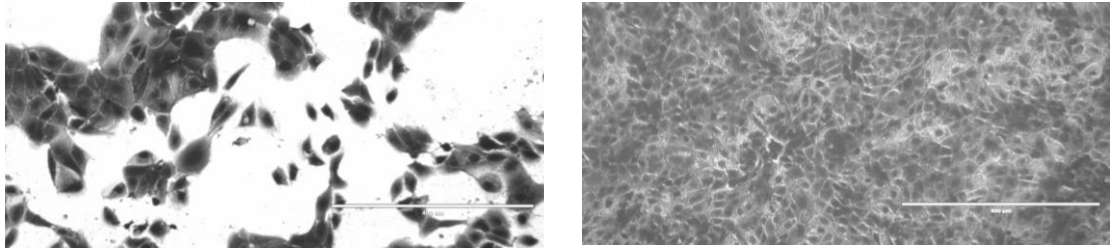
**Figure 3.5: Results from Extract/Virus Removal Assay. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine. -Fruit + Virus = Extracts added at 0 hour, removed at 1 hour, then oseltamivir-resistant H1N1 added for remainder of incubation time. -Virus + Fruit = oseltamivir-resistant H1N1 added at 0 hour, removed at 1 hour, then extracts added for remainder of incubation time. Results represent mean  $\pm$  standard error of data in triplicate. \* = significant difference from virus alone,  $p < 0.05$ .**



**Figure 3.6: Results from Extract/Virus Removal Assay. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine. -Fruit + Virus = Extracts added at 0 hour, removed at 6 hours, then oseltamivir-resistant H1N1 added for remainder of incubation time. -Virus + Fruit = oseltamivir-resistant H1N1 added at 0 hour, removed at 6 hours, then extracts added for remainder of incubation time. Results represent mean  $\pm$  standard error of data in triplicate. \* = significant difference from virus alone,  $p < 0.05$ .**

**Table 3.5: Comparison of Toxic Dose, Effective Dose, and Therapeutic Index of Extracts.**  
**TD<sub>50</sub>** = concentration that results in 50% of the maximal cytotoxic effect produced by extract; **EC<sub>50</sub>** = concentration that results in 50% of the maximal inhibitory effect produced by extract; therapeutic index = ratio of TD<sub>50</sub>/EC<sub>50</sub>.

<b>Extract</b>	<b>TD<sub>50</sub> (µg phenolics/mL)</b>	<b>EC<sub>50</sub> (µg phenolics/mL)</b>	<b>Therapeutic Index</b>
<b>Sambucol<sup>®</sup></b>	452	19	23
<b>Sambucus<sup>®</sup></b>	302	6.3	47
<b>Muscadine</b>	4790	29	166



**Figure 3.7: Plaque Assay Performed on MDCK Cells Incubated with Oseltamivir-Resistant H1N1 and 40  $\mu\text{g}$  phenolics/mL Muscadine. Left: MDCK cells with virus alone, right: MDCK cells with virus and muscadine.**

## CHAPTER FOUR

### EFFECT OF MUSCADINE CONCENTRATE AND COMMERCIAL ELDERBERRY PREPARATION ON WILD-TYPE H1N1 AND H3N2 INFLUENZA VIRUSES *IN* *VITRO*

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## **Abstract**

Based on evidence that elderberry and muscadine extracts inhibit cytotoxicity and improves cell viability when incubated with Tamiflu<sup>®</sup>-resistant H1N1 virus, we studied the effect of these extracts on wild-type H1N1 and H3N2 viruses. Both these viruses are influenza A viruses included in the annual vaccine, indicating that they are consistently of greatest concern in the population. In order to determine whether these extracts may be of benefit in these endemic viruses, we conducted experiments similar to those conducted with Tamiflu<sup>®</sup>-resistant H1N1. This involved adding Sambucol<sup>®</sup> and Sambucus<sup>®</sup> (two commercial elderberry products) and muscadine to Manin-Darby canine kidney cells at various timepoints relative to the addition of the H1N1 or H3N2 virus. Oseltamivir (Tamiflu<sup>®</sup>) was also used as a positive control. After 48 hours, the CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay was used to measure the amount of lactate dehydrogenase released due to cell lysis as a marker for cytotoxicity. All three extracts showed inhibition of cytotoxicity at various timepoints and concentrations with both viruses. Additionally, cytotoxicity was inhibited in studies in which extracts or virus were removed prior to addition of the other component, indicating the extracts may not require the physical presence of virus in order to show benefit to cells. Overall, these data suggest that elderberry and muscadine may have benefit in wild-type H1N1 and H3N2 influenza, viruses most likely to be infectious during the annual influenza season. They also may act as an alternative to Tamiflu<sup>®</sup> in instances in which a patient is intolerant to or cannot obtain the pharmaceutical agent.

## **Introduction**

Of the three influenza types (A, B, and C), influenza A is the one most commonly infecting humans. It also confers the greatest risk of the three, being capable of creating epidemics and pandemics. Influenza B, though infectious year-round rather than seasonally like influenza A, has not been shown to create pandemics.(1) Nevertheless, the annual influenza vaccine includes both influenza A and B.(2)

Influenza A contains two membrane-associated enzymes responsible for infection: hemagglutinin and neuraminidase. Sixteen subtypes of hemagglutinin and nine subtypes of neuraminidase have been identified.(1) This results in 144 possible influenza A variations, with 116 variations having been isolated.(3) The strain of virus is identified based on the subtype of enzyme the virus contains; that is, H1N1 contains hemagglutinin 1 and neuraminidase 1. These subtypes may be developed due to the phenomenon known as antigenic shift. In this phenomenon, a non-human host (most commonly wild aquatic fowl) may be infected with two different subtypes of influenza A. Progeny virions from these viruses may develop a new subtype. If the resultant subtype is capable of being transmitted to humans, a pandemic, or global epidemic, may occur. Antigenic drift is a phenomenon in which new strains of an enzyme subtype may occur due to genetic mutations of the virus. These strains may evade a population's immunity to similar viruses, creating an epidemic.(1)

The 2009 H1N1 pandemic was typical in that the greatest concern was for young adults rather than young children, elderly, or immunocompromised patients who are most likely to fare poorly in epidemics. Pandemics also occur in multiple geographic regions, rather than a single population as with epidemics. Deaths in pandemics are also more

likely to be due to secondary infection or multiple organ failure, and will occur in young adults because this population is less likely to have been exposed to a previous virus similar to the current virus. The 2009 H1N1 virus was similar to that which caused the 1918 epidemic sometimes called “Spanish flu”. Viruses similar to this one circulated in the population until the 1950s, and thus older patients were more likely to have immunity to the 2009 virus. By 2010, the H1N1 pandemic was declared over, and H1N1 is now endemic in the population. The annual influenza vaccine has included the H1N1 and H3N2 viruses.(1)

Influenza A is most likely to be infectious during winter and early spring. Thus, in order for the vaccine to be available in time for patients receiving it to develop immunity, the vaccine must be developed well in advance of flu season. This requires prediction of which virus strains will be infectious that year. The 2003-2004 vaccine was not terribly well-matched to the strains of concern that year.(4) While more recent vaccines have been more effective, this remains a possibility for decreased vaccine effectiveness. Additionally, the 2004-2005 vaccine had limited availability, and patients who were at higher risk of infection or complications were given priority over the general population.(5) Thus, patients who do not receive the vaccine or who receive a less-effective vaccine are at risk of infection and require treatment options.

Currently, only four drugs from two different classes are available for the treatment of influenza A. Adamantanes (amantadine and rimantadine) are M2 proton channel blockers. The M2 proton channel is responsible for allowing hydrogen ions to enter the virus and thus change the pH which allows for viral uncoating and replication. Thus, the drugs that block this channel prevent this step of viral replication.(6) These drugs have

been associated with resistance and were not recommended for use during the 2009 H1N1 pandemic.(7) The other class of drugs is neuraminidase inhibitors. The neuraminidase enzyme is responsible for cleaving the neuraminic acid residues on the viral surface, thus releasing progeny virions which may infect other cells. Inhibitors of this enzyme thus prevent further viral infection.(8) Currently, the only neuraminidase inhibitors available are oseltamivir (Tamiflu<sup>®</sup>) and zanamivir (Relenza<sup>®</sup>).

Many influenza strains have become resistant to adamantane drugs, and thus these medications have fallen from favor in preference to the newer neuraminidase inhibitors. Indeed, adamantanes were not used during the 2009 H1N1 pandemic due to resistance issues, and these drugs are no longer recommended for influenza prophylaxis or treatment, regardless of strain.(9, 10) Neuraminidase inhibitors, however, being newer medications, have no generic versions available, which may preclude their use in certain patient populations. Adverse effects may also render their use impossible: oseltamivir may cause neurologic issues and zanamivir, being an inhaled dosage form, may cause bronchospasm.(11, 12) Thus, zanamivir must be used with extreme caution in patients with underlying pulmonary conditions. The inactive ingredients of zanamivir include lactose, which also prevents its use in patients with dairy allergies. As such, oseltamivir tends to be first-line therapy, yet resistance oseltamivir is being reported.(13) These issues make it conceivably possible that a patient may be left with no treatment options upon contracting a resistant strain of influenza.

Various plant-based therapies and compounds have been studied in combination with influenza A. Studies have shown that plant-based phenolics may inhibit influenza infection by various mechanisms, including inhibition of hemagglutinin and

neuraminidase(14-19). Phenolics may also inhibit viral replication by inhibiting the PI3K/AKT pathway or simply provide antioxidant protection to cells against virus-mediated damage.(14, 16, 20) Because of this variety in virus-inhibiting mechanisms and the variety of phenolics contained in plant-based therapies, natural products such as elderberry and muscadine may be an ideal alternative to neuraminidase inhibitors.

Many studies on the effect of phenolic compounds on influenza infection involve more than one influenza strain. This is not only due to the variety of strains that may be of concern during the annual flu season, but also because hemagglutinin and neuraminidase isozymes may respond slightly differently to a given treatment. The neuraminidase enzyme in particular may be divided into two categories: group 1 (including N1 neuraminidase) and group 2 (including N2 neuraminidase), which have slight differences in the catalytic center. This accounts for greater resistance of N1 strains to oseltamivir than N2 strains, including H3N2; the catalytic center of N2 neuraminidase is less apt to affect binding to oseltamivir as a result of mutations. Because influenza treatment must be started within 48 hours of symptoms appearing, it may not be possible to identify the strain and drug sensitivity of a patient's viral infection. Thus, identification of products that inhibit multiple influenza strains by multiple mechanisms is desirable. For these reasons, H1N1 and H3N2 were selected for this study, not only because they are endemic in the population, but because each isozyme contained in the virus is different. Thus, inhibitory effects seen with these viruses may be applied to other viruses with the same isozyme; that is, products that inhibit the neuraminidase enzyme of H1N1 may inhibit H5N1, products that inhibit H3N2 may inhibit H9N2, etc.

## **Materials and Methods**

### **Materials**

Manin-Darby canine kidney cells were obtained from ATCC (Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM), phosphate-buffered saline (PBS) were obtained from Thermo Scientific (Suwanee, GA). Gentamicin was obtained from MP Biomedicals (Santa Ana, CA); TPCK-trypsin was obtained from Worthington (Lakewood, NJ). The CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay kit was obtained from Promega<sup>®</sup> (Madison, WI). The viral strains, A/Mississippi/3/2001 and A/55/NY/2004 (H1N1 and H3N2), respectively, were propagated using chicken eggs. Sambucol<sup>®</sup> is manufactured by Pharmicare US Inc.; Sambucus<sup>®</sup> is manufactured by Nature's Way Products, Inc.; both were obtained from a local health food store. Muscadine juice concentrate was generously provided by Muscadine Products Corporation, Wray, GA. Oseltamivir carboxylate (2 mM) was generously donated by Dr. S. Mark Tompkins.

### **Plaque Assay**

In order to calculate the plaque-forming units for the influenza strains to be used,  $5 \times 10^5$  Manin-Darby canine kidney (MDCK) cells were incubated with DMEM + 5% FBS + 1% L-glutamine + 0.1% gentamicin growth medium in 12 well plates for 24 hours and grown to confluency. The H1N1 was subjected to 10-fold serial dilution in MEM infection media with 1 $\mu$ g/mL TPCK-treated trypsin. The growth media was aspirated from each well of the 12-well plate and rinsed with PBS. Dilutions of the H1N1 virus was then added to each well of the 12-well plate at 100  $\mu$ L volumes. The plate was then incubated at 37°C with 5% CO<sub>2</sub> for 2 to 3 hours, after which the infection medium was

aspirated and 2 mL 1.2% Avicel overlay treated with 1:1000 TPCK-trypsin was added to each well and incubated again for 72 hours. After 72 hours, the overlay was aspirated and the cells rinsed with 1x PBS and fixed with cold acetone: methanol (60:40) for 10 minutes. After this fixing, the acetone: methanol solution was removed and the cells allowed to air dry. At this point, 1 to 2 mL Crystal Violet Solution was added to each well and incubated for 10 minutes at room temperature. Upon removal of the Crystal Violet and rinsing with deionized water, plaques visible in the violet stain were counted. The calculation of the virus titer was then performed as follows with a correction factor of 10:

$$(\text{Number of plaques})(\text{Dilution log})(\text{Correction factor}) = \text{Plaque forming units (pfu) per mL}$$

### **Cell Conditions and Time Points**

MDCK cells grown to confluency in a 96-well plate were used for the viral assays, with serial dilutions of the elderberry and muscadine extracts added to the cells at various time points relative to addition of 100 pfu virus. Cell conditions included extract with the virus, extract alone, virus alone, media alone, and oseltamivir with and without virus as positive control in the case of the wild-type virus. The cell plates were incubated at 37°C for 48 hours after virus addition, then subjected to the CytoTox 96<sup>®</sup> and hemagglutination assays to determine the effect of the extract on the cells and the virus.

Various timepoints were selected for the experiments described. Pre-incubation studies were performed by incubating three volumetric dilutions (1:40, 1:160, and 1:640) of extracts with the virus for one hour before adding them to the cells, and by adding extract to the cells one hour before addition with the virus; extracts and virus were also

added simultaneously to the cells. Post-virus addition assays were performed in which the middle dilution (i.e. 1:160) of extract was added 1, 6, and 24 hours after the virus was added. Analysis of the total phenolics content of the extracts revealed that the 1:160 dilution was approximately equal to 40 µg phenolics/mL for muscadine. As such, 40 µg/mL phenolics concentration was selected for a study in which the extracts were added at 0 hours, removed at 6, then the virus added, and in which the virus was added at 0 hours, removed at 6 hours, then the extract added for the remainder of the 48 hour incubation.

