INVESTIGATION OF ALBUMIN-PHENOLIC BINDING AND ITS ROLE IN BIOACCESSIBILITY AND BIOACTIVITY OF ABSORBED PHENOLIC COMPOUNDS FROM SUMAC (*Rhus coriaria*)

A Dissertation

by

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ABSTRACT

Phenolic compounds circulate to their sites of action bound to human serum albumin (HSA), whether injected into the bloodstream or absorbed from the human GI tract, and consequently, molecular interactions with albumin may influence phenolic biological activity. HSA associates with a variety of ligands, endogenous and exogenous, and serves as the conveyor to their sites of action, metabolism or elimination, though this physiological aspect is often neglected in literature reviews and experimentation. Thus, the objective of the following studies was to investigate the role of the albumin-phenolic interaction in preclinical and clinical models. To address the variability commonly associated with food and nutrition studies, gallotannins from *Rhus coriaria* (sumac) were used because gallotannin bonds are hydrolyzable by commercial enzymes and sumac is a rich source of hydrolyzable tannins, thereby making the product of sumac gallotannin hydrolysis, gallic acid (GA) and its metabolite methyl gallate (MG), more easily targeted and enriched in sumac tea study treatments. Three hypotheses were tested: (1) the biophysical interaction between GA, MG and albumin in a fluorescence quenching assay, (2) the effect of albumin-phenolic interaction on GA, MG antiproliferative activity in a HepG2 cancer cell model, and (3) the effect of albumin-phenolic interaction on the evaluation of a food processing technique in a pilot human clinical trial. The results of the biophysical study showed that MG associated with albumin significantly more than GA (MG, K_{SV}= 9.97E+03 ± 7.01E+01; GA, K_{SV}= 1.35E+03 ± 1.55E+01, $p<0.05$) and this result was attributed to the greater hydrophobic character of MG provided by the methyl

group substitution about the phenolic ring. The results of the *in vitro* antiproliferative activity assay (MTT) in HepG2 cells showed that MG was significantly more potent ($p<0.05$) when assayed alone (MG IC_{50} = 310 ± 52 µM, GA IC_{50} = 436 ± 25 µM, but significantly less potent ($p<0.05$) when combined with HSA (MG+HSA IC_{50} = 5167 ± 289 µM, GA+HSA IC_{50} = 2466 ± 340 µM). Accordingly, the human clinical trial LC-MS method performance analyses revealed that the albumin-rich, human plasma matrix suppressed the signal of the internal standard (IS) ethyl gallate (EG) significantly more ($p<0.05$) than in mobile phase. However, a significant increase in GA absorption was still observed between tea treatments hydrolyzed with tannase (Pre-Treated and Co-Consumed treatments) and the unhydrolyzed control. In summary, these results suggest that phenolic bioactivity and bioavailability are dependent upon albumin-binding.

DEDICATION

I dedicate this dissertation to my mom, dad and older brother. So much of who I am comes from the times we've shared together. And, I saw that more and more during this journey. I wouldn't be me without you!

ACKNOWLEDGEMENTS

I would like to thank the chair of my dissertation committee, Dr. Stephen Talcott, along with the members of my dissertation committee, Dr. Susanne Mertens-Talcott, Dr. Giuliana Noratto and Dr. Elena Castell-Perez. I greatly appreciate your comments and suggestions on my experimental designs, protocols and manuscripts throughout the course of this research. I would also like to thank all of the faculty and staff in the Department of Food Science here at Texas A&M University.

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This achievement is such a milestone for me and serves as an endcap to over a decade's long pursuit of a career in STEM. It all started when my older brother encouraged me to apply to a summer research program for high school seniors. That summer I worked in a lab at UT Southwestern helping a graduate student finish her dissertation and after that I knew I wanted to keep going in the STEM field. That experience led to more summer research internships and finally an industry position with a biotech company after graduation. When I was working in industry with my Bachelor's in chemistry, I just knew I wanted a deeper knowledge of science and biotechnology. I'm so glad that I found Texas A&M and had the opportunity to study here in College Station, TX. I truly value my time in the lab, developing methods, troubleshooting, reaching out to other scientists and delving into the literature. I will always remember my experience in Aggieland!

Finally, I'd like to thank the good people of Bryan/College Station. I'm glad I got to know you and your town. Also, a special thanks to the First UMC church congregation for blessing me with your smiling faces and lovely voices every Sunday.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Stephen Talcott, Dr. Susanne Mertens-Talcott and Dr. Giuliana Noratto of the Department of Food Science & Technology and Dr. Elena Castell-Perez of the Department of Biological and Agricultural Engineering.

All other work conducted for the dissertation was completed by the student independently.

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1. INTRODUCTION

Secondary plant compounds have the potential to provide therapy to a variety of populations with health disorders. The phenolic class of secondary plant compounds demonstrates a wide range of therapeutic effects in diabetes, obesity, cancer and neurological disease (D'Archivio, Filesi, Varì, Scazzocchio, & Masella, 2010). A recurring feature of the pharmacokinetics of secondary plant compounds is large variances in clinical endpoints due to a number of food-related, human, and methodological factors (Sayed, Khurana, & Godugu, 2019). The food matrix interferences that modulate oral absorption, including conjugation to macromolecules and inhibition by other matrix components, have been reviewed previously (Vitali Čepo, Radić, Turčić, Anić, Komar, & Šalov, 2020). Also, the myriad of overlapping physiological processes that occur post consumption such as hepatic and colonic metabolism have been described (Koudoufio, Desjardins, Feldman, Spahis, Delvin, & Levy, 2020). Finally, the shortcomings of research methods and technologies, namely the inability of preclinical *in vitro* studies to simulate the complexity of *in vivo* systems, compliance of human volunteers in clinical trials or stability of phenolic compounds in study treatments have been discussed in the literature. (Gleichenhagen & Schieber, 2016). Taken together, secondary plant compounds often fail clinical trials because of the variation contributed by the aforementioned factors, though phenolics demonstrate promise as lead molecules in drug development. Of all the phenolic classes, gallotannins represent an easily controlled group due to the hydrolyzable nature of its linkages (Serrano, Puupponen-Pimiä, Dauer, Aura, & Saura-Calixto, 2009).

Enrichment of gallotannin hydrolytic product gallic acid and its metabolites including methyl gallate provides a targeted approach to studies of biological behavior that overcomes variation from the aforementioned factors. Furthermore, food substances that are abundant in gallotannin are widely available, including the genus and species *Rhus coriaria* found in the sumac family.

An under-explored factor in phenolic bioavailability studies is the phenolic-albumin interaction. Human serum albumin (HSA) comprises the majority of blood proteins and may be considered the first compartment to which polyphenols migrate after absorption from the intestines (Li & Hagerman, 2013). Albumin is a large carrier protein (66.6 kD) equipped with multiple hydrophobic pockets naturally suited for transport of different ligands such as growth factors, lipids, and synthetic drugs (Peters, 1996). Because of its centrality to absorption, metabolism, distribution and excretion (ADME), HSA potentially influences the bioavailability, bioaccessibility and bioactivity of absorbed compounds. The terms ‘bioavailability’ and ‘bioaccessibility’ are inter-related (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009). The term bioavailability can be traced back to the field of pharmacology and is defined as the fraction of an oral dose that is available in systemic circulation relative to an intravenously administered dose (Tozer & Rowland, 2006). However, researchers in other fields use the term bioavailability generally to describe plasma concentrations after oral administration or bioactivity of a compound in a matrix relative to a pure standard. The term ‘bioavailability’ encompasses the term ‘bioaccessibility’, a reflection of the chemical form of the compound that garners a biological effect (Carbonell-Capella, Buniowska, Barba, Esteve,

& Frígola, 2014). In this dissertation, the terms “bioavailability” and “bioaccessibility’ are used interchangeably to describe the effects of albumin-binding on the behavior of phenolic compounds in the human body.

The central hypothesis of this dissertation is that albumin-phenolic interactions are central to ADME studies of phenolics in the field of pharmacokinetics and pharmacodynamics. The objectives of this research were:

1. To investigate protein-ligand binding between HSA and phenolic compounds by fluorescence quenching methodology. This biophysical study analyzes the binding interactions between low-molecular weight phenolic compounds and HSA and relates this effect to phenolic structure.
2. To evaluate the effects of albumin binding on the anti-proliferative activities of phenolics in HepG2 liver cancer cells. This study set out to investigate the usefulness of adding HSA to study treatments in pre-clinical *in vitro* studies of anticancer potential, namely, effects of cell viability, cell growth, and expression of associated genes and proteins.
3. To improve sumac gallotannin bioaccessibility through pre-treatment with tannin acyl hydrolase in a clinical study. The clinical study examines the role of phenolic-albumin binding between the enzymatic product of hydrolyzable tannins, gallic acid, and human serum albumin (HSA) in the determination of gallic acid absorption by LC-MS methodology.