### **CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay**

The pathogenesis of influenza involves virus-mediated cell death.(21) Death of infected cells not only directly damages tissues, but appears to be responsible for an inflammatory response that results in respiratory symptoms and other effects associated with influenza infection.(22) Thus, measuring inhibition of cell death indicates the ability of a treatment to prevent tissue damage and other viral effects. The CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay is commonly used to determine the effect of treatment on influenza-induced cytotoxicity.(23-25) This assay involves the reaction of a substrate with lactate dehydrogenase released into the supernatant due to cell lysis.(26) To perform this assay, 50 microliters supernatant are transferred from the cell plate to a 96-well round-bottom plate and combined with 50 microliters assay buffer. After incubation at room temperature for 30 minutes, 50 microliters stop solution are added to the wells and the absorbance of the plates are read at 490 nm. The absorbance values are used to reflect the amount of lactate dehydrogenase released from lysed cells and are a marker for cytotoxicity. Total cell lysis was induced in control wells and maximum

absorbance associated with these wells was found to be approximately 4. Higher absorbance indicates greater release of lactate dehydrogenase from lysed cells, with lower absorbance reflecting greater cell viability and integrity.

### **Statistical Analysis**

All numerical data were subjected to the D'Agostino-Pearson method to determine normality, and outliers were revealed using Grubbs' test. The data were then subjected to one-way ANOVA with post-hoc testing by Tukey's test.

### **Results**

#### **Pre-Incubation of Extracts with Virus Prior to Addition to Cells**

When the extracts were incubated with each virus for one hour prior to addition to cells, all extracts, including oseltamivir, significantly inhibited H1N1-mediated cytotoxicity at 1:40, and muscadine and oseltamivir inhibited cytotoxicity at 1:160. Oseltamivir approached significant inhibition at 1:640. (Figure 4.1) This same assay showed no significance against H3N2, though similar trends were maintained, with all extracts approaching significance at 1:40 and 1:160, and muscadine and oseltamivir approaching significance at 1:640. (Figure 4.2) When the extracts were added to cells one hour prior to viral addition (without extracts being removed), Sambucol<sup>®</sup> and oseltamivir significantly inhibited H1N1-mediated cytotoxicity at 1:40, with Sambucus<sup>®</sup> approaching significance at this concentration. Significance was not observed at lower concentrations against H1N1, though oseltamivir approached significance at 1:160 and 1:640, and muscadine approached significance at 1:640. (Figure 4.3) Using similar assay conditions, Sambucol<sup>®</sup> and oseltamivir significantly inhibited H3N2-mediated

cytotoxicity at 1:40, and muscadine and oseltamivir inhibited cytotoxicity at 1:160. Only oseltamivir inhibited cytotoxicity at 1:640. (Figure 4.4)

### **Simultaneous Addition of Extracts and Virus**

When extracts were added at the same time as the virus, all extracts showed significant inhibition of H1N1-mediated cytotoxicity at 1:40 and 1:160. Muscadine and oseltamivir also inhibited cytotoxicity at 1:640. At 1:40, Sambucol<sup>®</sup>, Sambucus<sup>®</sup>, and oseltamivir showed significantly lower cytotoxicity compared to muscadine.

Oseltamivir showed significantly lower cytotoxicity than both elderberry products at 1:160 and 1:640, with muscadine also showing significantly lower cytotoxicity at 1:640. (Figure 4.5) All extracts significantly inhibited cytotoxicity compared to H3N2 at 1:40 and 1:160, and oseltamivir inhibited cytotoxicity at 1:640. (Figure 4.6)

### **Addition of Extracts Up To 24 Hours After Addition of Virus**

Extracts at 1:160 dilution were added 1, 6, and 24 hours after addition of virus without washing away the virus in order to determine whether the extracts may have the capacity to overcome cell damage due to the virus. When extracts were added one hour after the virus, all extracts inhibited H1N1-mediated cytotoxicity, while only muscadine inhibited H3N2-mediated cytotoxicity. In the H3N2 assay, muscadine significantly inhibited cytotoxicity compared to the other extracts, including oseltamivir. (Figure 4.7) When extracts were added 6 hours after the virus, all fruit extracts significantly inhibited cytotoxicity compared to the H1N1 control and the oseltamivir positive control. For the H3N2 assay, muscadine and oseltamivir significantly inhibited cytotoxicity compared to virus alone; muscadine showed significantly lower cytotoxicity compared to the other extracts. (Figure 4.8) When extracts were added 24 hours after the virus, all extracts

showed inhibition of cytotoxicity compared to H1N1 alone, with muscadine showing significantly lower cytotoxicity compared to oseltamivir. Muscadine inhibited cytotoxicity not only compared to the H3N2 control, but compared to all other treatments. (Figure 4.9)

### **Removal of Extract and Addition of Virus; Removal of Virus and Addition of Extract**

Elderberry and muscadine extracts contain tannins, compounds which may precipitate proteins, including viral enzymes. In order to control for this, a set of experiments were designed in which the virus was added to the cells, removed six hours later and cells washed and then the fruit extracts were added, or the extracts were added to cells, removed six hours later and cells washed and then the virus was added. In this case, the cells were never exposed to both the virus and the extracts simultaneously. In order to directly compare the effect of the extracts based on phenolic content, these experiments were performed with extracts at 40 µg phenolics extracts per mL.

Oseltamivir was not used as a positive control in this set of experiments, as oseltamivir contains no precipitating or phenolic compounds. When extracts were added, removed at six hours, then the virus added, all extracts approached significance compared to viral control with the H3N2 assay, with muscadine showing significantly lower cytotoxicity compared to Sambucol<sup>®</sup>. No significant inhibition was seen with the H1N1 virus.

(Figure 4.10) When the virus was added for six hours, removed, then extracts added, all extracts inhibited cytotoxicity compared to both viruses, and Sambucol<sup>®</sup> showed significantly lower cytotoxicity than muscadine in the H1N1 assay. (Figure 4.11)

## **Discussion**

These results presented in this communication demonstrate that under certain experimental conditions elderberry and muscadine juice products show cytoprotection in both H1N1 and H3N2 infection. Most interesting, the fruit extracts were able to provide cytoprotection when added 1, 6 and 24 hours after H1N1 infection. These experiments were performed at the same phenolic concentration for all three extracts inhibited cytotoxicity at these time points. In contrast, only muscadine was able to provide cytoprotection in cells infected with H3N2 influenza strain of virus. These results demonstrate that these extracts can inhibit cytotoxicity up to 24 hours after viral infection, with muscadine showing particular benefit. It is interesting to note that oseltamivir, under these conditions, provides very little cytoprotection against the H3N2 virus when added after the initial viral infection; in contrast, muscadine provided an average of approximately 50% cytoprotection when added at these three timepoints. When the fruit extracts were added to cells and then removed prior to viral infection, no cytoprotection effects were observed. In contrast, when the virus was incubated with the cells for six hours and then removed, both the fruit extracts were able to provide significant cytoprotection against the effects of H1N1 and H3N2 viruses. These results demonstrate that the cytoprotective effects of these fruit extracts are not the result of a direct interaction of virus and the contents of these fruit extracts.

Roscheck, et al. found elderberry flavonoids to approach the efficacy of oseltamivir in vitro; a quercetin-related compound was found to have an EC<sub>50</sub> of 0.36  $\mu$ M compared to 0.32  $\mu$ M for oseltamivir against the H1N1 virus in an MTT cell viability assay.(27) A study involving a combination of elderberry and green tea found 50%

inhibition of cytopathic effect at 1:20.(28) While the methods for our experiments were different, it is worth noting that many of our studies showed significant inhibition of cytotoxicity at concentrations as low as 1:640. These researchers also found greatest effect when the treatment was combined with the virus directly, whereas our research showed effect hours after addition of virus, and the virus need not be present for the extract to produce benefits. Smee, et al. also found the green tea/elderberry product produce benefit when combined with oseltamivir in vivo.(28) Our findings also support a study conducted by Zakay-Rones, et al., which found Sambucol<sup>®</sup> to be effective against both the H1N1 and H3N2 viruses in vitro, though no positive control was used.(29)

On occasion, the effects of elderberry and muscadine varied between H1N1 and H3N2. Many other studies have used multiple influenza strains, especially strains with different isozymes, to study the effects of phenolics on these viruses, including both H1N1 and H3N2.(14, 16-19) Indeed, the green tea/elderberry study cited above found two different H3N2 strains to have quite different responses to treatment; one strain was especially sensitive and another especially resistant.(28) Various studies have found phenolics to bind neuraminidase in a different manner from oseltamivir and zanamivir, suggesting phenolic compounds may improve outcomes in combination with neuraminidase inhibitors, as combining products with different mechanisms of action is associated with greater benefit.(18, 30, 31) Indeed, studies have shown phenolic compounds to be synergistic with both oseltamivir and zanamivir.(17, 32) Again, the effect of elderberry and muscadine on influenza-induced cytotoxicity at various timepoints suggests these products may have various mechanisms of influenza inhibition, including hemagglutinin inhibition, neuraminidase inhibition, and general protection of

cell viability. Thus, products such as elderberry and muscadine, which contain several different phenolic compounds, may have particular benefit against various influenza strains, either alone or in combination with current influenza therapy.

Overall, our findings contribute significantly to the literature regarding the effect of elderberry against influenza. These findings support other data indicating that elderberry is effective against H1N1 and H3N2 and introduce muscadine as a potential alternative to oseltamivir in both H1N1 and H3N2.

### **Conclusions**

As stated above, neuraminidase inhibitors are currently the preferred treatment for influenza, yet these products may not be a viable treatment option for all patients.

Elderberry and muscadine showed inhibition of cytotoxicity and improvement of cell viability at various concentrations and timepoints, indicating elderberry or muscadine products may be a viable alternative to oseltamivir for patients for whom Tamiflu<sup>®</sup> may be cost-prohibitive. Overall, it appears elderberry and muscadine may have a role in the prevention or treatment of H1N1 and H3N2 influenza infection.