2. LITERATURE REVIEW

2.1. Phenolic Compounds of Sumac

2.1.1. Background

Phenolic compounds naturally occur in a variety of plant species used for food such as fruits, vegetables, grains, nuts, and spices. Sumac or *Rhus coriaria* is a plant in the Anacardiacea family that is native to the Mediterranean geographical region (Kossah et al., 2009). It grows as flowering shrub and its fruits are dried, milled and have been popularized in Middle Eastern beverages and spices (Asgarpanah & Saati, 2014). Early records of sumac commercial cultivation from the Royal Botanic Gardens indicate that sumac was produced along the rocky hillsides of Palermo, Sicily for its high tannin content to tan leather and dye fabric. ("Sumach. (Rhus Coriaria, L.)," 1895). Sumac is also known for its ethno-medicinal properties such as cholesterol reduction and high antioxidant capacity (Rayne & Mazza, 2007). The variety of uses of sumac in Mediterranean and Middle Eastern cuisine, leather, textiles production and medicine derive from its phenolic content. For example, the astringent taste of sumac beverages come from gallotannins; application of sumac in leather and fabric tanning is also due to the tannin content; the deep red color of sumac powder arises from anthocyanins; and, the medicinal properties are due to the presence of compounds like quercetin and its metabolites like protocatechuic acid (Abu-Reida, Jamous, & Ali-Shtayeh, 2014). Overall, sumac is comprised of a variety of phenolic compounds including flavonoids, tannins and phenolic acids which confer an array of culinary, commercial and cultural uses.

2.1.2. Structural Description

Sumac contains phenolics in the flavonoid, tannin and phenolic acid classes and each exhibit different structural features. Generally, flavonoid compounds are distinguished by their aglycone structure, the form without the sugar moiety, and include differences in hydroxyl (-OH) and methoxyl (-OCH_3) group substitution sites (Figure 1).

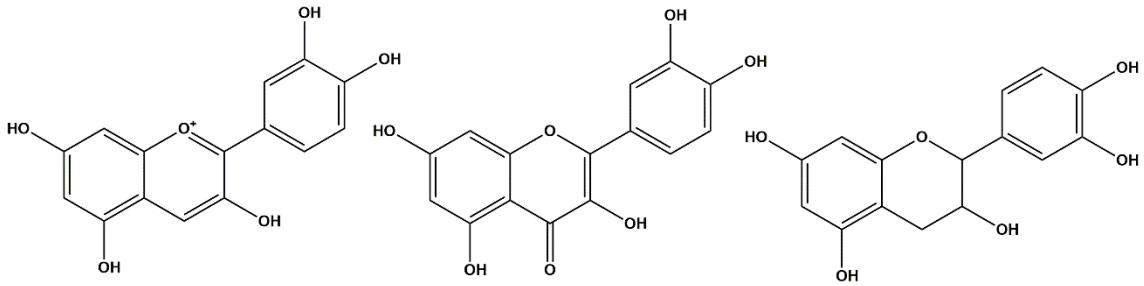

Figure 1. Structure of the 3 major classes of flavonoids: a. cyanidin, an anthocyanin; b. quercetin, a flavonol; c. catechin, a flavn-3-ol.

The 3 major classes of flavonoids are anthocyanins, flavonols and flavan-3-ols and their structural features include the following: the A ring and C ring occur as two aromatic rings fused together; the C ring possesses an oxygen heteroatom which is designated as the 1 position, dictating the numbering of the other carbon atoms in the fused A and C ring; the B ring is connected to the C ring by a carbon-carbon bond at the 2 position of the C ring (Del Rio, Borges, & Crozier, 2010). Interestingly, cyanidin, quercetin and catechin have

identical structures at the A ring, very similar structures in the B ring and differ the most at the 2, 3, and 4 position in the C ring. Tannins consist of 3 subclasses including

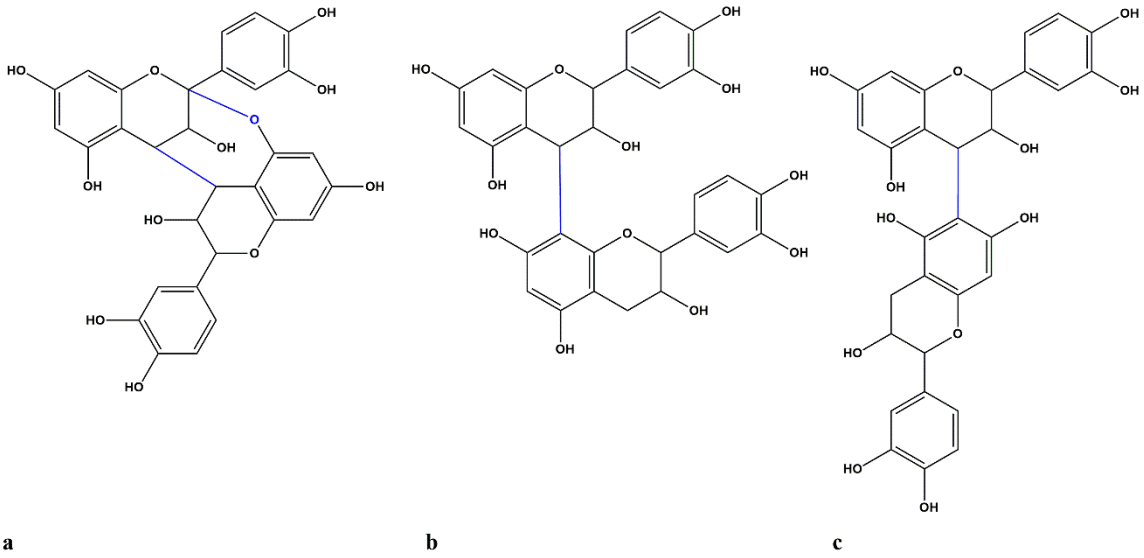

Figure 2. Structure of the 2 types of condensed tannins, proanthocyanidins: a. A-type linkage; b. and c. B-type linkage.

condensed tannins, gallotannins, ellagitannins, and their structural features include the following: the condensed tannins, also known as proanthocyanidins, are catechin flavan-3-ol polymers comprised of A-type (C4 → C8 and C2 → C7) and B-type (C4 → C8 or C4 → C6) linkages (Figure 2); gallotannins are polymers of gallic acid connected by m-depside bonds with a central glucose moiety that has its hydroxyl groups substituted with galloyl groups (Figure 3); ellagitannins are polymers of ellagic acid or two C-C linked galloyl moieties (Figure 4) (Smeriglio, Barreca, Bellocco, & Trombetta, 2017). Phenolic

acids are hydroxybenzoic acid and hydroxycinnamic acid derivatives with -OH and -OCH_3 substitutions in the aromatic ring or carboxylic acid moiety (Figure 5) (N. Kumar & Goel, 2019). These compounds occur at different concentration levels and often times as

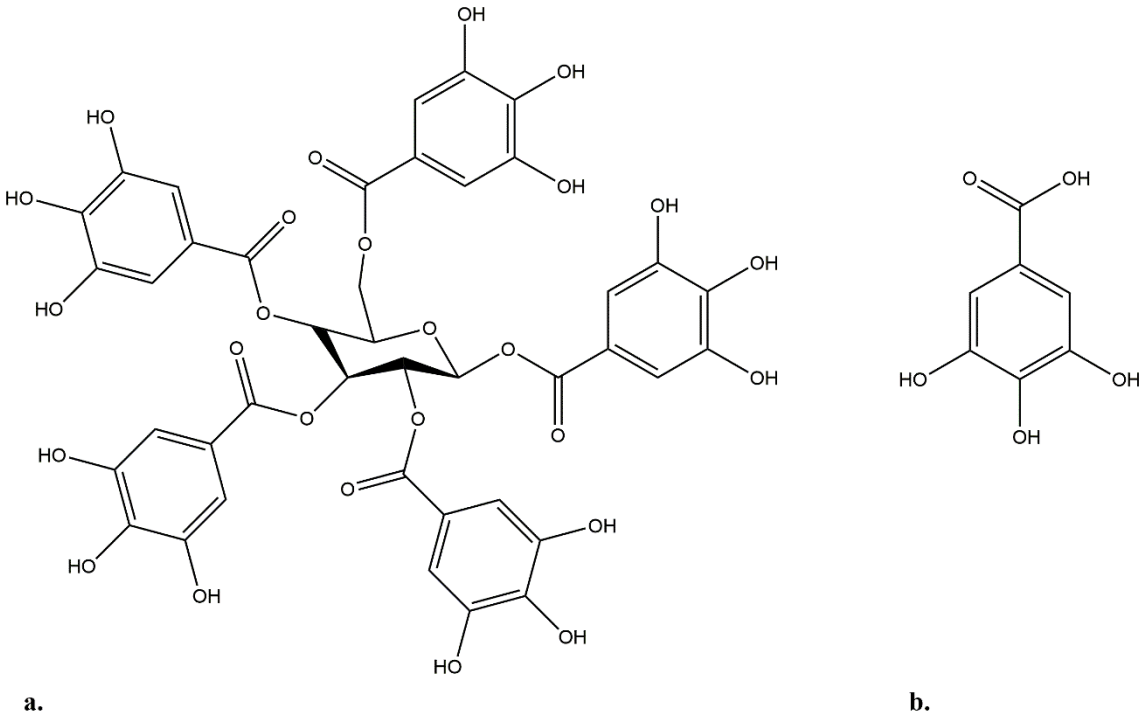

Figure 3. Structure of a typical gallotannin (a) and its constituent (b) : a. pentagalloyl glucose (1,2,3,4, 6-penta-*O*-galloyl-β-D-glucopyranose); b. gallic acid.

conjugates to other macromolecules and other polyphenols. Such chemical complexity creates challenges in *in vivo* and clinical studies of the biological activities of polyphenols within their natural fruit matrix. Thus, it is desirable to target a polyphenol class that can

be controlled and of all the polyphenol classes found in sumac, gallotannins are strong candidates for bioactivity studies because their *m*-depside bonds are hydrolyzble by commercial esterases. Thus, sumac is an attractive food substance to utilize in human intervention studies because the gallotannin hydrolytic product, gallic acid, can be well-characterized in study treatments and thereby better linked to its physiological behavior *in vivo*.

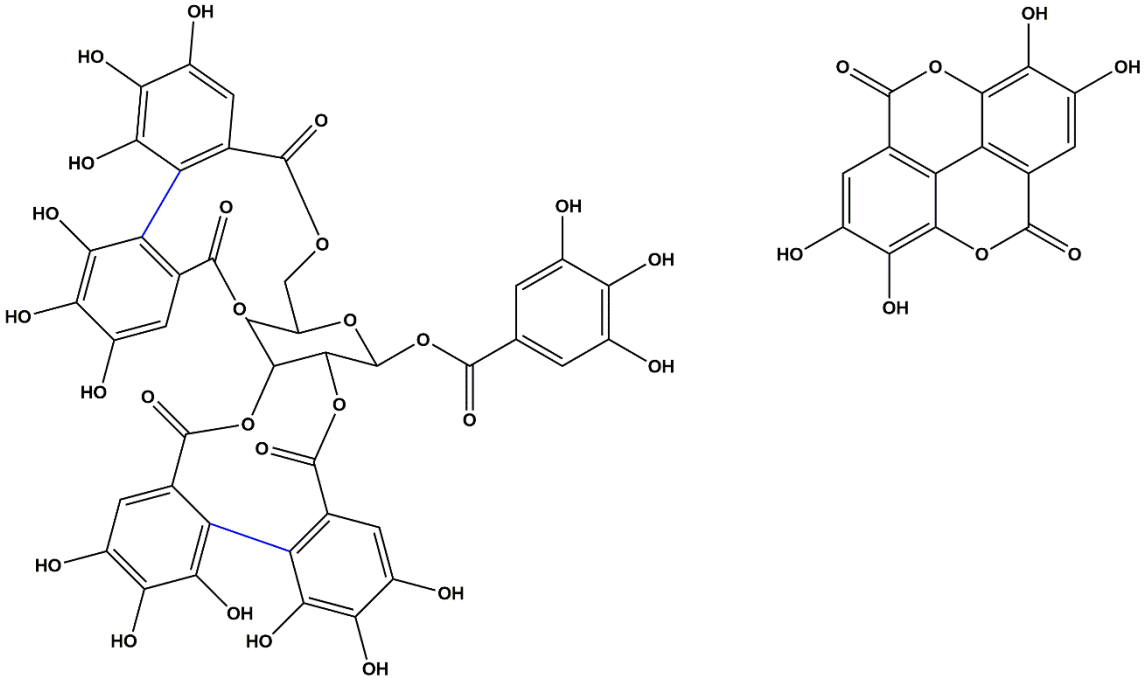

Figure 4. Structure of a typical ellagitannin (a) and its constituent (b): a. casuaricitin; b. ellagic acid.

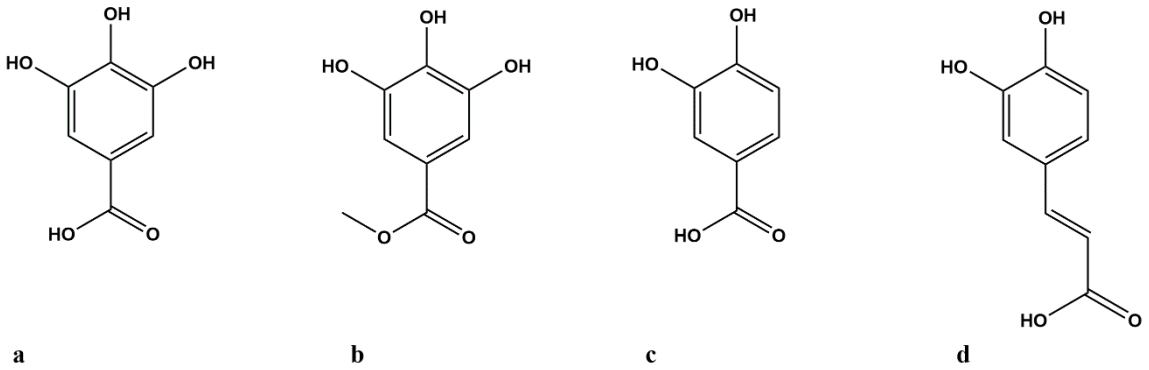

Figure 5. Structures of phenolic acids: a. gallic acid, a hydroxybenzoic acid; b. methyl gallate, a CH3-substituted hydroxybenzoic acid; c. protocatechuic acid, a hydroxybenzoic acid; d. caffeic acid, a hydroxycinnamic acid.

2.1.3. Physiochemical Properties

A number of studies have examined the physiochemical properties of sumac fruits. Acidity, pH, moisture content, nutrient composition and organoleptic properties have been determined by several researchers (Abu-Reidah, Ali-Shtayeh, Jamous, Arraez-Roman, & Segura-Carretero, 2015; Al-Bataina, Maslat, & Al-Kofahil, 2003; Kizil & Turk, 2010; Kossah et al., 2009; Mazaheri Tehrani M, 2017; Özcan & Haciseferogullari, 2004). For example, these studies found *Rhus coriaria* pH to range between 2.7-3.7 and reported malic acid as the predominant organic acid found in the fruit. In addition, *Rhus coriaria* has been classified as a low moisture substance with a water content between 6-11%. Data from several sources have measured protein content between 2-3% and identified leucine, threonine, and lysine as predominant amino acids in the fruit. Similarly, the lipid content has been measured at 8-18% consisting of predominantly monounsaturated fatty acids like oleic and linoleic acid in addition to the saturated palmitic acid. Also, these studies have

shown that the fiber content of sumac is 15-22% and its mineral composition is comprised predominantly of the minerals calcium, iron, and phosphorus and the vitamins pyroxidine, cyanocobalamin, nicotinamide, and biotin. Moreover, the organoleptic properties of sumac, such as its deep red color and astringent taste, are due to the anthocyanins and gallotannin content, respectively. These physiochemical properties favor sumac as a test product in human dietary intervention trials as it exhibits a diverse array of nutrients and a unique flavor.

2.2. Biological Activities of Sumac Phenolics

2.2.1. Antioxidant Activities

The literature on the antioxidant activity of *Rhus coriaria* has demonstrated that sumac is comparable or more effective than other antioxidants used in the food processing industry and that it demonstrates protective effects against oxidative stress related disorders. The oxidation of lipids in food products results in rancid odors that decrease food quality, prompting researchers to screen various plants for their ability to scavenge or quench the free radicals which cause oxidative damage (Pokorný, 1991). In studies on sunflower oil stabilization, sumac extracts were found to be just as potent as rosemary and sage extracts (Akgul & Ozcan, 1995) and more potent than BHT and BHA (Bozan, Kosar, Tunalier, Öztürk, & Baser, 2003) by DPPH free radical scavenging test. Sumac extracts have also demonstrated antioxidant activity equivalent to α-tocopherol and researchers attributed this effect to its anthocyanin and gallotannin content (Kosar, Bozan, Temelli, & Baser, 2007). The oxidation of lipids in food products has also been associated with health disorders such as heart disease and carcinogenesis (Yagi, 1987) and sumac extracts have

demonstrated antioxidant activity against *in vitro* models of oxidative stress as well. In a study on oxidative damage in rat liver cells, an aqueous sumac extract (75 and 100 ppm) significantly increased mitochondrial membrane integrity, reduced lipid peroxidation levels and ROS generation while restoring glutathione levels in response to induction by cumene hydroperoxide (Pourahmad, Eskandari, Shakibaei, & Kamalinejad, 2010). Similar protective effects were observed in a human clinical trial of sumac antioxidant activity in which 3 g/day sumac was administered to healthy volunteers and resulted in a 40% increase in glutathione S-transferase plasma levels (Chakraborty et al., 2009). The application of sumac as an antioxidant ingredient and therapeutic agent supports future investigations on the behavior of its active compounds under physiological conditions. Such studies will drive commercial development of sumac products.

2.2.2. Chemopreventive Activities

A comprehensive investigation of the chemopreventive activities of sumac has been published highlighting its anticancer potential. Treatment with an ethanolic sumac extract resulted in a reduction in metastasis and growth of MDA-MB-231 cells, a type of triple negative breast cancer cell (TNBC) that does not express the receptors targeted by several commercial chemopreventive agents (El Hasasna et al., 2016). In this study, MDA-MB-231 breast cancer cell invasion was reduced by 10 and 50 ug/mL of sumac extract and cell migration was reduced by 200 ug/mL of sumac extract. The mechanism proposed in this study was attributed to three functions: inhibition of fibronectin, an extracellular matrix component which facilitates cell motility or spread of cancer from one site to another, inhibition of COX-2, the progenitor of hormone-like prostaglandin E2 which

signals an increase in cell division producing more cells and inhibition of STAT3, a regulatory molecule active in signal transduction of cell survival and proliferation pathways (Golias, Charalabopoulos, & Charalabopoulos, 2004; Johnston & Grandis, 2011). This result coordinated with a reduction in the production of nitric oxide (NO), a molecule that demonstrates tumor activating activity through the induction of cell proliferation and migration (Mocellin, Bronte, & Nitti, 2007; Wink, Ridnour, Hussain, & Harris, 2008). Further confirmation of the antitumor potential of sumac was demonstrated by a reduction in the necrosis factor TNF-alpha, a death receptor ligand active in cell autophagy, a survival process upregulated in cancer cells (Djavaheri-Mergny et al., 2006; White, 2015). Similarly, the sumac extract reduced the expression of pro-inflammatory cytokines IL-6 and IL-8 and its transcription factor NFκB, highlighting the role of inflammation in cancer progression (Mantovani, 2005). Thus, sumac exhibits a wide-range of inhibitory effects on cancer progression which supports future development in pharmacokinetics of its bioactive components.