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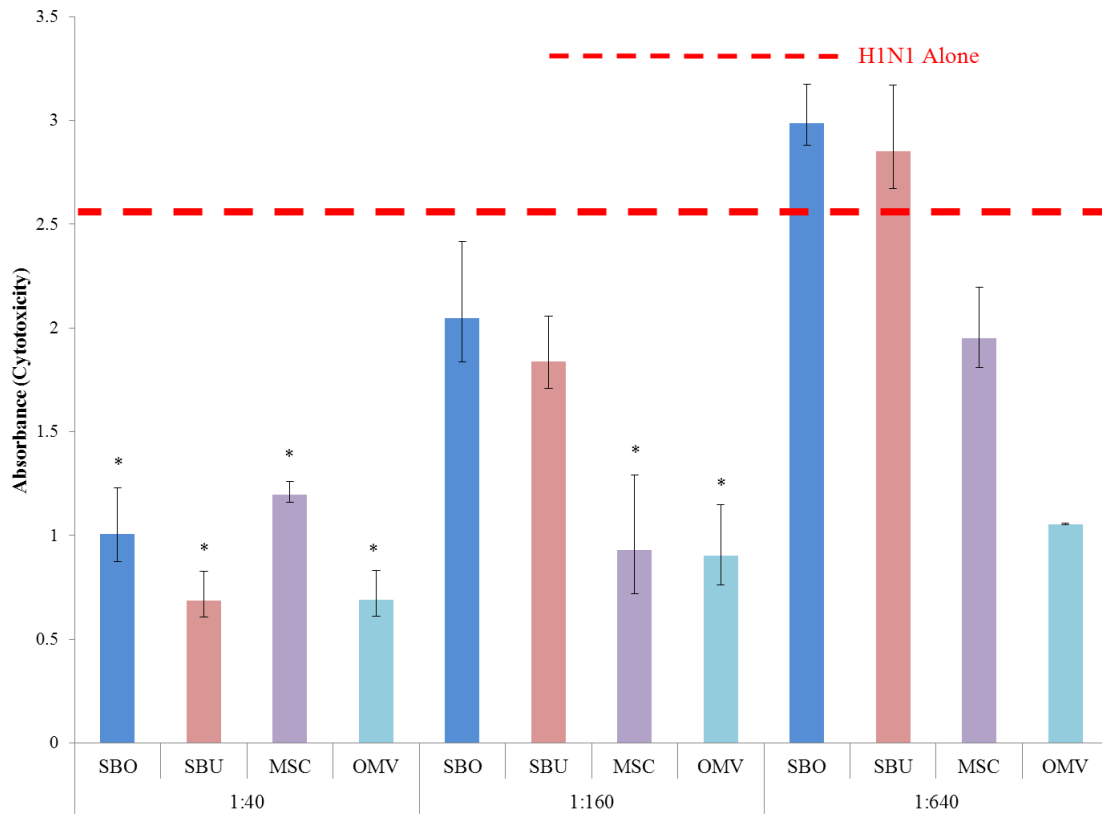
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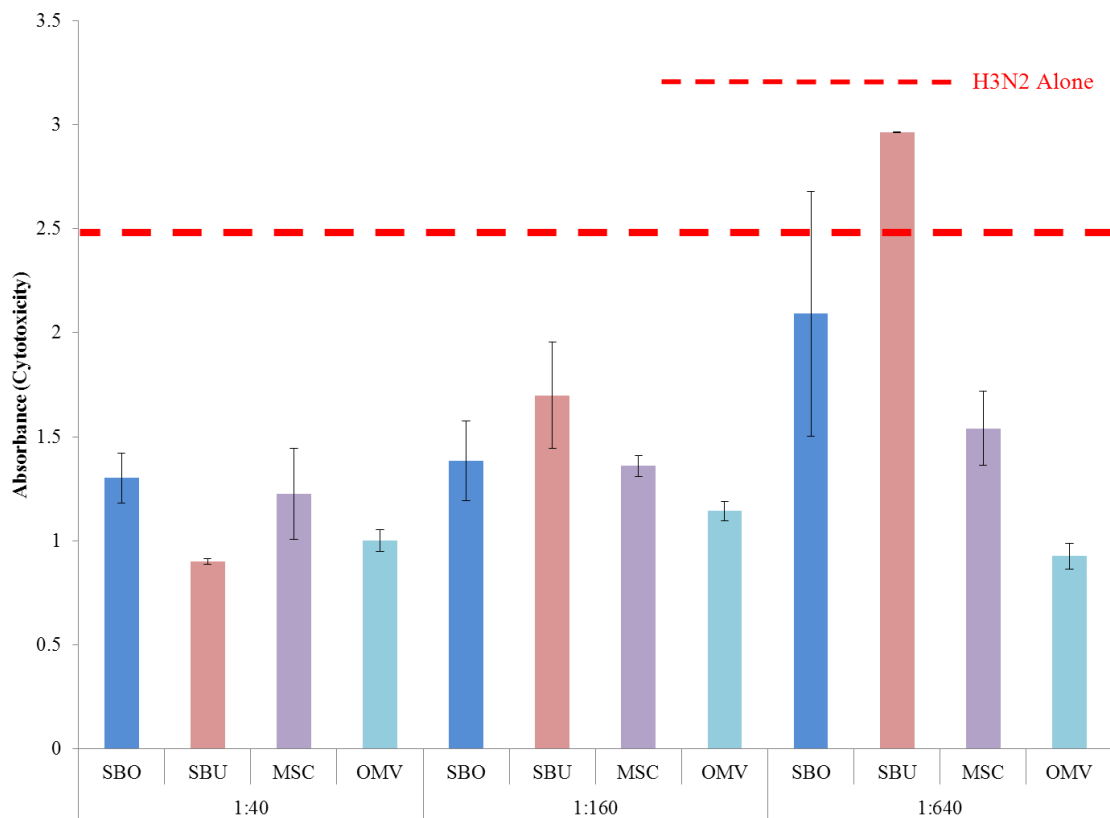
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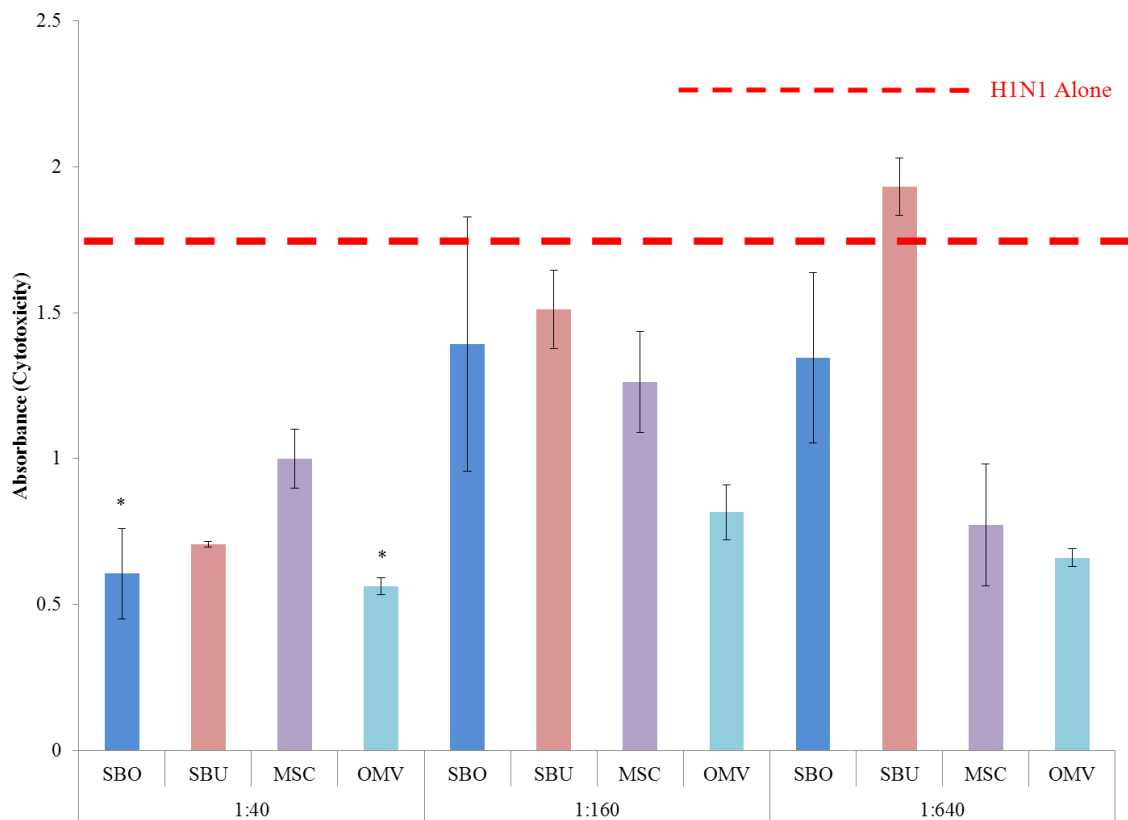
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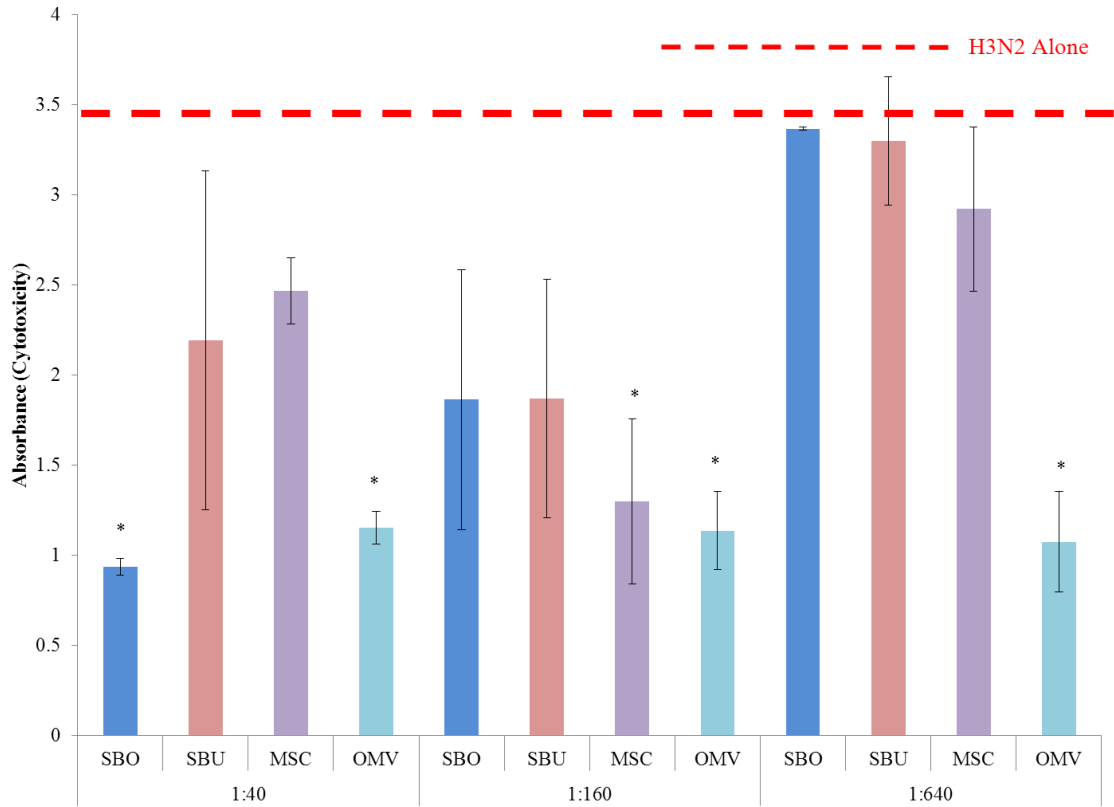
**Figure 4.1 Effect of Pre-Incubation on H1N1-Induced Cytotoxicity. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine, OMV = oseltamivir carboxylate. Extracts incubated with H1N1 virus for one hour prior to addition to MDCK cells. Results as mean  $\pm$  standard error absorbance for data in triplicate. \* = significant difference from virus alone,  $p < 0.05$ .**



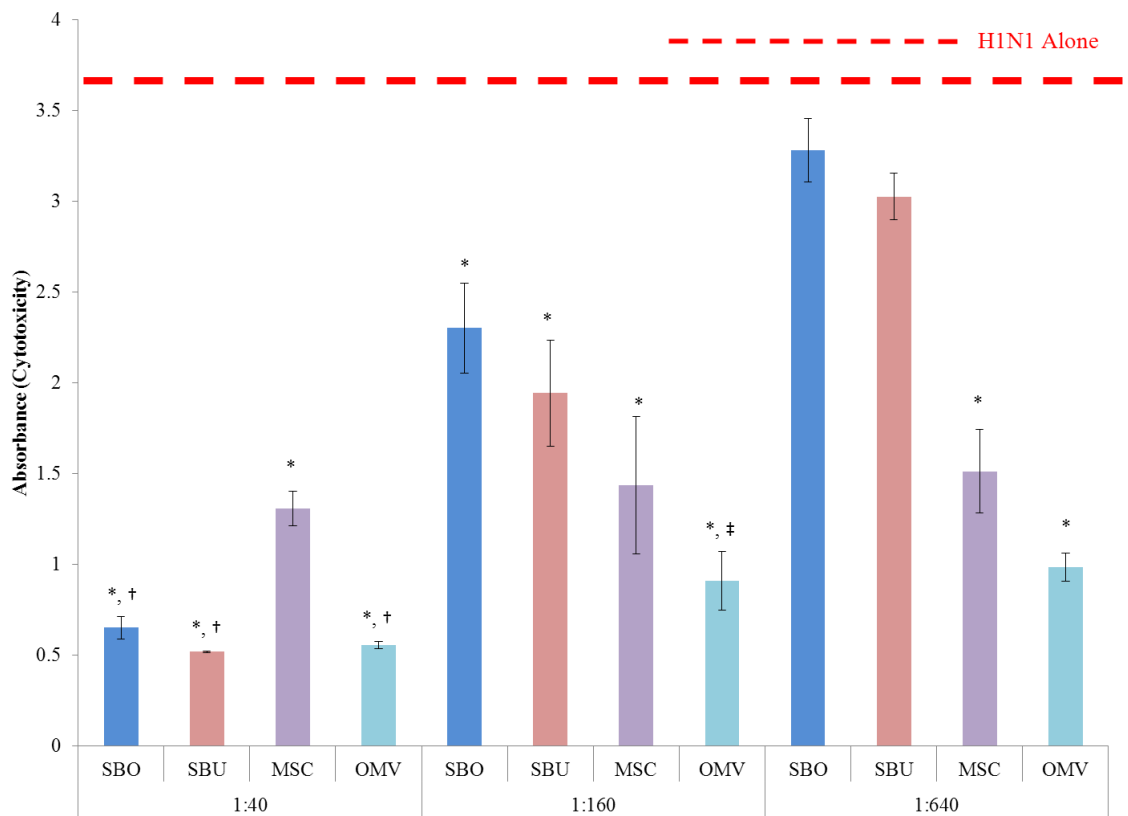
**Figure 4.2 Effect of Pre-Incubation on H3N2-Induced Cytotoxicity. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine, OMV = oseltamivir carboxylate. Extracts incubated with H3N2 virus for one hour prior to addition to MDCK cells. Results as mean  $\pm$  standard error absorbance for data in triplicate. No significant differences,  $p > 0.05$ .**



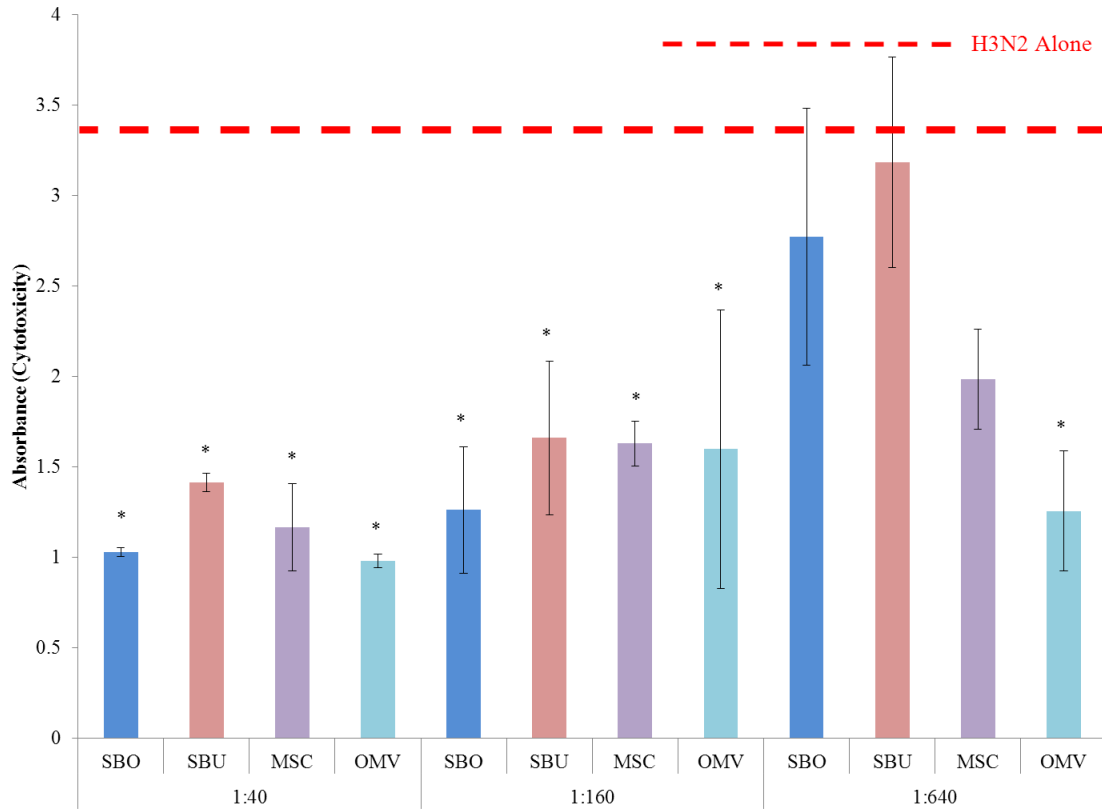
**Figure 4.3 Effect of Extract Pre-Incubation on H1N1-Induced Cytotoxicity. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine, OMV = oseltamivir carboxylate. Extracts added to MDCK cells one hour before H1N1 virus added. Results as mean  $\pm$  standard error absorbance for data in triplicate. \* = significant difference from virus alone,  $p < 0.05$ .**



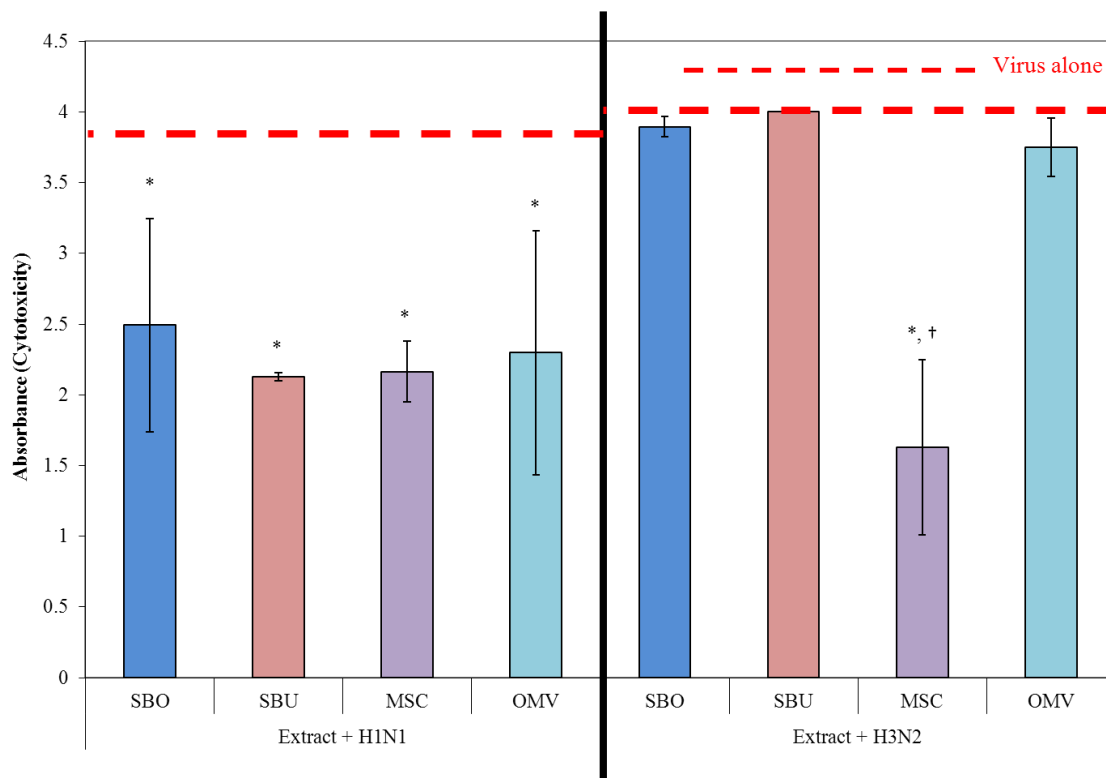
**Figure 4.4 Effect of Extract Pre-Incubation on H3N2-Induced Cytotoxicity. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine, OMV = oseltamivir carboxylate. Extracts added to MDCK cells one hour before H3N2 virus added. Results as mean  $\pm$  standard error absorbance for data in triplicate. \* = significant difference from virus alone.**