2.2.3. Blood Sugar and Blood Lipid Lowering Activities

Previous animal and human studies have begun to investigate the effect of sumac consumption on biomarkers of diseases such as diabetes and hyperlipidemia. In a study on anti-diabetic potential, 200 and 400 mg/kg of lyophilized ethanolic sumac extract was administered to diabetic rats and a 24% decrease in postprandial blood glucose was found (Mohammadi, Montasser Kouhsari, & Monavar Feshani, 2010). Similarly, an average of 14% decrease in post prandial glucose was detected after 250 and 500 mg/kg lyophilized ethanolic sumac extract was administered to diabetic rats in a separate study (Doğan &

Çelik, 2016). Consistent with these results was the significant decrease ($p<0.05$) in blood sugar levels of 41 diabetic patients who consumed 3 g dried sumac powder daily for 3 months (Shidfar et al., 2014). In addition, sumac demonstrated positive effects on blood lipid profiles in several animal and human studies. Rabbit studies showed that sumac supplementation to a final concentration of 1.5% and 2% in the diet decreased cholesterol levels significantly, $p<0.05$ (Capcarova et al., 2012; Madihi et al., 2013). Methanolic sumac extract at 5 and 10 g/kg reduced ($p<0.05$) cholesterol levels in broiler chickens (Golzadeh, Farhoomand, & Daneshyar, 2012). Additionally, methanolic sumac extract at 100 and 200 mg/kg decreased ($p<0.05$) total cholesterol of rats (Shafiei, Nobakht, & Moazzam, 2011). Overall, sumac administration to animals and humans consistently demonstrates therapeutic effects in diabetes and hyperlipidemia disease states. These results were consistent with significant reductions in serum total cholesterol and triglyceride levels of 72 adolescents with dyslipidemia after 30 day consumption of 1.5 g sumac powder (Sabzghabaee, Kelishadi, Golshiri, Ghannadi, & Badri, 2014). Human clinical studies with 1 g sumac powder showed similar results of improved blood lipid profiles (Asgary et al., 2018; Hajmohammadi et al., 2018). While it is useful to have agreement of results, much of these studies on sumac *in vivo* effects are limited to standard clinical endpoints, namely blood sugar and blood lipid levels, and do not draw connections between the consumption of sumac, absorption of its bioactive constituents, or its modulation of disease biomarkers. Thus, several questions remain to be answered regarding the pharmacological dose-response of sumac consumption in animal and human studies.

2.3. Influencing Factors in Bioavailability and Efficacy of Phenolic Compounds

2.3.1. ADME and First-Pass Effects

When phenolic compounds are ingested, several sites within the gastrointestinal tract metabolize and eliminate the original compound resulting in the poor oral bioavailability profiles associated with phenolics in food matrices. Oral bioavailability of phenolic compounds is the fraction of the ingested compounds that reach systemic circulation relative to an intravenously administered compound (Porrini & Riso, 2008; Ribas-Agusti, Martin-Belloso, Soliva-Fortuny, & Elez-Martinez, 2018). The absorption, distribution, metabolism and excretion (ADME) processes include the transport dynamics and first-pass metabolism that influence phenolic bioactivity. After consumption of phenolic compounds in food, extensive biotransformation and elimination occurs by the liver, small intestine, colon microbiota and kidneys (Figure 6) (Back & Rogers, 1987; Teng et al., 2012; Xue, Ying, Zhang, Meng, Ying, & Kang, 2014). The enzymes of the small intestine epithelial cells and liver cells that modify absorbed compounds during phase I and phase II metabolism have been well-established (Caldwell, Gardner, & Swales, 1995; George, 1981; Hartiala, 1973). In phase I metabolism, several enzymes located in both the endoplasmic reticulum microsome and mitochondria remove functional groups: alcohol dehydrogenase, monoamine oxidase, hydroxylase, demethylase, deaminase, esterase, deacetylase and ß-glucosidase. In phase II metabolism, liver cells add functional groups: glucuronide, sulfate or methyl substitutions. Interplay between the liver and intestine in enterohepatic recirculation has also been well-established (Smith, 1966) and consists of five key processes: absorption into the intestinal epithelium, transportation

to the liver by the hepatic portal vein, biotransformation in the liver by phase I or II mechanisms, excretion in the bile and return to the small intestine as a different product. Similarly, in colon metabolism, microbiota have been found to express various enzymes such as dehydroxylase, esterase or deacetylase which remove hydroxyl and acetyl groups, sever ester bonds in addition to enzymes that that perform ring fission (Mikov, 1994; Stevens & Maier, 2016). The kidney glomerulus functions as an eliminating organ that filters the hydrophilic products of liver and intestinal metabolism. Overall, the result of ADME processes is that the phenolic profile of the ingested food product does not match the plasma profile of absorbed compounds from that product, thereby complicating the work of finding a dose-response.

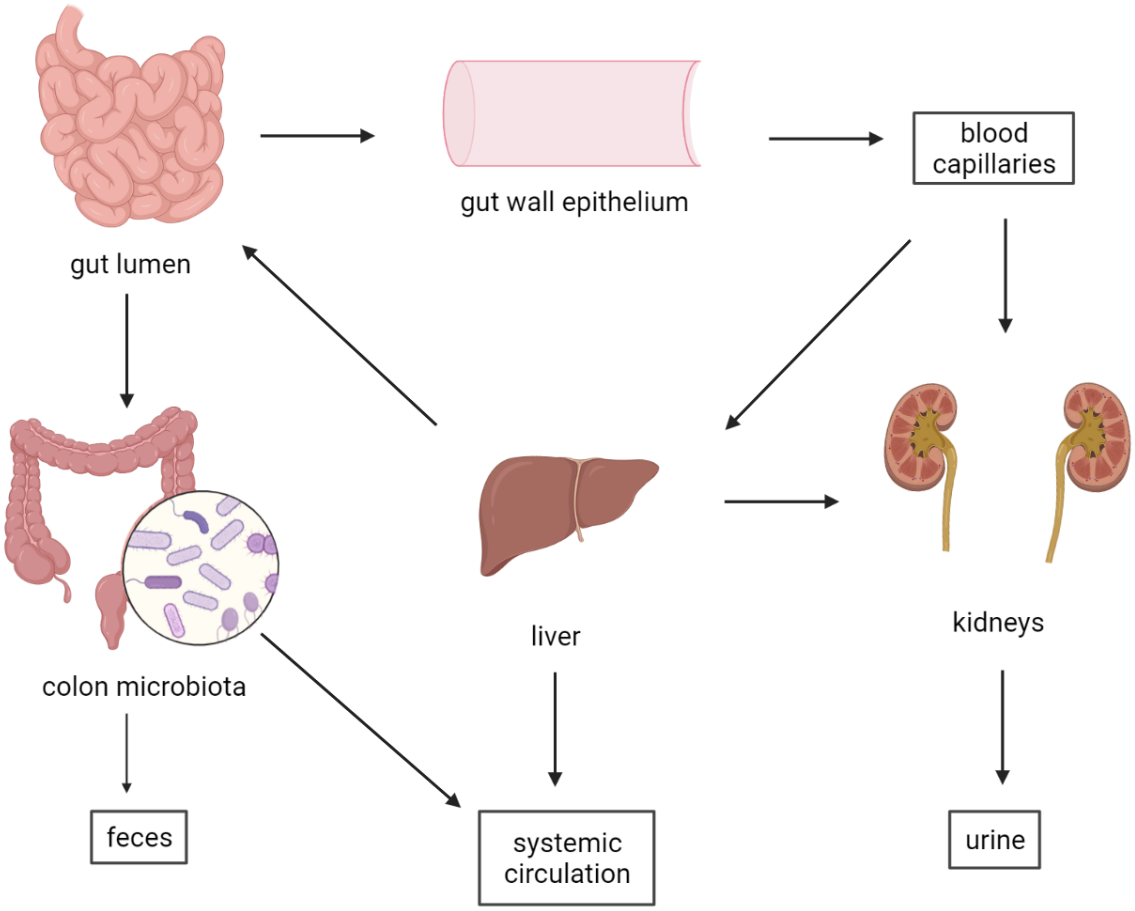

Figure 6. Sites of first pass metabolism in ADME processes. Created with BioRender.com

2.3.2. Albumin-binding

ADME factors that influence the phenolic dose-response have been reviewed in many studies but much less attention has been paid to the role of plasma protein binding in phenolic bioactivity and efficacy. Human serum albumin (HSA) is the most abundant plasma protein in circulation and it serves as the carrier that conveys all absorbed compounds to their sites of metabolism, elimination and action (Peters, 1996). The binding

of polyphenol compounds to albumin occurs in the hydrophobic pockets of subdomain IIA (Figure 7) (Sudlow, Birkett, & Wade, 1975). Preclinical drug efficacy studies have focused on albumin-binding because it modulates the drug concentration in systemic circulation that is available for bioactivity, and thus informs the development of an effective dosage concentration (Vorum, 1999). Albumin-binding determines whether absorbed compounds are eliminated by liver metabolizing enzymes and kidney glomerular filtration or if they are stabilized in the blood compartment by association with the circulatory protein (Yamasaki, Chuang, Maruyama, & Otagiri, 2013). Less clearance from hepatic or renal elimination extends the half-life of the compound and may contribute to greater availability in the body. To date, only a limited number of investigations have focused on the role of albumin binding in bioactivity of therapeutic compounds (Audus, Knaub, Guillot, & Schaeffer, 1992; Fleischer, Shurmantine, Luxon, & Forker, 1986; Luxon, Forker, & Falany, 1980) and none of these were designed to characterize preclinical safety and efficacy of phenolics. Though it is well-known that absorbed compounds circulate bound to albumin in vivo (Goldstein, 1949; Meyer & Guttman, 1968), in vitro studies do not include albumin binding in the experimental design of preclinical studies. Moreover, if these preclinical studies are the foundation of future clinical trials, then it is reasonable to suggest that the in vitro models imitate physiological conditions as much as possible. This gap in knowledge indicates a need to understand the relationship between albumin binding and phenolic bioactivity as it is possible that albumin binding influences efficacy of absorbed compounds at their sites of action. Overall, studies in the relation of albumin-binding and in vivo phenolic bioactivities are

needed to address the dose-response gap that contributes to poor translation between in vitro studies and human clinical trials.

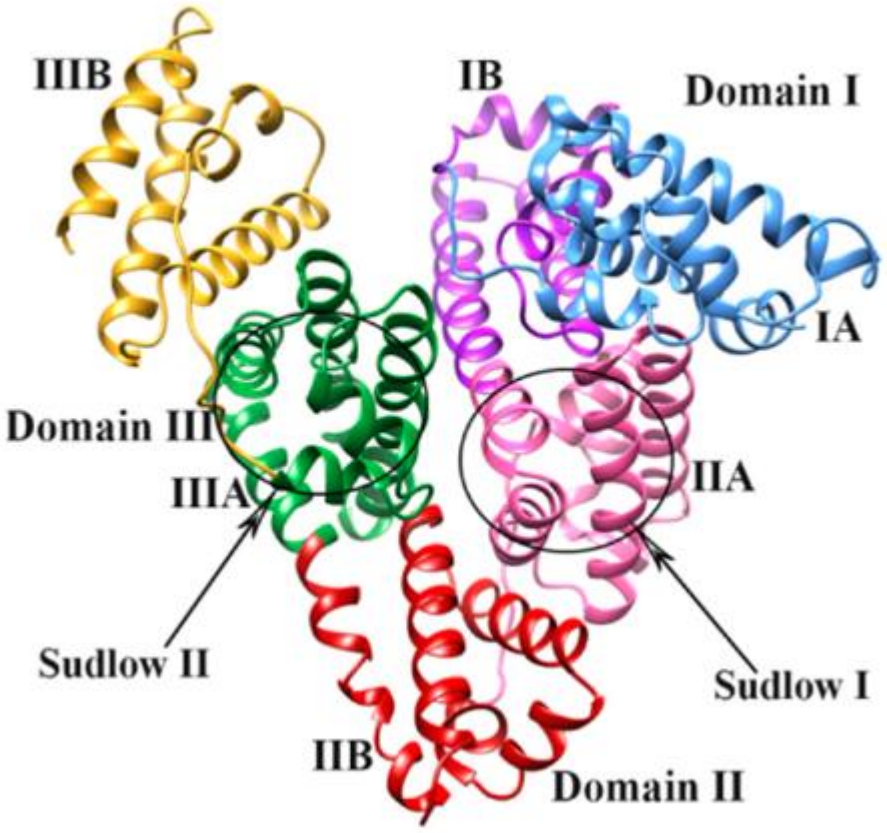

Figure 7. Sudlow binding sites and subdomains of HSA. Reprinted with permission from Lakshmi, Mondal, Ramadas, & Natarajan, 2017.

The literature on albumin binding of flavonoids has proposed that flavonoid-albumin complex formation prolongs flavonoid antioxidant activity *in vivo*, effectively

extending the therapeutic effect. By drawing on the comprehensive studies of quercetin metabolites in rat and human plasma, (C. Manach et al., 1998; C. Manach et al., 1995; Claudine Manach, Texier, Régérat, Agullo, Demigné, & Rémésy, 1996; Morand, Crespy, Manach, Besson, Demigne, & Remesy, 1998), it was hypothesized in a review of flavonoid-albumin interactions that flavonoid bioactivity *in vivo* occurs in an albumin-bound form (Dangles, Dufour, Manach, Morand, & Remesy, 2001). The studies on quercetin metabolites in rat plasma demonstrated that the physiological disposition of quercetin and its metabolites is bound to albumin. This was demonstrated by a bathochromic shift in absorbance maxima of rat plasma post consumption of quercetin compared to quercetin alone. The absorbance of pure quercetin was 375 nm but the absorbance detected in rat plasma was 411 nm and this shift was also found in quercetin-spiked plasma. (C. Manach et al., 1995; Claudine Manach et al., 1996). Furthermore, in the review of flavonoid-albumin interactions the observation that rat and human plasma metabolites retained antioxidant properties relative to pure quercetin led to the proposed hypothesis that albumin binding may contribute to flavonoid bioactivities. Currently, the hypothesis in the field is that albumin-phenolic binding may offer insight into the molecular mechanisms of phenolic bioactivity and investigational studies providing empirical evidence are needed.

The majority of empirical evidence on the use of albumin binding to enhance the efficacy phenolics is in the field of drug delivery formulations. Nanoparticle-based drug delivery studies have demonstrated increased phenolic bioavailability and bioactivity in *in vitro* and *in vivo* studies. A study on the polyphenols catechin and epicatechin showed

that encapsulation in albumin nanoparticles increased the *in vitro* cellular inhibition capacity, highlighting the role of albumin association as a bioavailability enhancer (Yadav, Kumar, Kumari, & Yadav, 2014). Also, in a study on curcumin bioactivity, researchers attributed the increase in *in vitro* curcumin antitumor activity to its formulation in an albumin nanoparticle encapsulation and endothelial transport via an albumin receptor-mediated pathway (T. H. Kim et al., 2011). Likewise, in a mouse study on resveratrol antitumor activity, use of albumin in nanoparticle formulation improved resveratrol antioxidant activity by extending its half-life and allowing resveratrol to accumulate at its site of action (Xu et al., 2018). Thus, the phenolic-albumin binding interaction has a demonstrated record of application to bioavailability and bioactivity enhancement studies in nanoparticle formulations. In these experiments , albumin was pre-processed into micelles with nano-sized polymers that enhanced the stability and functionality of the drugs used (Qu, Chu, Shi, Peng, & Qian, 2017; Wang et al., 2014). While these studies highlight the use of modified albumin in drug delivery technology, there are no experiments that evaluate the role of unmodified albumin in drug delivery or similar preclinical studies. Overall, the successful use of a modified albumin in nanoparticle form suggest that the unmodified form of albumin may also have enhancing properties.

3. DETERMINATION OF PROTEIN-LIGAND BINDING INTERACTION BETWEEN LOW MOLECULAR WEIGHT PHENOLICS AND HSA BY FLUORESCENCE QUENCHING ASSAY

3.1. Introduction

Consumption of secondary plant compounds like polyphenols introduces into circulation bioactive metabolites that have demonstrated protective effects against oxidation, inflammation and metabolic diseases. Among the many bioactivities of polyphenols are their ability to associate with proteins both dietary and biological. The protein-polyphenol interaction is a well-studied phenomenon in the area of food product development and food applications. For example, the protein-polyphenol interaction has demonstrated changes in polyphenol antioxidant activity and has been applied to the delivery of pharmaceutical formulations (Buitimea-Cantúa, Gutiérrez-Uribe, & Serna-Saldívar, 2018; Livney, 2010; J. Xiao, Mao, Yang, Zhao, Zhang, & Yamamoto, 2011). The mechanisms responsible for the binding properties are hydrophobic interactions and hydrogen bonding as noncovalent interactions responsible for stabilizing the protein-polyphenol interaction (Charlton et al., 2002; Le Bourvellec & Renard, 2012; Pal, Saha, Hossain, Dey, & Kumar, 2012; Rawel, Meidtner, & Kroll, 2005). Protein-polyphenol interactions were suggested to mediate the bioactivity of polyphenols post-consumption, thereby creating new lines of research on metabolite-protein interactions (Li et al., 2013; J. B. Xiao & Kai, 2012). Since many polyphenols were shown to be unstable in the gastrointestinal tract, due to catabolism by intestinal microflora and conjugation by liver

enzymes, it is believed that the breakdown products are responsible for the health benefits associated with polyphenol consumption (Duda-Chodak, Tarko, Satora, & Sroka, 2015; Winter et al., 2017; Xiang et al., 2017). For example, colonic metabolism of polyphenols such as flavonols, catechins, and anthocyanins creates smaller products such as benzoic acids, benzyl alcohols, hydroxyphenyl propionates, and hydroxyphenyl acetates (Appeldoorn, Vincken, Aura, Hollman, & Gruppen, 2009; Juániz et al., 2016). Therefore, it is important to study the interactions of native, unmetabolized polyphenolics and their colonic or liver metabolites with biological proteins involved in digestion, absorption, metabolism, and excretion. While previous studies have investigated the interaction between native polyphenols found in plants and proteins such as pancreatic lipase (Moreno-Cordova et al., 2020) and α-glucosidase (Liu et al., 2020), the interaction between the metabolites of ingested polyphenols and circulatory proteins in human serum are poorly characterized.

The most abundant biological protein is human serum albumin (HSA). It is the most represented protein in blood circulation at 34-54 g/L (Dangles et al., 2001; Krebs, 1950; Larsen, Kuhlmann, Hvam, & Howard, 2016; Sleep, Cameron, & Evans, 2013). HSA functions as a carrier protein in the blood with many ligand-binding sites and has one tryptophan residue (Trp-214) located in subdomain 2A which is a common binding site for drugs (Meyer & Guttman, 1968; Sudlow, Birkett, & Wade, 1975). This tryptophan residue has fluorescence activity at 295 nm and is used to study protein ligand interactions by fluorescence spectroscopy (Ali, Amina, Al-Lohedan, & Al Musayeib, 2017; Balaei & Ghobadi, 2019; Lakshmi, Mondal, Ramadas, & Natarajan, 2017; Vanekova et al., 2019).