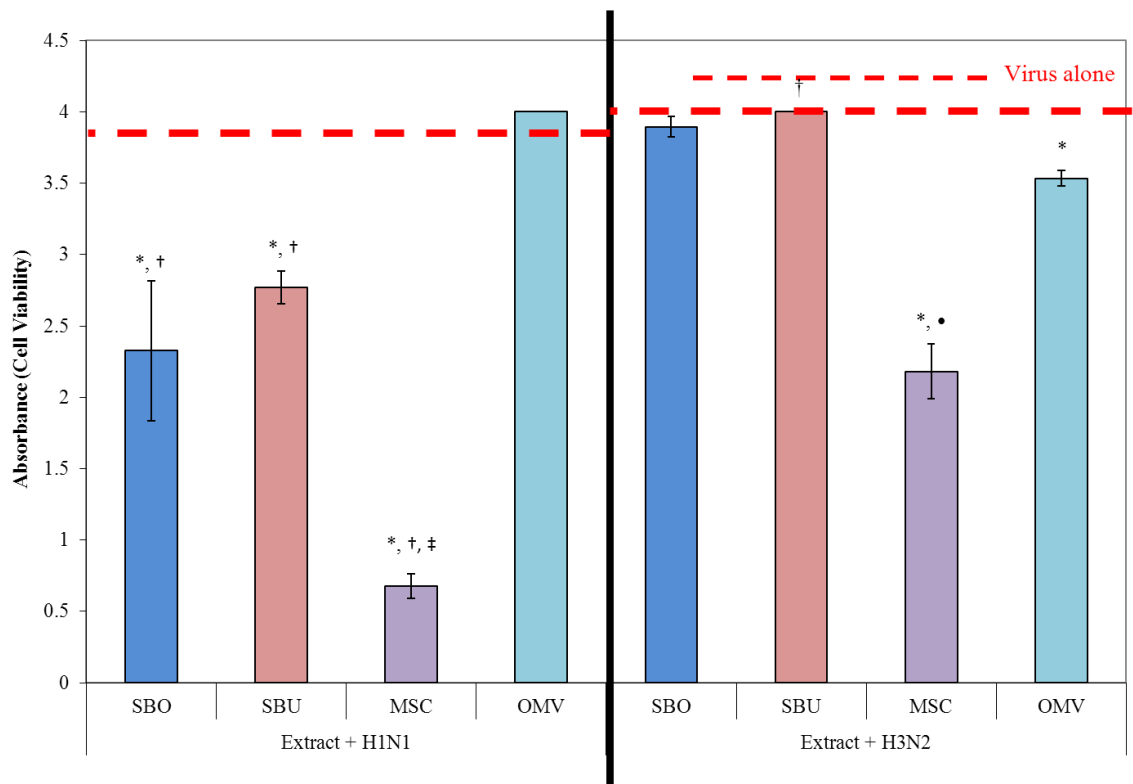
**Figure 4.5 Effect of Simultaneous Addition on H1N1-Induced Cytotoxicity.** SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine, OMV = oseltamivir carboxylate. Extracts and H1N1 virus added simultaneously to MDCK cells. Results as mean  $\pm$  standard error absorbance for data in triplicate. \* = significant difference from virus alone, † = significant difference from muscadine 1:40, ‡ = significant difference from Sambucol<sup>®</sup>, Sambucus<sup>®</sup> 1:640,  $p < 0.05$ .



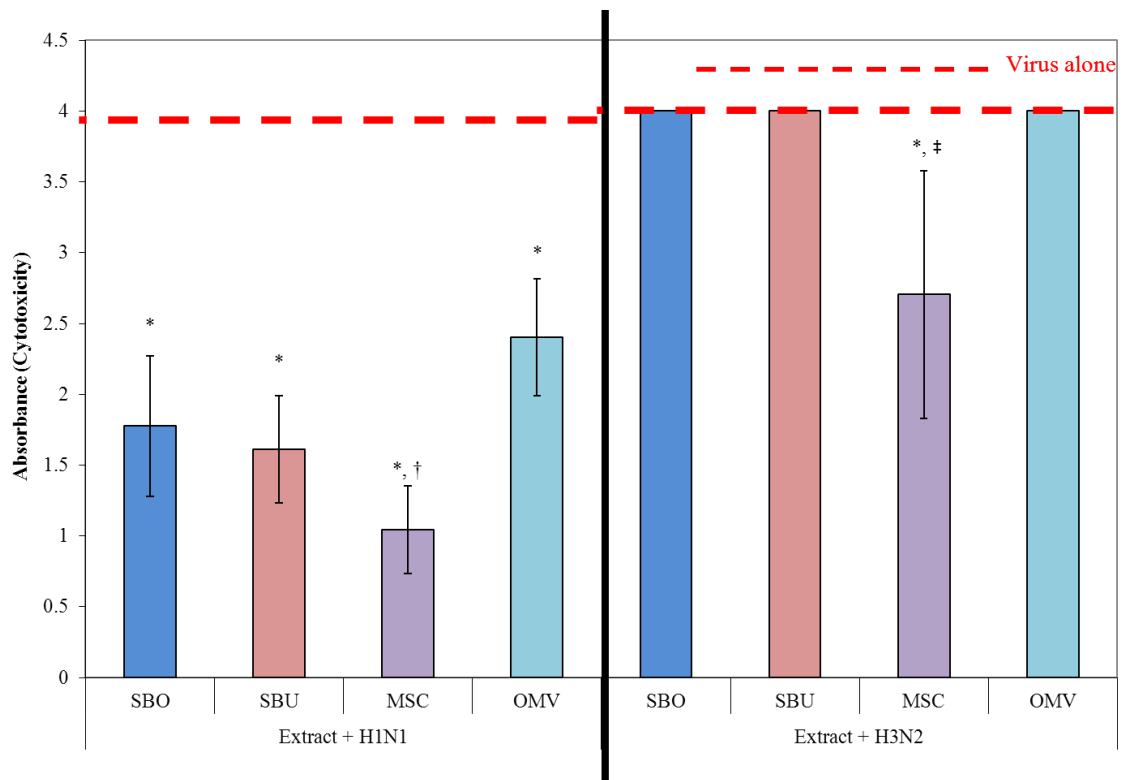
**Figure 4.6 Effect of Simultaneous Addition on H3N2-Induced Cytotoxicity. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine, OMV = oseltamivir carboxylate. Extracts and H3N2 virus added simultaneously to MDCK cells. Results as mean  $\pm$  standard error absorbance for data in triplicate. \* = significant difference from virus alone,  $p < 0.05$ .**



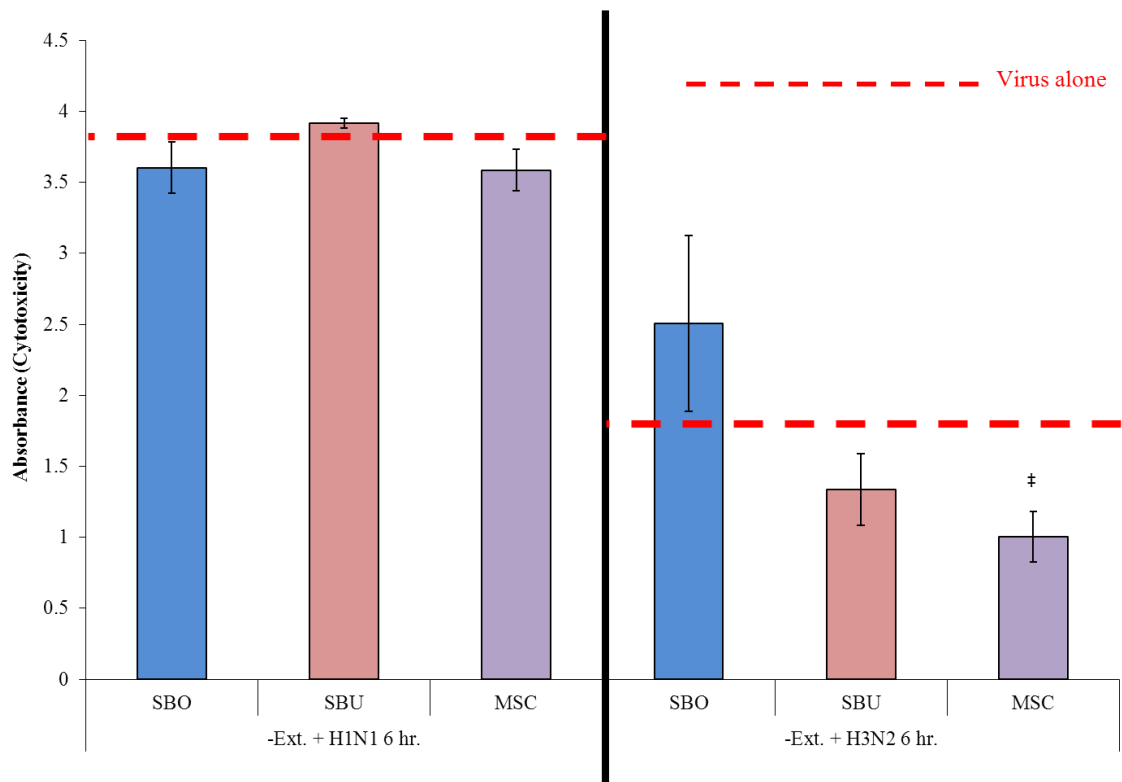
**Figure 4.7: Effect of 1 Hour Post-Viral Addition of Extracts (1:160) on Virus-Induced Cytotoxicity. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine, OMV = oseltamivir carboxylate. Extracts added 1 hour after addition of H1N1 or H3N2 virus to MDCK cells. Results as mean  $\pm$  standard error absorbance for data in triplicate. \* = significant difference from virus alone, † = significant difference from Sambucol<sup>®</sup>, Sambucus<sup>®</sup>, and oseltamivir,  $p < 0.05$ .**



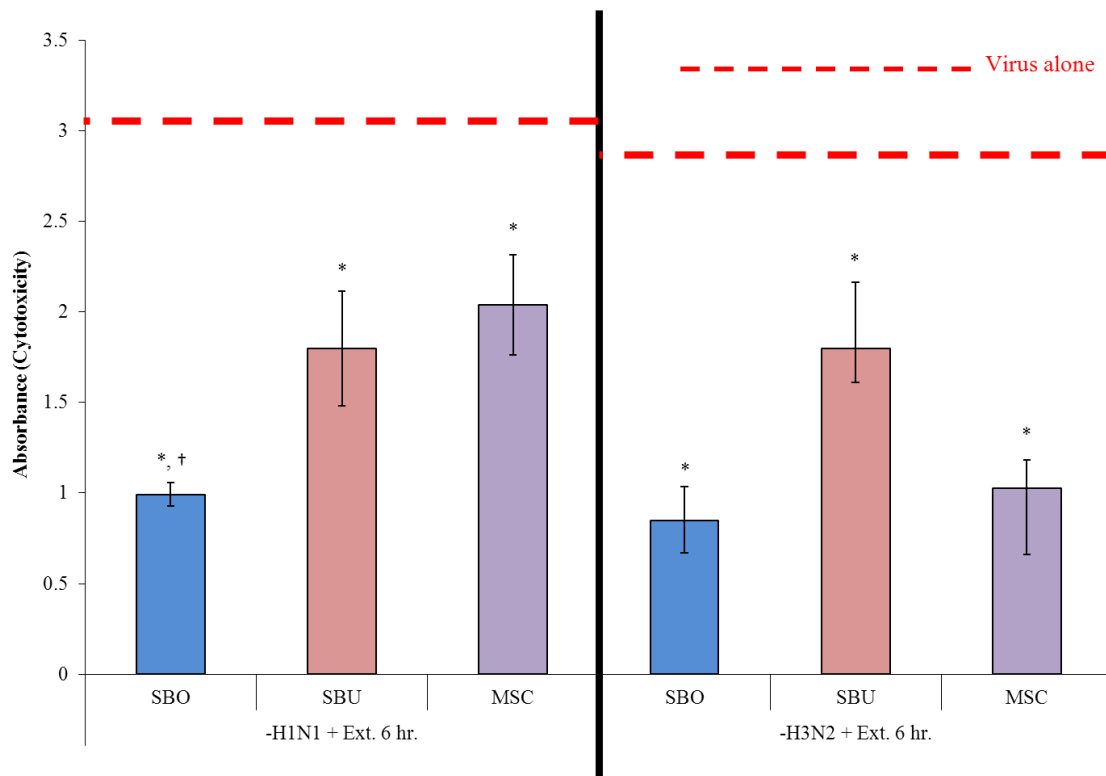
**Figure 4.8: Effect of 6 Hour Post-Viral Addition of Extracts (1:160) on Virus-Induced Cytotoxicity. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine, OMV = oseltamivir carboxylate. Extracts added 6 hours after addition of H1N1 or H3N2 virus to MDCK cells. Results as mean  $\pm$  standard error absorbance for data in triplicate. \* = significant difference from virus alone, ‡ = significant difference from Sambucol<sup>®</sup> and Sambucus<sup>®</sup>, † = significant difference from oseltamivir, • = significant difference from Sambucol<sup>®</sup>, Sambucus<sup>®</sup>, and oseltamivir  $p < 0.05$ .**



**Figure 4.9: Effect of 24 Hour Post-Viral Addition of Extracts (1:160) on Virus-Induced Cytotoxicity. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine, OMV = oseltamivir carboxylate. Extracts added 24 hours after addition of H1N1 or H3N2 virus to MDCK cells. Results as mean  $\pm$  standard error absorbance for data in triplicate. \* = significant difference from virus alone, † = significant difference from oseltamivir, ‡ = significant difference from Sambucol<sup>®</sup>, Sambucus<sup>®</sup>, and oseltamivir,  $p < 0.05$ .**



**Figure 4.10: Effect of Extract Removal, Viral Addition on Virus-Mediated Cytotoxicity.** SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine. Extracts at 40 µg phenolics/mL added at to MDCK cells 0 hour, removed at 6 hours, then virus added for remaining incubation time. Results as mean ± standard error absorbance for data in triplicate. ‡ = significant difference from Sambucol<sup>®</sup>, p<0.05.