Protein fluorescence activity is sensitive to changes in its environment, and as a result changes in HSA emission spectra are due to changes in the environment of Trp-214 (Royer, 2006). Fluorescence quenching theory indicates that molecular contact causes quenching of a protein's fluorescence properties (Lakowicz, 2006a). The Stern-Volmer theory of fluorescence quenching describes the rate at which a compound deactivates an excited fluorophore and provides the Stern-Volmer constant, K_{SV}, the product of the quenching rate constant, k_q, and the lifetime of the fluorophore in its excited state, τ, when fluorescence emission intensity is plotted as a function of quencher concentration (Lakowicz, 2006b). According to the model, two possible mechanisms responsible for quenching include interactions by complex formation (static quenching) or diffusion-limited interaction (collisional quenching) demonstrated by a linear relationship between fluorescence emission intensity and quencher concentration. Furthermore, static and collisional quenching are distinguished by the size of k_q, where $k_q \leq 10^{10}$ ($M^{-1}s^{-1}$) for a collisional process. Thus, experiments with fluorescent-active biological proteins and their ligands have the potential to provide information about binding interactions.

There is conjecture in the literature that the interaction between polyphenol metabolites and HSA is important for polyphenol bioactivity and bioavailability (Li et al., 2013; J. B. Xiao et al., 2012), however this interaction has not been the subject of study. These studies determined the role of protein-ligand binding between HSA and several parent polyphenols and polyphenolic metabolites. Changes in HSA fluorescence intensity were measured upon interaction with the polyphenols quercetin, methyl gallate, gallic acid

protocatechuic acid, (-)-epicatechin, 3-(3-hydroxyphenyl) propionic acid, and 4-hydroxyphenyl acetic acid (Figure 8).

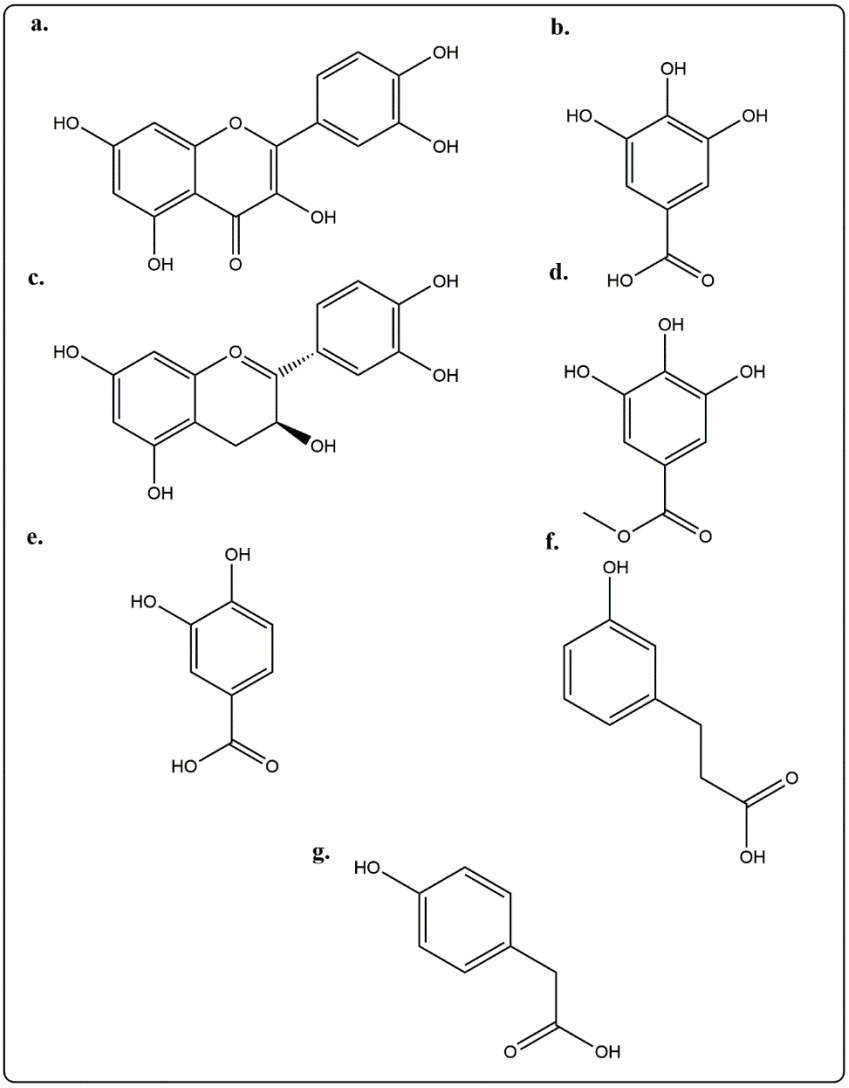

Figure 8. Structure of phenolics. a) quercetin, b) gallic acid, c) (-)-epicatechin, d) methyl gallate, e) protocatechuic acid, f) 3-(3-hydroxyphenyl) propionic acid, and g) 4-hydroxyphenyl acetic acid.

3.2. Materials and Methods

3.2.1. Chemicals

Phenolic standards, human serum albumin (lyophilized powder ≥97%) and methanol (MeOH) with 0.1% formic acid (HPLC grade ≥ 99.9%) were purchased from Sigma Aldrich. 1X PBS (pH 7.4) was prepared from 10X PBS powder purchased from Fisher BioReagents. Nanopure water was prepared from a Thermo Scientific Barnstead Nanopure filter system and acidified with 88% formic acid purchased from Fischer Chemicals. Nanopure water solution was made to a final concentration of 0.1% formic acid.

3.2.2. Sample Preparation

Stock solutions of each phenolic (10 mM) were prepared in acidified MeOH diluted with acidified water to give a final acidified water:MeOH of 10:90 (v/v) in order to solubilize phenolic standards. Quercetin, however, required 30% MeOH for solubilization to give a final acidified water:MeOH of 70:30 (v/v). A dilution series (0 – 5600 µM) of each polyphenol stock solution was prepared (1 mL) with acidified water as the diluent. The final concentration of acidified MeOH in the stock solutions was 0-5.6% except for the quercetin dilution series which had 0-16.8% MeOH (0.1% formic acid). A stock solution of HSA (25 µM) was prepared in 1X PBS (pH7.4).

3.2.3. Instrument and Parameters

All fluorescence intensities were recorded on a BMG LabTech (Cary, NC, USA) ClarioStar Plus microplate plate reader in spectral scan mode. The monochromator was set to excite at 295 nm and scan emission from 320-450 nm with an excitation filter (295-

10, BMG LabTech) and an LP dichroic filter (LP 309, BMG LabTech). A 96-well plate (Corning Inc., Corning, NY, USA) with black sides and clear bottom was used for all fluorescence measurements.

3.2.4. Fluorescence Quenching Assay

For each quenching experiment, 175 µL of HSA was added to 25 µL of phenolic. After combination on the microplate, final concentrations were as follows: phenolics were 0-700 µM, HSA was 22 µM, MeOH was 0-0.7% or 0-2.1% in the case of quercetin. Experiments were ran in triplicate including a blank containing only 175 µL PBS, and 25 µL acidified water. Each plate was gently shaken for 2 minutes by hand before fluorescence emission spectrum (320-450 nm) was recorded. Two control experiments were run as follows: (1) the intrinsic fluorescence of each phenolic was measured by combining 175 µL 1X PBS and 25 µL phenolic at each dilution concentration and (2) the effect of acidified MeOH on HSA fluorescence was measured by combining 175 µL HSA and 25 µL acidified MeOH at each dilution concentration (0-5.6% and 0-16.8%) with acidified water used as the diluent.

3.2.5. Stern-Volmer Model and Data Analysis

The fluorescent property of the single tryptophan residue in the 2A binding site of HSA enables changes in HSA fluorescence emission spectra to be representative of changes in the local environment of Trp-214 (Royer, 2006). To obtain the HSA emission spectrum, the data was plotted as average fluorescence intensity (FI) versus wavelength (320-450 nm) (Appendix A). Inner filter effects due to intrinsic fluorescence of phenolic compounds were assessed and subtracted from HSA emission spectra. No effect of

acidified MeOH was observed (Appendix B). To obtain Stern-Volmer plots, the Stern-Volmer equation (**Equation 1**) was fit to HSA emission maxima as a function of polyphenol concentration, where F_0 was the HSA emission max without the polyphenolic quencher (Q) and F was HSA emission max at each quencher concentration. The slope of the line was determined to obtain the Stern-Volmer constant, K_{SV} (M^{-1}). To obtain the Stern-Volmer bimolecular rate constant, k_q ($M^{-1}s^{-1}$), **Equation 2** and the HSA fluorescence lifetime value of 7.1 x 10^{-9} s (determined previously (Amiri, Jankeje, & Albani, 2010)) were used.

Equation 1

$$\frac{F_0}{F} = 1 + K_{SV}[Q]$$

Equation 2

$$K_{SV} = k_q \times \tau$$

3.2.6. Statistics

Fluorescence emission spectra were measured in triplicate and mean emission max (± standard error) was used in Stern-Volmer calculations. Stern-Volmer parameters K_{SV} and k_q were reported at mean ± standard error. Analysis of variance (ANOVA) was

measured at alpha=0.05 significance level and Tukey's multiple comparison test was the post-test used to compare means.

3.3. Results and Discussion

The findings in this study provide evidence for the first time of a binding interaction between the metabolites of polyphenols and the circulatory protein human serum albumin (HSA). Methyl gallate, gallic acid and protocatechuic acid demonstrated protein-ligand binding to HSA by a static quenching mechanism. Each of these polyphenols demonstrated a strong correlation ($R^2 > 0.96$) to the Stern-Volmer quenching model, and each of their quenching rate constants (k_q) were greater than the maximum possible quenching rate constant of a collision-induced interaction ($\sim 10^{10}$ M-1s-1), suggesting that the interaction was complex formation. In addition, 3-(3-hydroxyphenyl) propionic acid and 3,4-dihydroxyphenyl acetic acid demonstrated the lowest binding affinity to HSA with quenching rate constants (k_q) within the limits of collision-based quenching. Lastly, interaction with polyphenols demonstrated perturbations to HSA conformation indicated by bidirectional shifts in HSA emission maxima.

3.3.1. Fluorescence Quenching Tests and Quenching Mechanism

Polyphenol-albumin binding was investigated using fluorescence spectroscopy to determine polyphenol metabolite quenching constants (k_q). To investigate the binding interaction between polyphenols and HSA, a fluorescence quenching study was performed. HSA (22 µM) was combined with polyphenols in a dilution series (0-700 µM) and changes in HSA fluorescence emission spectra were measured with the Stern-Volmer model (Equation 1 and Equation 2). All compounds tested demonstrated a high correlation

coefficient (R^2 > 0.93) between F_0/F and polyphenol concentration (Q) except 4 hydroxyphenyl acetic acid (R^2 = 0.25) (Figure 9 - Figure 15). The K_{SV}'s (Table 1) of QC samples quercetin (3.84×10^4 M^{-1}) and (-)-epicatechin (5.77×10^2 M^{-1}) were consistent with a previous study on flavonoids and BSA in which quercetin (53×10^4 M^{-1}) demonstrated a higher K_{SV}, greater binding affinity, than (-)-epicatechin (13.8×10^3 M^{-1}) (Papadopoulou, Green, & Frazier, 2005). Although previous studies report K_{SV}'s of greater magnitude (Papadopoulou et al., 2005) (Skrt, Benedik, Podlipnik, & Ulrih, 2012) (Soares, Mateus, & de Freitas, 2007), this study utilized HSA rather than BSA which has a single tryptophan residue (Trp-214), one-half of the tryptophan residues in BSA (Trp-134 and Trp-213), thereby explaining the lesser fluorescence quenching response. Thus, the fit of the Stern-Volmer model to the fluorescence spectra was consistent with the formation of a binding interaction between Trp-214 of HSA and polyphenol metabolites

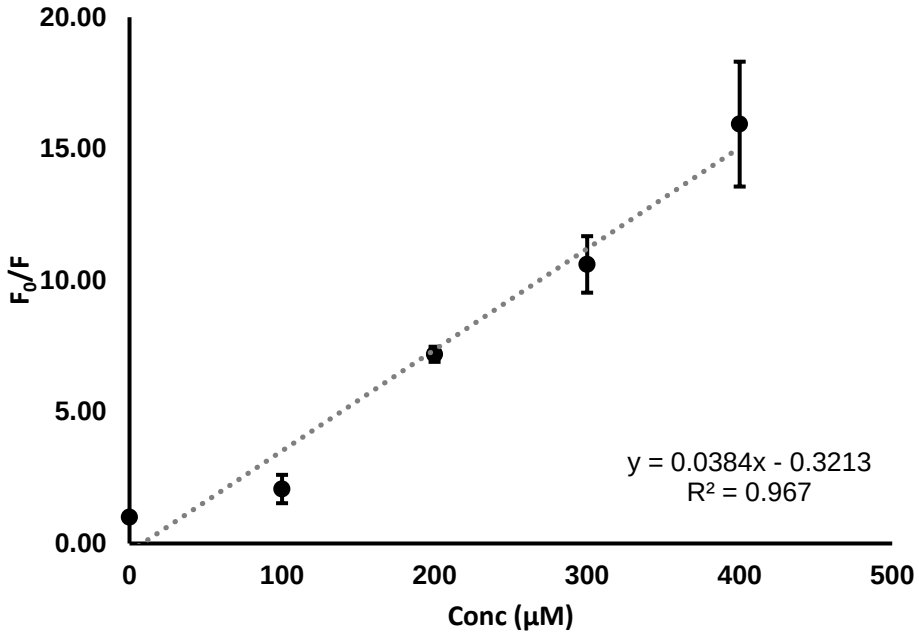

Figure 9. Representative Stern-Volmer plot of HSA relative fluorescence (F0/F) caused by binding with quercetin (0-700 µM).

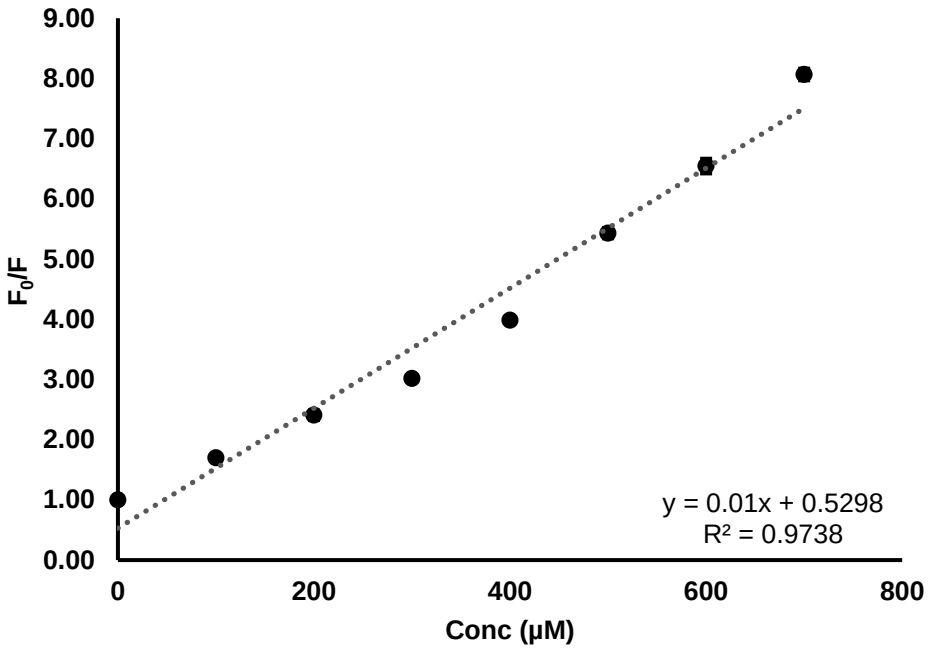

Figure 10. Representative Stern-Volmer plot of HSA relative fluorescence (F0/F) caused by binding with methyl gallate (0-700 µM).

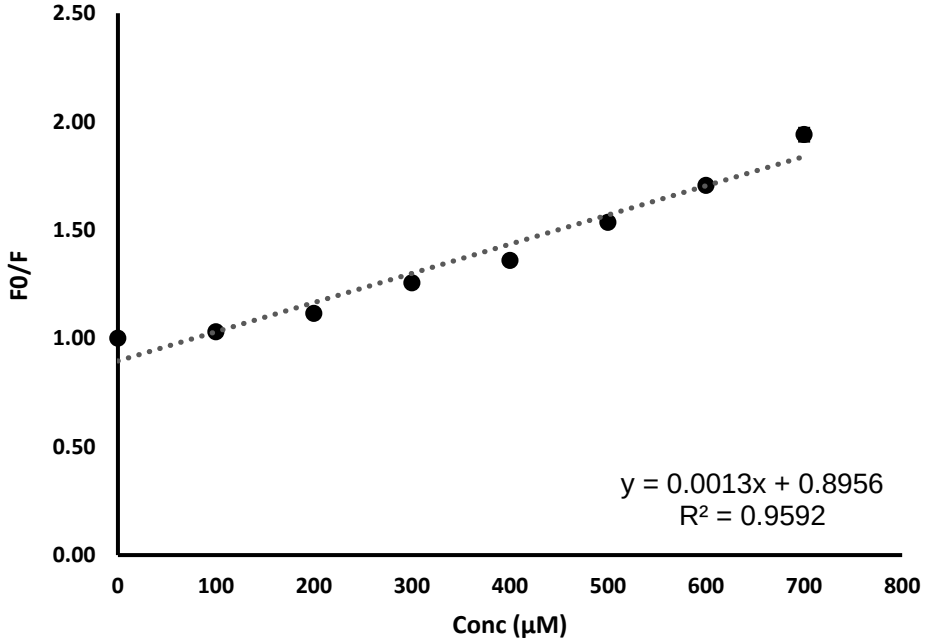

Figure 11. Representative Stern-Volmer plot of HSA relative fluorescence (F0/F) caused by binding with gallic acid (0-700 µM).