**Figure 4.11: Results from Viral Removal , Extract Addition on Virus-Mediated Cytotoxicity. SBO = Sambucol<sup>®</sup>, SBU = Sambucus<sup>®</sup>, MSC = muscadine. Virus added at to MDCK cells 0 hour, removed at 6 hours, then extracts at 40  $\mu$ g phenolics/mL added for remaining incubation time. Results as mean  $\pm$  standard error absorbance for data in triplicate. \* = significant difference from virus alone, † = significant difference from muscadine,  $p < 0.05$ .**

## CHAPTER FIVE

### EFFECT OF TWO STYLES OF CRAFT BEER ON CELLS INFECTED WITH OSELTAMIVIR-RESISTANT H1N1

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## **Abstract**

With increasing resistance of influenza viruses to current pharmaceutical therapies, interest is turning to natural products that might provide antiviral activity. Various plant-based phenolic compounds have been shown to have anti-influenza effects, and beverages that provide high amounts of phenolics, such as craft beer, may prevent influenza infection. Two major ingredients of beer, hops and barley, both contain phenolic compounds that have been shown to inhibit influenza activity. We studied the effects of two beers from two different styles on Tamiflu<sup>®</sup>-resistant H1N1, a virus which is of particular concern due to its seasonal infectiveness and resistance to first-line therapy. We selected American pale ale, a beer high in hops, and stout, a beer high in barley, in order to study the contribution of these ingredients to beer's effect against influenza. Our studies revealed that Stone Pale Ale inhibited virus-mediated cytotoxicity at 1:10 volumetric concentrations, and Oskar Blues Dale's Pale Ale inhibited cytotoxicity at 1:20 and 40 µg phenolics/mL. Samuel Adams Cream Stout and North Coast Old No. 38 Stout inhibited cytotoxicity at 1:10 and 1:20; North Coast Old No. 38 Stout also inhibited cytotoxicity at 40 µg phenolics/mL. Because American pale ale is high in hops and stout high in barley, these results indicate that both beer ingredients may contribute to anti-influenza effects. These results suggest that craft beers may act, along with other phenolics present in the diet, to lessen or prevent the complications of influenza infection.

## **Introduction**

Influenza viruses, particularly the H1N1 virus that emerged in 2009, have been showing increasing resistance to pharmaceutical treatment options.(1) This is of particular concern, as the H1N1 virus has become endemic and is included in the annual influenza vaccine that seeks to prevent infection of the most common strains of influenza.(2) Even so, the influenza vaccine may not prevent all cases of influenza infection, due to contraindicating medical conditions, allergy or other adverse reaction to the vaccine, lack of availability, or mis-prediction of the influenza strains most likely to cause infection.(3-5) When influenza infections do occur, the treatment options include Tamiflu® (oseltamivir) and Relenza® (zanamivir).(6, 7) These medications act via a single mechanism of action; they inhibit neuraminidase, the influenza enzyme responsible for cleaving the sialic acid component of the virion and releasing the virus from the host cell. Thus, these medications prevent progeny virions from being released from the host cell and infecting other cells. Both medications are expensive and have no generic options available, limiting their use in certain patient populations. Additionally, these drugs have certain alarming side effects which may prevent their use. Relenza® in particular may not be of use in many patients due to bronchospasm, which occurs most commonly in patients with underlying respiratory issues, the very population that is at higher risk of contracting influenza.(8) Thus, many patients are limited to the use of Tamiflu® for influenza infection. However, because H1N1 has been associated with instances of Tamiflu® resistance, it is theoretically possible that patients with H1N1 may have no treatment options available.(1)

In addition to neuraminidase, two other enzymes may be inhibited in order to treat influenza infection: hemagglutinin and matrix proteins. Hemagglutinin is responsible for the binding and entry of the influenza virus to host cells, a mechanism in which neuraminidase has also been implicated recently.(9-11) The matrix protein is involved in the transport of hydrogen ions, allowing for the change in pH, which is necessary for the uncoating of the virus and the invasion of host cells.(12) Inhibitors of the matrix protein M2 are available but no longer recommended due to adverse effects and increasing viral resistance.(13-15)

Concerns regarding drug resistance have led many to turn to natural products and other options to treat influenza. Recent research into elderberry products has identified phenolic compounds that have been shown to have activity against the influenza virus.(16-19) Clearly, identification of a high phenolic, plant-based product that can be included in the diet to prevent influenza infection would be highly desirable, and a product that is highly palatable to most consumers would be ideal.

There are various mechanisms by which phenolics may inhibit influenza activity. Elderberry phenolics have been shown to inhibit hemagglutinin, as have phenolic compounds from various other plants.(16, 20-24) This is of particular interest, as no hemagglutinin inhibitors are currently indicated for influenza treatment, although research into inhibition of this enzyme is developing.(25) Also worth noting is that several of these hemagglutinin inhibitors are phenolics found in tea, a beverage that, along with beer, contributes significantly to phenolic intake in the American diet.(26) Additionally, some of these same phenolics also inhibit neuraminidase, as do various other phenolics.(27-29)

As mentioned above, beer, along with beverages such as tea and wine, contribute significantly to the amount of phenolics consumed by the typical American. Craft beer is widely and increasingly consumed, and as such represents a greater contributor to the amount of phenolics consumed.(30) Beer contains two major plants that contribute to its phenolic content: hops and barley. Hop provides a bitter taste to beer, and beer styles with significant amounts of hops include American pale ale, India pale ale, and imperial India pale ale. These beers tend to be lighter in color and may have a grassy or citrus-like bitter flavor due to the hop content. Beers containing barley tend to be darker in color, appearing dark brown to black. Barley may be processed by malting, kilning, and/or roasting in order to create various flavors, including caramel, coffee, or chocolate. Certain darker beers, such as porters and stouts, may also have coffee or chocolate added to them in order to enhance these flavors. Thus craft beer may have phenolic content due to hops and barley as well as added ingredients such as fruit, spices, coffee, and chocolate.(31)

Xanthohumol and related compounds are found exclusively in hops; therefore, beer is virtually the sole source of these compounds. While research is very limited, it is possible that xanthohumol may have anti-viral properties, although anti-influenza effects have yet to be established.(32-37) Limited data also suggests that barley phenolics may inhibit influenza activity. Ferulic acid, which has limited evidence of influenza inhibition, is contained in barley, especially roasted barley.(38, 39) Barley also contains chlorogenic acid and caffeic acids, which have been shown to inhibit neuraminidase, and thus, prevent viral infection.(40-42) All together, these functional ingredients of craft beer suggest that this beverage may have a role in prevention of influenza infection.

Because craft beer is widely and increasingly consumed in the U.S., identification of functional characteristics of this beverage would be ideal, as it would be readily integrated into typical American consumption.(30)

In order to study the effects of craft beer on influenza, two different styles of beer were selected: American pale ale and stout. American Pale Ale is a “lighter” beer, closer to the more widely consumed commercial beers than other craft beer. It is also higher in hops than darker beer like stout. Stouts are higher in roasted barley, and will thus be higher in ferulic acid and chlorogenic acid. Selection of these styles allowed for study of how hops and barley may each contribute to anti-influenza activity. Two beers from each style were selected. Because the utility of functional beverages against influenza is greatest in viruses likely to be infectious and without simple treatment, Tamiflu®-resistant H1N1 was selected for these studies.

## **Materials and Methods**

### **Materials**

Manin-Darby canine kidney cells were obtained from ATCC (Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM), phosphate-buffered saline (PBS) were obtained from Thermo Scientific (Suwanee, GA). Gentamicin was obtained from MP Biomedicals (Santa Ana, CA); TPCK-trypsin was obtained from Worthington (Lakewood, NJ). The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit was obtained from Promega (Madison, WI). The viral strain, A/Mississippi/3/2001, was propagated using chicken eggs. The Folin-Ciocalteu reagent, TPTZ (2, 4, 6-tri (2-pyridyl)-s-triazine, ferrous sulfate heptahydrate, and anhydrous

ferric chloride were purchased from Sigma Chemical Company (St. Louis, MO). Beers were purchased from Five Points Bottle Shop in Athens, GA.

### **Folin-Ciocalteu Method for Determining Total Phenolic Concentration**

Three volumetric dilutions and one phenolic concentration of craft beer were selected for these studies. Previous research on elderberry and muscadine found that a concentration of 40 µg phenolics/mL to be effective against the Tamiflu®-resistant H1N1 virus. Thus, the selected beers were subjected to the Folin-Ciocalteu assay(43) to determine the total phenolic content. Briefly, for the Folin-Ciocalteu method of determining total phenolic content, 20 µL of beer was combined with 1.58 mL of deionized water, 100 µL of Folin-Ciocalteu reagent, and 300 of µL sodium carbonate and incubated at room temperature for 45 minutes. Absorbance was measured at 765 nm and results were expressed in gallic acid equivalents per milliliter based on a standard curve.

### **Plaque Assay**

In order to calculate the plaque-forming units for the H1N1 strains to be used, 5 x 10<sup>5</sup> Manin-Darby canine kidney (MDCK) cells were incubated with DMEM + 5% FBS + 1% L-glutamine + 0.1% gentamicin growth medium in 12 well plates for 24 hours and grown to confluency. The H1N1 virus was subjected to 10-fold serial dilution in MEM infection media with 1µg/mL TPCK-treated trypsin. The growth media was aspirated from each well of the 12-well plate and rinsed with PBS. Dilutions of the H1N1 virus was then added to each well of the 12-well plate at 100 µL volumes. The plate was then incubated at 37°C with 5% CO<sub>2</sub> for 2 to 3 hours, after which the infection medium was aspirated and 2 mL 1.2% Avicel overlay treated with 1:1000 TPCK-trypsin was added to each well and incubated again for 72 hours. After 72 hours, the overlay was aspirated

and the cells rinsed with 1x PBS and fixed with cold acetone: methanol (60:40) for 10 minutes. After this fixing, the acetone: methanol solution was removed and the cells allowed to air dry. At this point, 1 to 2 mL Crystal Violet Solution was added to each well and incubated for 10 minutes at room temperature. Upon removal of the Crystal Violet and rinsing with deionized water, plaques visible in the violet stain were counted. The calculation of the virus titer was then performed as follows with a correction factor of 10:

(Number of plaques)(Dilution log)(Correction factor) = Plaque forming units (pfu) per mL

### **Cell Assays for Determination of Effect of Craft Beer on Oseltamivir-Resistant H1N1 Activity and Cytotoxicity**

Craft beer at concentrations of 1:10, 1:20, 1:40, and 40 µg phenolics per mL were added to MDCK cells grown to confluency in a 96-well plate along with 100 pfu oseltamivir-resistant H1N1. Ethanol control wells were also prepared to account for any effect of alcohol in craft beer. The cell plates were incubated for 48 hours before being subjected to the CytoTox 96® Non-Radioactive Cytotoxicity Assay and the hemagglutination assay. The pathogenesis of influenza involves virus-mediated cell death.(44) Death of infected cells not only directly damages tissues, but appears to be responsible for an inflammatory response that results in respiratory symptoms and other effects associated with influenza infection.(45) Measuring inhibition of cell death indicates the ability of a treatment to prevent tissue damage and other viral effects. The CytoTox 96® Non-Radioactive Cytotoxicity Assay is commonly used to determine the effect of treatment on influenza-induced cytotoxicity.(46-48) This assay involves the

reaction of a substrate with lactate dehydrogenase released into the supernatant due to cell lysis.(49) To perform this assay, 50 microliters supernatant are transferred from the cell plate to a 96-well round-bottom plate and combined with 50 microliters assay buffer. After incubation at room temperature for 30 minutes, 50 microliters stop solution are added to the wells and the absorbance of the plates are read at 490 nm. The absorbance values reflect the amount of lactate dehydrogenase released from lysed cells and are a marker for cytotoxicity. Maximum cell lysis was induced in control wells and maximum absorbance associated with these wells was found to be approximately 4. Higher absorbance indicates greater release of lactate dehydrogenase from lysed cells, with lower absorbance reflecting greater cellular viability and integrity.

The hemagglutination assay involved removal of 50  $\mu$ L supernatant from the 96-well plates and transfer to 96-well round-bottomed plates. Next, 50  $\mu$ L 0.5% red blood cells added to each well. The plates were incubated for 30 minutes to 1 hour at room temperature, at which point the plates were tilted to observe whether the red blood cells were agglutinated, i.e. formed a lattice due to hemagglutinin activity. Cloudy wells indicated hemagglutinin activity, whereas dots forming at the bottom of the well indicated no hemagglutinin activity.

### **Statistical Analysis**

All data were subjected to the D'Agostino-Pearson method to determine normality, and outliers were revealed using Grubbs' test. The data were then subjected to one-way ANOVA with post-hoc testing by Tukey's test.

## **Results**

The results of the American pale ale studies showed that Stone Pale Ale significantly inhibited virus-mediated cytotoxicity at the 1:10 dilution compared to virus alone, and Oskar Blues Dale's Pale Ale inhibited cytotoxicity at 1:20 and 40 µg phenolics/mL. Both American pale ales inhibited cytotoxicity by approximately 40% to 50% at 1:10 and 1:20; Oskar Blues Dale's Pale Ale inhibited cytotoxicity by approximately 62% at 40 µg phenolics/mL. (Figure 5.1) Oskar Blues Dale's Pale Ale inhibits hemagglutination at 1:10 and 40 µg phenolics/mL (in two out of three wells). (Table 5.1) Both Sam Adams Cream Stout and North Coast Old No. 38 Stout significantly inhibited cytotoxicity at 1:10 and 1:20, and North Coast Old No. 38 Stout inhibited cytotoxicity at 40 µg phenolics/mL. Both stouts inhibited cytotoxicity by approximately 40% to 70% at 1:10 and 1:20. (Figure 5.2) The hemagglutination assay indicated that Sam Adams Cream Stout 1:10 inhibited hemagglutinin activity, as did both stouts at 40 µg phenolics/mL. (Table 5.2)

## **Discussion**

These results indicate that higher concentrations of certain craft beers, i.e. 1:10 and 1:20, may show cytoprotection against Tamiflu®-resistant H1N1. One American pale ale inhibited cytotoxicity at the 1:10 concentration, and the other APA showed inhibition at 1:20 and 40 µg phenolics/mL. These results were borne out by the hemagglutinin assay. Both stouts showed inhibition of cytotoxicity at the 1:10 and 1:20 concentrations, with one stout inhibiting cytotoxicity at 40 µg/mL. Only one stout showed inhibition of hemagglutinin activity at the 1:10 and 40 µg phenolics/mL. Overall, then, it appears that some craft beers may show cytoprotection against this virus,

supporting their use as a functional beverage for prevention of viral infection. Because these beers are very different in style, with one being higher in hops and the other higher in barley, these data suggest both hop and barley phenolics may provide protection against this virus. No other studies have shown evidence of craft beer being a functional food in this regard, but these findings are consistent with studies that have shown products high in phenolics and antioxidants to have benefit against influenza and other viruses.

As the average volume of distribution of extracellular fluid is 15 liters, two 12 ounce servings of beer daily would approximate a 1:20 dilution. As previously mentioned, beer is a highly-consumed antioxidant beverage, along with coffee, tea, and wine, delivering a large amount of phenolics to the daily American diet.(26) Beer contains certain phenolics, notably caffeic acid and chlorogenic acid, which have been studied for anti-influenza effects.(40-42) Chlorogenic acid has been shown to inhibit H5N1 neuraminidase in a manner similar to oseltamivir.(41) It is worth noting that, as the neuraminidase enzyme of H5N1 is vulnerable to the same H274Y mutation that accounted for the oseltamivir resistance of the H1N1 strain used in this study, craft beer may show activity against oseltamivir-resistant H5N1 as well.(50) Furthermore, caffeic acid derivatives have been shown to inhibit neuraminidase enzymes against both N1 and N2 neuraminidase enzymes, suggesting that beverages containing caffeic acid, such as craft beer, may have activity against various influenza strains, due to inhibition of multiple influenza-associated isozymes.(40) Indeed, a study of a treatment containing green tea showed inhibition of multiple influenza strains, including multiple isolates of H1N1, H5N1, and H3N2.(51) This activity was attributed to catechins in green tea,

which were found to inhibit influenza via various mechanisms, including inhibition of adsorption due to hemagglutinin activity. Catechins have been found in beer and its barley component, and this may account for the hemagglutination effects seen with certain concentrations of beer studied here.(52, 53)

### **Conclusions**

Craft beer appears to be a viable functional beverage for prevention of viral infection. This is encouraging, as many patients prefer such palatable beverages as beer over other functional options. Because craft beer is so widely consumed, this may allow patients to select particular beers that may prevent influenza infection during the annual influenza season. As studies have shown that phenolics inhibit influenza via various mechanisms, it is likely the beverages such as beer, which contain a variety of phenolics, may prevent influenza infection due to multiple mechanisms of inhibition.

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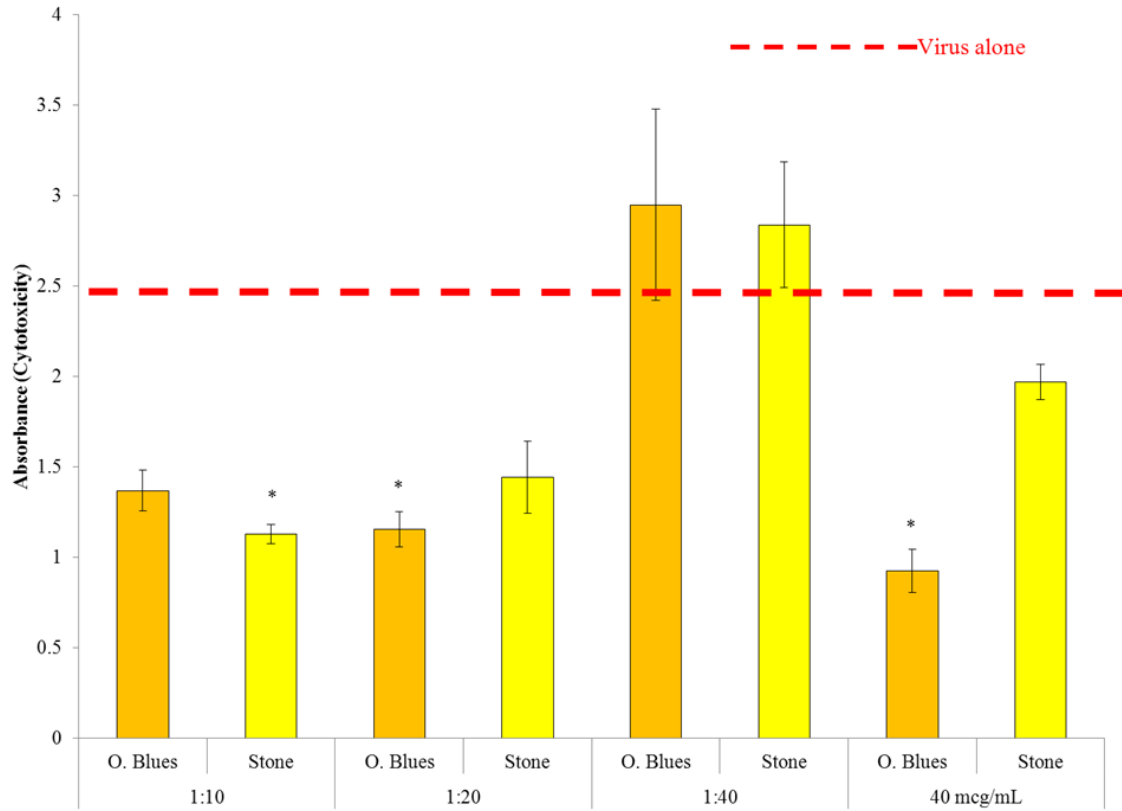
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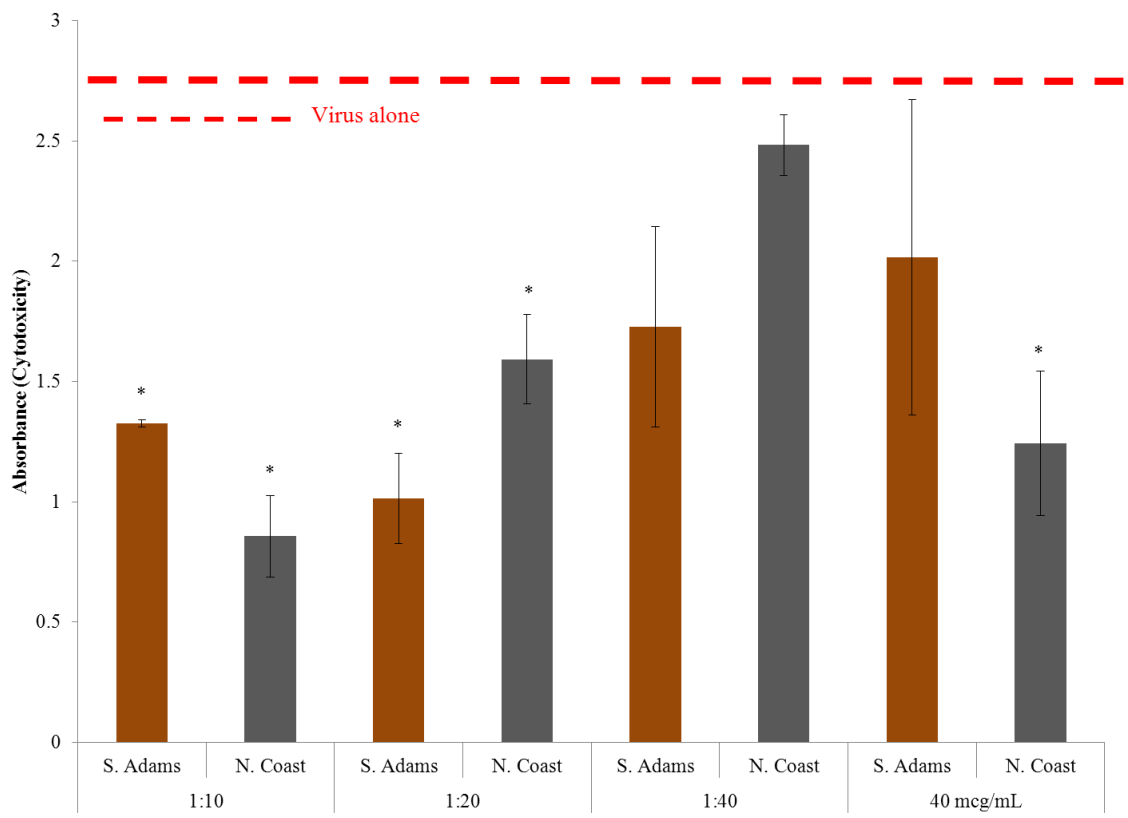
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**Figure 5.1: Inhibition of Influenza-Induced Cytotoxicity by American Pale Ale. O. Blues = Oskar Blues Dale's Pale Ale; Stone = Stone Pale Ale. Results as mean  $\pm$  standard error craft beer for data in triplicate. \*=significant difference from virus alone,  $p < 0.05$ .**

**Table 5.1: Effect of American Pale Ale on Influenza Hemagglutinin Assay. O. Blues = Oskar Blues Dale's Pale Ale; Stone = Stone Pale Ale. ND = no dot observed (i.e. hemagglutinin activity) • = dot formed (i.e. inhibition of hemagglutinin activity)**

1:10		1:20		1:40		40 mcg/mL	
O. Blues	Stone	O. Blues	Stone	O. Blues	Stone	O. Blues	Stone
•	ND	ND	•	ND	ND	ND	ND
•	ND	ND	ND	ND	ND	•	ND
•	ND	ND	ND	ND	ND	•	ND



**Figure 5.2: Inhibition of Influenza-Induced Cytotoxicity by Stout. S. Adams = Sam Adams Cream Stout; N. Coast = North Coast Old No. 38 Stout. Results as mean  $\pm$  standard error craft beer for data in triplicate. \*=significant difference from virus alone,  $p < 0.05$ .**

**Table 5.2: Effect of Stout on Influenza Hemagglutinin Assay. S. Adams = Sam Adams Cream Stout; N. Coast = North Coast Old No. 38 Stout. ND = no dot observed (i.e. hemagglutinin activity) • = dot formed (i.e. inhibition of hemagglutinin activity).**

1:10		1:20		1:40		40 mcg/mL	
S. Adams	N. Coast	S. Adams	N. Coast	S. Adams	N. Coast	S. Adams	N. Coast
•	•	ND	ND	ND	ND	•	ND
•	ND	ND	ND	ND	ND	•	•
•	ND	ND	ND	ND	ND	•	•

CHAPTER SIX  
INHIBITION OF PROTEIN GLYCATION BY CRAFT BEER

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## **Abstract**

Protein glycation is a non-enzymatic process in which amino residues of proteins react with sugar groups, resulting in cross-linking of proteins that can cause many complications associated with Type 2 diabetes. Various fruits and vegetables have been found to inhibit protein glycation, usually due to their phenolic compounds. Craft beer, which has been shown to contain significant amounts of phenolic compounds, has been increasing in market share of late. Because of the high volume in which craft beer is consumed, it has the potential to be an excellent phenolic delivery system. To this end, we endeavored to determine whether craft beer might inhibit protein glycation and thus be a part of a functional diet to prevent diabetic complications. Of the craft beer styles studied (American pale ale, porter, stout, Imperial India pale ale, and pumpkin beer) all beers significantly inhibited protein glycation on a volumetric (4  $\mu\text{L}/\text{mL}$ ) basis, and all but pumpkin beer inhibited glycation based on phenolic content (4  $\mu\text{g}$  phenols/ $\text{mL}$ ). All beers significantly inhibited protein glycation based on antioxidant content, showing significant inhibition compared to both control and a commercial American domestic. A wider range of porters was studied and found to inhibit protein glycation based on volume (again, 4 $\mu\text{L}/\text{mL}$ ). A porter and Imperial India pale ale also inhibited dicarbonyl formation, a highly reactive compound formed in the process of protein glycation. The studies demonstrate that craft beer inhibits protein glycation, showing clear superiority in this regard over commercial American domestic beer. Because craft beer is a good delivery device for phenolics, selecting craft beer over commercial beer may have a role in a functional diet to prevent diabetic complications.

## **Introduction**

Hyperglycemia, a hallmark of diabetes mellitus, is associated with various health complications. Acute hyperglycemia has been shown to induce reversible oxidative stress, whereas chronic hyperglycemia can cause irreversible modifications to proteins and vasculature.(1) One of these modifications to proteins is known as protein glycation. Protein glycation, the non-enzymatic reaction of sugars with proteins, has been implicated in various complications of diabetes mellitus.(2) This reaction is associated with oxidative stress, and thus antioxidants have been implicated as a means of preventing glycation-associated diabetic complications.(3)

Protein glycation involves the reaction of a reducing sugar (glucose, fructose, galactose, mannose, or ribose) with the amino group of endogenous proteins.(4) This initial reaction yields a Schiff base, which undergoes rearrangement to an Amadori product. In the second stage of protein glycation, the Amadori product is degraded to dicarbonyl compounds, which are more reactive than the parent sugar.(3) These reactive products may either cross-link proteins directly, or may form advanced glycation endproducts, and are associated with the production of reactive oxygen species.(3, 5) The dicarbonyl compounds are primarily responsible for inter- and intra-crosslinking of proteins, which produce structural and functional changes of these proteins.(5) Clearly, this process can affect endogenous proteins and their role in the body.

Various phenolic compounds have been shown to inhibit protein glycation. Foods high in phenolics, including fruit, spices, and grains have been shown to inhibit protein glycation.(5-8) They have also been implicated in improvement in other pathways

associated with hyperglycemia, including IL-1 $\beta$  activation and generation of reactive oxygen species by monocytes.(1)

Craft beer, or beer from any brewery producing less than six million barrels a year(9) is made from hops and barley, grains shown to have significant phenolic content.(10, 11) Hop provides a bitter taste to beer, and beer styles with significant amounts of hops include American pale ale, India pale ale, and Imperial India pale ale. These beers tend to be lighter in color and may have a grassy or citrus-like bitter flavor due to the hop content. Beers containing barley tend to be darker in color, appearing dark brown to black. Barley may be processed by malting, kilning, and/or roasting in order to create various flavors, including caramel, coffee, or chocolate. Certain darker beers, such as porters and stouts, may also have coffee or chocolate added to them in order to enhance these flavors.(12) Thus craft beer may have phenolic content due to hops and barley as well as added ingredients such as fruit, spices, coffee, and chocolate. Craft beer is high in gallic acid and ferulic acid, compounds shown to inhibit protein glycation.(13, 14) Certain styles of craft beer, such as pumpkin beer, contain spices, which again have been shown to inhibit protein glycation.(8) Coffee, an excellent source of caffeic acid and chlorogenic acid, is also present in some craft beers, also potentially contributing to an effect on protein glycation.(15) All these ingredients contribute to the high content of phenolics in craft beer. Additionally, craft beer represents a wide range of beer styles, and hence a wide range of phenolic compounds. Thus, craft beer may be an excellent functional beverage, due to its potential to inhibit protein glycation and the volume in which it tends to be consumed (typically twelve ounces for American craft beer). As phenolics have been shown to inhibit protein glycation, beverages such as craft beer,

which contribute significantly to phenolic intake, may help prevent protein glycation and thus certain diabetic complications.(29)

## **Materials and Methods**

### **Beer Selection**

The styles of beer used for this study were American Pale Ale (APA), porter, stout, Imperial India pale ale (IPA), and pumpkin beer. (Table 5.1) All beers were purchased from the same store in Athens, GA. A commercial American beer was also acquired for comparison purposes.

### **Total Phenolic Content and Antioxidant Power Assays**

In order to determine the phenolic and antioxidant contents of craft beer, the beers were subjected to the Folin-Ciocalteu and Ferric-Reducing Antioxidant Power (FRAP) assays.(16, 17) Briefly, for the Folin-Ciocalteu method of determining total phenolic content, 20  $\mu$ L beer was combined with 1.58 mL deionized water, 100  $\mu$ L Folin-Ciocalteu reagent, and 300  $\mu$ L sodium carbonate and incubated at room temperature for 45 minutes. Absorbance was measured at 765 nm and results were expressed in gallic acid equivalents. For the FRAP assay, the reagent was made fresh daily by combining 2.5 mL 20 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 2.5 mL 10 mM TPTZ (in 40 mM HCl), and 25 mL of 300 mM acetate buffer. The beer samples were added at 30  $\mu$ L and combined with 300  $\mu$ L reagent, incubated for 5 minutes, and then 340  $\mu$ L deionized water added. Absorbance was measured at 593 nm and results expressed as mmol Fe (II) equivalents per L based on the  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  standard curve.

## **Protein Glycation Assay**

In order to study the effects of craft beer on protein glycation, the methods by McPherson, et al. were modified.(18) Briefly, 10 mg/mL bovine serum albumin was incubated with 250 mM fructose in 200 mM phosphate buffer (pH 7.4) in the presence of 0.02% sodium azide and various volumetric, phenolic, and antioxidant concentrations of each beer sample for 3 days at 37 °C. The fluorescent products formed by the reaction of sugar and amino groups were then detected at excitation/emission wavelength pair of 370 nm/440 nm with a Perkin-Elmer LS 55 Luminescence Spectrophotometer. Beer was added on a volumetric basis at 4 $\mu$ L/mL, a phenolic basis at 4  $\mu$ g phenolics/mL, and an antioxidant basis at 60 nmol Fe(II) equivalents/mL. Fluorescence of each sample was corrected by subtracting the fluorescence of albumin incubated with each beer. A dicarbonyl assay was also performed under these same incubation conditions using a modified protein carbonyl colorimetric assay(19) using 2,4-dinitrophenylhydrazine (DNPH). In these experiments, 30  $\mu$ L of beer was added on both days 1 and 4 of the incubation period and the samples analyzed on day 5. The DNPH reacts with protein carbonyls and generates a hydrazine product that can be quantified at an absorbance of 375 nm. This assay was done in order to determine whether the beers can inhibit the formation of dicarbonyl intermediates in protein glycation. All samples were analyzed in triplicate and the results analyzed via one-way ANOVA with post-hoc testing by Tukey's test.

## **Results**

When beer was added on a volumetric (4 $\mu$ L/mL) basis, all beers inhibited protein glycation significantly compared to control, with porters producing the greatest

inhibition, with a greater than 40% decrease of the control fluorescence. (Figure 5.1)

When beer was added on the basis of phenolics, all beer except the Great Divide Hercules Double IPA and Weyerbacher Imperial Pumpkin Ale significantly inhibited protein glycation, with the double IPA and pumpkin beer having essentially no effect on fluorescence. (Figure 5.2) Beer based on antioxidant content showed significant inhibition with all beers compared to control and to the major commercial American beer, with most beers decreasing fluorescence by approximately 30% to 40%, whereas the commercial beer increased fluorescence by approximately 12% over controls. (Figure 5.3) Because porter showed the greatest inhibition of protein glycation on a volumetric basis, five additional porters were analyzed and all significantly inhibited protein glycation, decreasing fluorescence by at least 15%, with Founders' Porter showing greatest inhibition at approximately 37% decrease in fluorescence. (Figure 5.4) Because Founders' Porter most consistently inhibited protein glycation, it was selected for the dicarbonyl study, along with Imperial IPA, which showed less inhibition than American Pale Ale (APA), despite being more highly hopped than APA. Both these beers showed significant inhibition compared to control, with similar levels of inhibition being achieved at 77% and 76% of control, respectively. (Figure 5.5)

## **Discussion**

Protein glycation, a normal part of the aging process, is accelerated in diabetes mellitus. It is of particular concern with the proteins collagen and lens crystallin, which show very slow turnover.(20) Protein glycation as recognized as early as the 1900s, and while it was known to have a role in food chemistry, it wasn't until the 1970s and 1980s that it was found to be a biomarker, with hemoglobin A1c found to be a means of

monitoring glycemic control in diabetic patients.(21-23) The Amadori reaction products generated by rearrangement of the Schiff base tend to accumulate on proteins and form fluorescent glycation products. Dicarbonyl intermediates are formed from degraded glucose reacting with Amadori products. Dicarbonyls can themselves cross-link proteins or may undergo dehydration to Amadori dione or Amadori ene-diones that are extremely reactive and prone to cross-linking.(24-26)

Protein glycation of collagen can cause vascular damage, resulting in atherosclerosis, coronary disease, renal damage, retinopathy, and impaired circulation, all of which are well-known complications of diabetes mellitus.(20) Because of the wide-ranging implications of protein glycation, dietary intervention to prevent this glycation is of increasing interest. The role of diet in preventing Type 2 diabetes mellitus and in controlling diabetic complications is well-established.(27) Because interest in craft beer is increasing and beer is an excellent delivery system of antioxidants due to the volume at which it is consumed, this beverage has the potential to be included as part of a diet to prevent diabetic complications.

American pale ale has a low to medium malt flavor (often describe as caramel-like) and a medium to medium-high hop character (i.e. bitterness, citrus, etc.). It is often considered to be one of the craft beer styles closest to the more widely-consumed commercial American beers. India pale ale has medium-high to very high hop bitterness, and medium maltiness. Imperial IPA has higher alcohol content than IPA and very high hop bitterness. According to the Brewers Association 2013 Beer Style Guidelines, “The intention of this style of beer is to exhibit the fresh and bright character of hops”.(12) Porter is dark brown to very dark in color, with acceptable roasted malt flavor, but not

roasted barley. Stout, a black-colored beer, should contain the flavor of roasted barley. Pumpkin beers must contain pumpkin or winter squash, which should be obvious in the flavor. They may contain the spices often associated with pumpkin flavor, but this is not necessary.(12)

All beers consistently inhibited protein glycation, with porters and stouts in particular showing excellent inhibition. Because both these beers contain high amounts of barley, one may infer that phenolics contained in barley (ferulic acid, chlorogenic acid, etc.) may contribute to the inhibition of protein glycation. The consistent inhibition of glycation by American pale ale is interesting, as APA shows much lower phenolic and antioxidant content than stout, yet it appears that hop- and barley-based phenolics inhibit protein glycation to a similar degree.

Since a large variety of phenolic compounds inhibit protein glycation, these experiments were performed using rather small concentration of craft beers (1:250 dilution v/v). Our laboratory has found that phenolic compounds that are excellent inhibitors of glycation are capable of producing significant inhibitory effects at concentrations of 5 µg phenolics/ml. Thus, we tailored the experimental conditions to differentiate those craft beers that have significant inhibitory activity from those beers that contain phenolics that are not capable of affecting glycation at such a low concentration. Hence, it is not that surprising that some beers like commercial American beer (at an antioxidant concentration of 60 nmol Fe[II]/mL) and Great Divide Hercules Double IPA and Weyerbacher Imperial Pumpkin Ale (at 4 µg phenolics/ml) did not produce any effect on glycation. Phenolics present in large variety of natural products are also incapable of inhibiting glycation at these concentrations, while others like the

ellagic acid present in muscadine can inhibit glycation by approximately 90% at 5  $\mu\text{g}$  phenolics/ml.(5)

The fact that porters and stouts consistently inhibited protein glycation to a greater degree than other beer styles again suggests that compounds that inhibit protein glycation may be present in higher amounts in porter and stout. Additionally, because craft beer inhibits glycation compared to commercial beer, it appears that these beers contain glycation-inhibiting compounds in greater quantities than commercial beer, thus demonstrating superiority of these craft beer styles over more widely-consumed beer.

### **Conclusions**

Taken together, these data indicate that craft beer may have a role as a functional beverage to prevent protein glycation and diabetic complications associated with it. While our lab has previously demonstrated porters and stouts have greater phenolic and antioxidant content than lighter beers, the fact that an American Pale Ale inhibited protein glycation is especially interesting; APA is the craft style closest to commercial American beer. As such, it is possible that consumers accustomed to commercial beer may more easily transition to APA rather than other beer styles. Additionally, craft beer consumers concerned about diabetic complications may also use these results to select certain beer styles over others. This research establishes the possibility of craft beer as a functional food to inhibit protein glycation and thus potentially prevent the development of complications due to Type 2 diabetes. Ideally, a craft beer can now be brewed with the specific intent of inhibiting protein glycation, choosing styles and ingredients that have been shown in this study and others to have beneficial effect in this regard.

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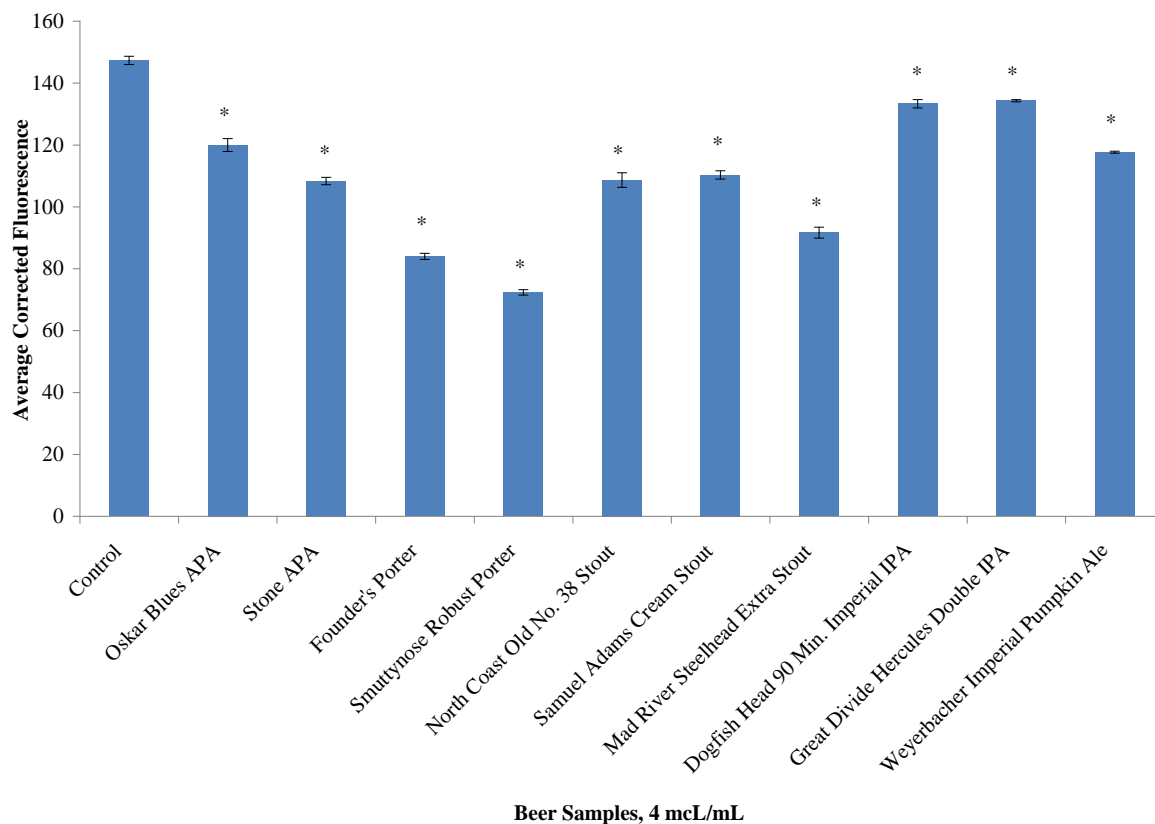
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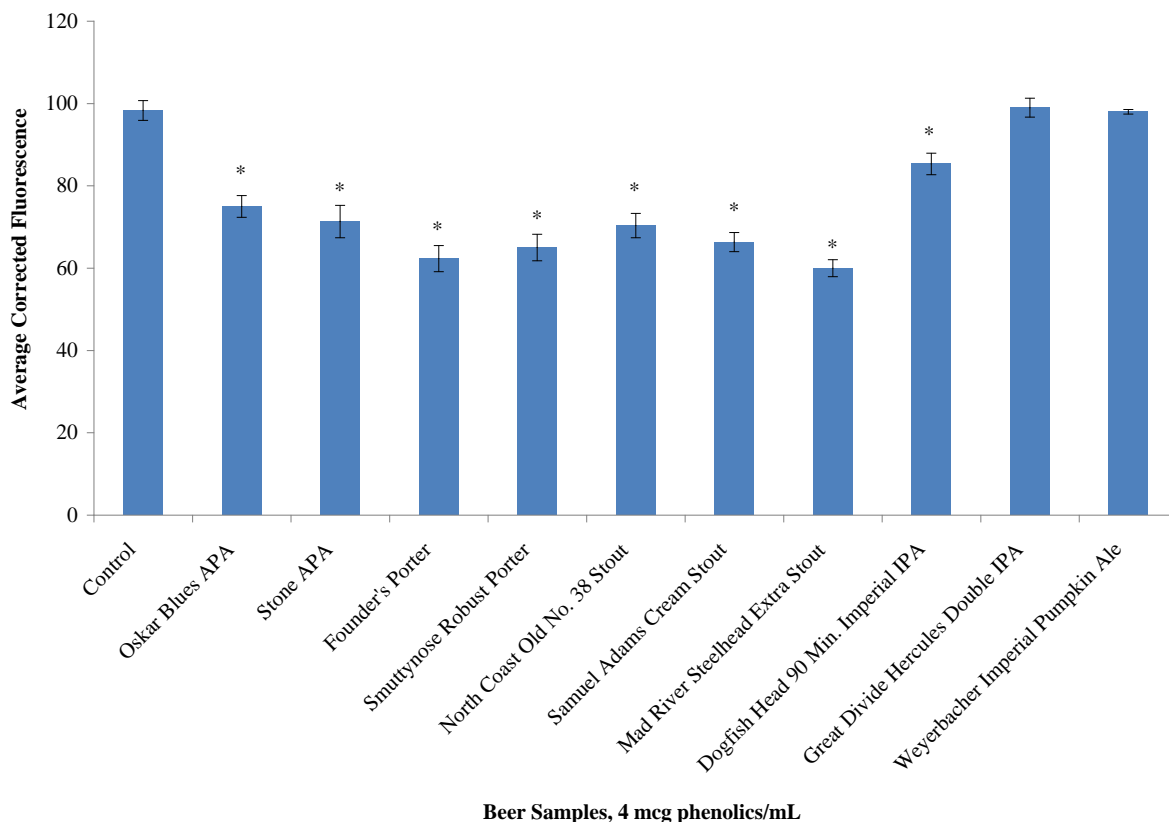
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**Table 5.1 Beers Selected for Analysis**

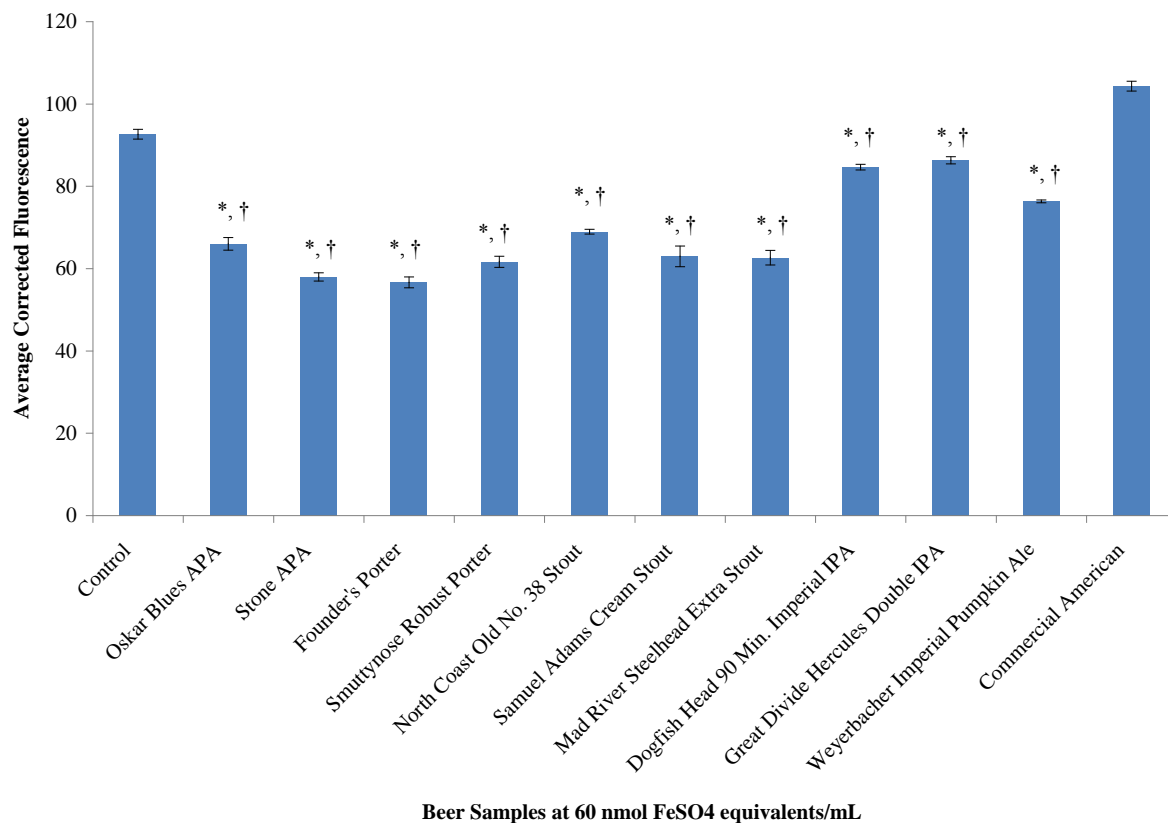
<b>Beer Style</b>	<b>Beer Name</b>
American Pale Ale	Oskar Blues Dales' Pale Ale Stone Pale Ale
Porter	Founders Porter Smuttynose Robust Porter
Stout	North Coast Old No. 38 Stout Samuel Adams Cream Stout Mad River Steelhead Extra Stout
Imperial India Pale Ale	Dogfish Head 90 Minute Imperial IPA Great Divide Double IPA
Pumpkin	Weyerbacher Imperial Pumpkin Ale



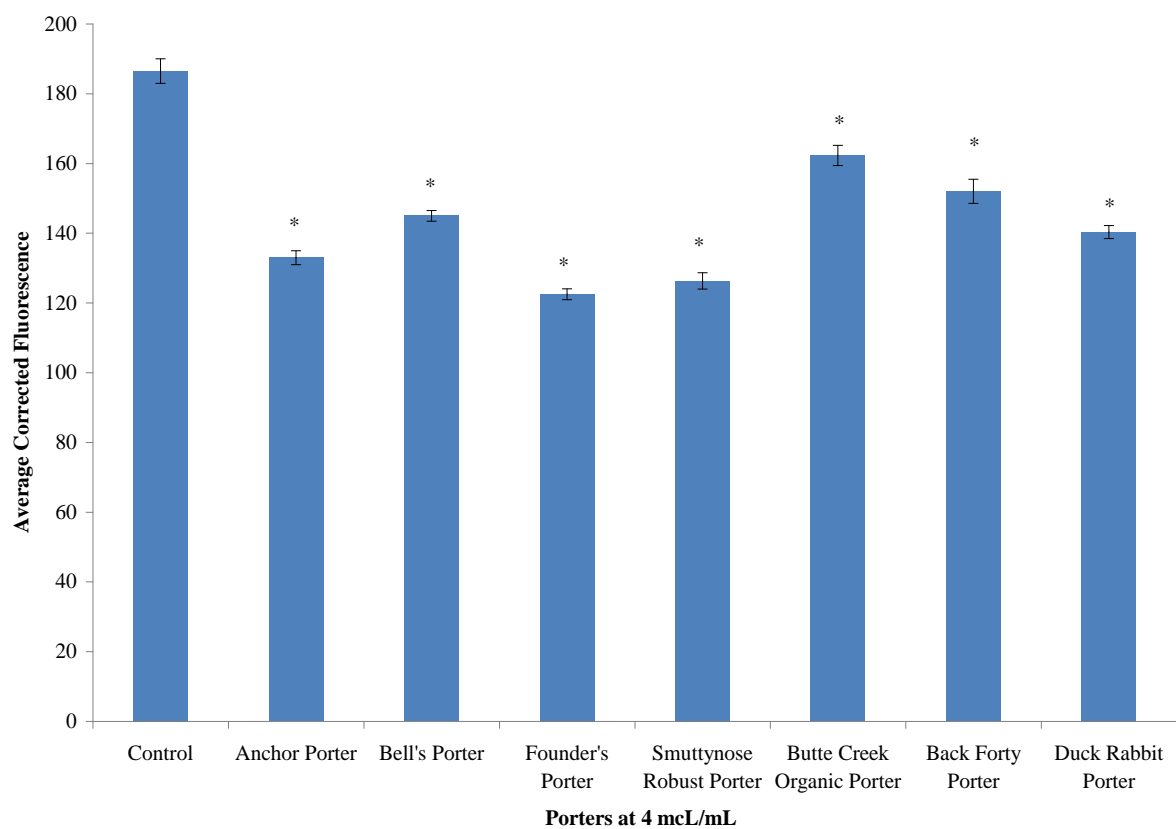
**Figure 5.1: Inhibition of albumin glycation by craft beer on the basis of volume.** Albumin (10 mg/mL) and fructose (250 mM) were incubated with 4  $\mu$ L/mL craft beer for 72 hours at 37°C. The fluorescence intensity was measured at 370/440 nm and represented as mean  $\pm$  standard error of triplicate readings. \* $p$ <0.05 when compared to control.



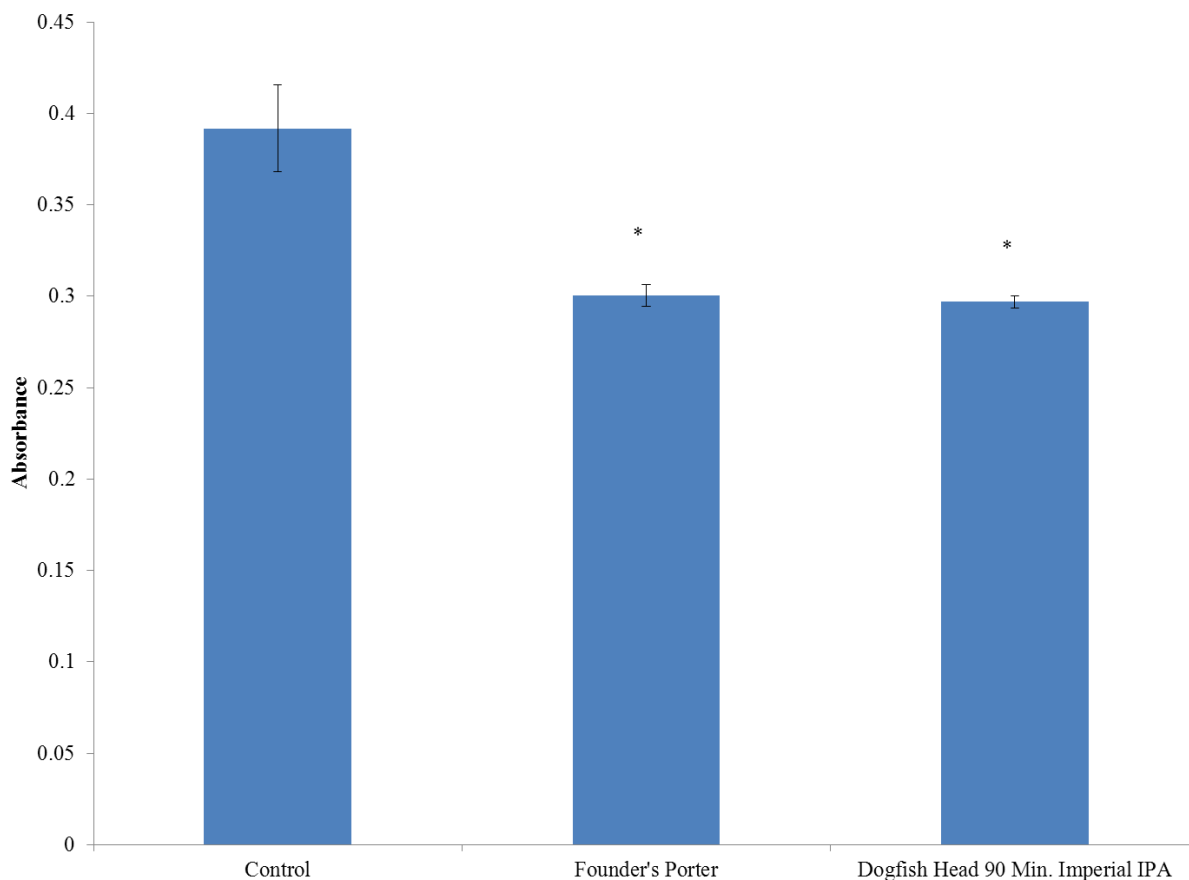
**Figure 5.2: Inhibition of albumin glycation by craft beer on the basis of phenolic content.** Albumin (10 mg/mL) and fructose (250 mM) were incubated with 4  $\mu$ g phenolics/mL craft beer for 72 hours at 37°C. The fluorescence intensity was measured at 370/440 nm and represented as mean  $\pm$  standard error of triplicate readings. \* $p < 0.05$  when compared to control.



**Figure 5.3: Inhibition of albumin glycation by craft beer on the basis of antioxidant content. Albumin (10 mg/mL) and fructose (250 mM) were incubated with 60 nmol FeSO<sub>4</sub> equivalents/mL craft beer for 72 hours at 37°C. The fluorescence intensity was measured at 370/440 nm and represented as mean ± standard error of triplicate readings. \* = significant difference compared to control, † = significant difference from commercial American beer, p<0.05.**



**Figure 5.4: Inhibition of albumin glycation by a selection of porters based on volume. Albumin (10 mg/mL) and fructose (250 mM) were incubated with 4  $\mu$ L/mL porter for 72 hours at 37°C. The fluorescence intensity was measured at 370/440 nm and represented as mean  $\pm$  standard error of triplicate readings. \* =  $p < 0.05$  when compared to control.**



**Figure 5.5: Effect of Founders' Porter and Dogfish Head 90 Minute Imperial IPA on dicarbonyl formation. After 5 days of incubation, dicarbonyl formation was determined by the DNPH colorimetric method. Results represent mean  $\pm$  standard error of triplicate readings. \* =  $p < 0.05$  when compared to control.**

## CHAPTER 7

### CONCLUSIONS AND RECOMMENDATIONS

Plant-based foods and nutraceutical preparations have been attracting attention in recent years due to a variety of factors, including increasing viral drug resistance and increasing obesity and inflammatory-related diseases. Foods and preparations high in polyphenols and other antioxidants have been shown to improve overall health, decrease the risk of inflammatory disease, and prevent virus infection.

Influenza A accounts for thousands of deaths annually, and is increasing in drug resistance. Current treatment options are limited, and, as treatment must be started within 48 hour of the appearance of symptoms, it may not be possible to identify the strain and drug sensitivity of the infecting virus. Thus, natural products that act in concert with pharmaceutical agents may increase the chances of viable immune response to influenza infection. Elderberry and muscadine, both high in phenolics, show activity against oseltamivir-resistant H1N1, wild-type H1N1, and H3N2. Because it may not be possible to ascertain the virus' sensitivity prior to initiating treatment, it may be prudent to treat patients with elderberry or muscadine preparations along with pharmaceutical treatments. This co-treatment may allow pharmaceutical treatment to be effective in patients infected with a drug-resistant strain of influenza, and, due to their wide therapeutic indices, would not cause harm to patients. For patients who cannot be treated with oseltamivir or zanamivir, elderberry and muscadine may be a viable alternative to these products.

Moderate alcohol consumption is associated with various health benefits, especially prevention of cardiovascular disease. Craft beer is higher in phenolics and antioxidants than major commercial brands of beer, with “darker” beers such as porter and stout having greater antioxidant capacity than “lighter” beer such as American pale ale. These beers are similar in antioxidant content to wine, an accepted functional beverage. The high antioxidant content of craft beer may account for functional properties. Not only do high-antioxidant beverages create improvements in general health, but may prevent infection of influenza and the development of diabetic complications due to protein glycation. Cytotoxicity due to oseltamivir-resistant H1N1 was inhibited by higher concentrations of both American pale ale and stout, indicating that regular consumption of these beverages may prevent influenza infection. Several craft beers also inhibited protein glycation, the process partially responsible for development of diabetic complications. Thus, craft beer appears to be a functional beverage for prevention of diabetic complications. Because craft beer is increasing in consumption in America, this recommendation would likely be easily followed by most patients.

In summary, elderberry and muscadine are recommended for combinatorial treatment with neuraminidase inhibitors for influenza infection, and craft beer is introduced as a functional beverage for delivery of antioxidants, prevention of influenza infection, and prevention of diabetic complications.