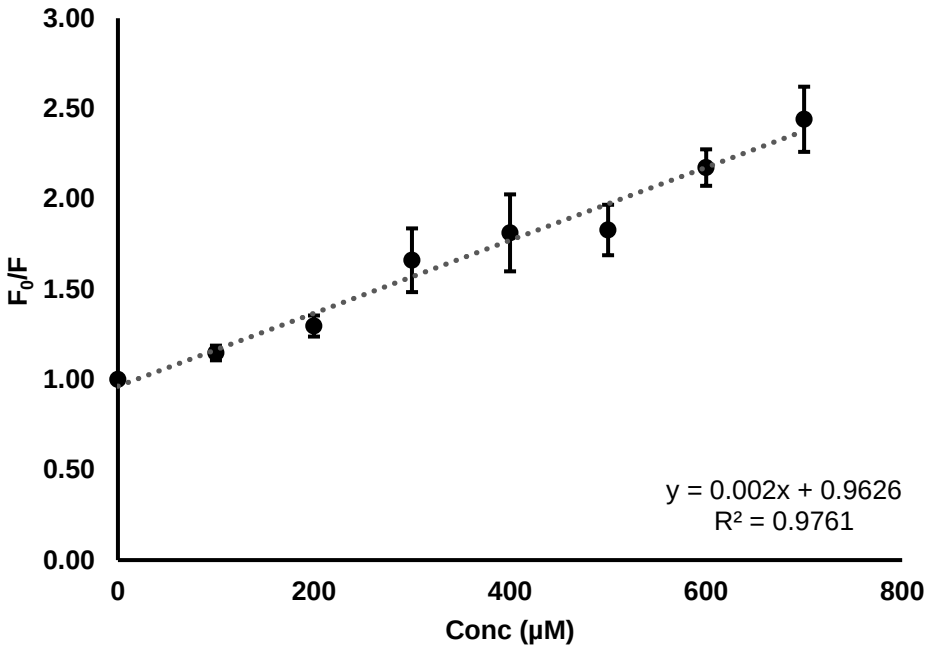

Figure 12. Representative Stern-Volmer plot of HSA relative fluorescence (F0/F) caused by binding with protocatechuic acid (0-700 µM).

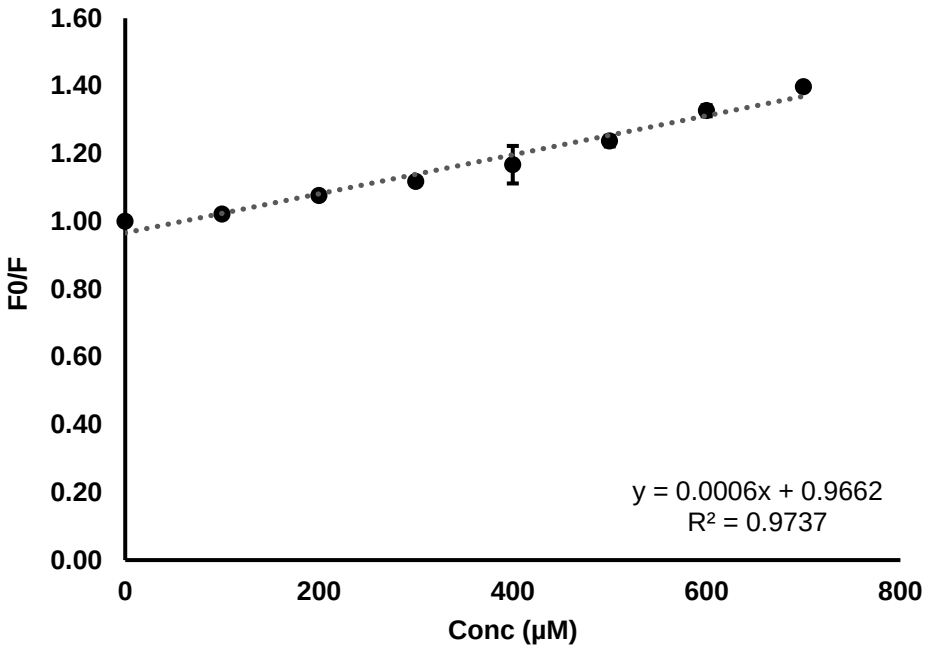

Figure 13. Representative Stern-Volmer plot of HSA relative fluorescence (F0/F) caused by binding with (-)-epicatechin (0-700 µM).

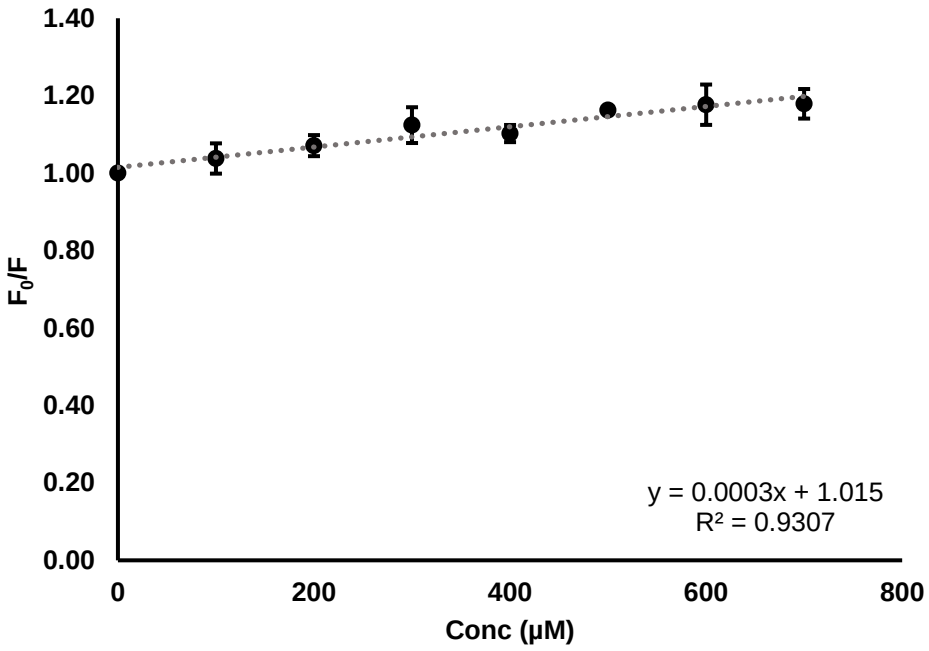

Figure 14. Representative Stern-Volmer plot of HSA relative fluorescence (F0/F) caused by binding with 4-hydroxyphenylacetic acid.

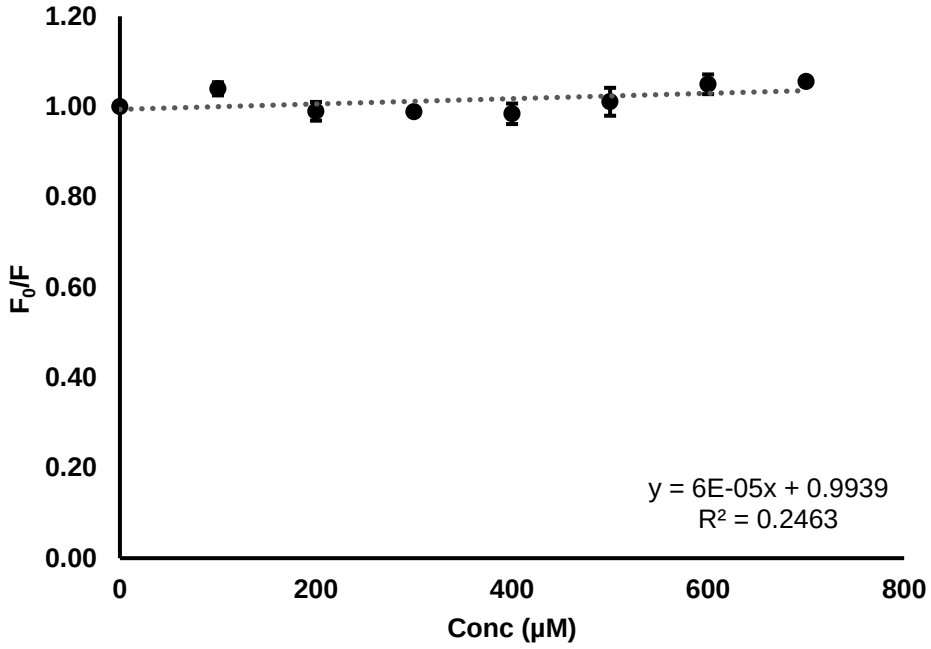

Figure 15. Representative Stern-Volmer plot of HSA relative fluorescence (F0/F) caused by binding with 3-(3-hydroxyphenyl) propionic acid.

Classification of the quenching mechanism of polyphenols may provide information about how polyphenols and HSA associate in blood circulation. In order to determine whether quenching occurred by protein-ligand complex formation (static quenching), or collision between protein and ligand molecules (dynamic quenching), the bimolecular quenching rate constant, k_q, was calculated according to Equation 2. The value used for fluorescence lifetime of HSA excitation was 7.7 ns, which has been experimentally determined previously (Amiri et al., 2010). Four of the 7 polyphenols tested, (quercetin, methyl gallate, gallic acid, and protocatechuic acid) exhibited quenching constants greater than the maximum quenching constant possible for a dynamic

quenching process, 10^{10} $M^{-1}s^{-1}$, and thus were identified as static quenchers (Table 1). However, 3 of the 8 polyphenols tested ((-)-epicatechin, 3-(3-hydroxyphenyl) propionic acid, and 4-hydroxyphenyl acetic acid) exhibited quenching constants below the maximum quenching constant possible for a dynamic quenching process, and thus were identified as dynamic quenchers (Table 1). Static quenching indicated a protein-ligand binding type of intermolecular interaction, in which a complex was formed between HSA and polyphenols, while dynamic quenching indicated that polyphenols quenched HSA fluorescence through ordinary collisions between molecules in a solution (Arroyo-Maya, Campos-Terán, Hernández-Arana, & McClements, 2016; Soares et al., 2007). The results of the Stern-Volmer quenching analysis showed that some HSA-polyphenol interactions are more than just collisional.

Table 1.Stern-Volmer (K_{SV}) and Bimolecular Quenching Rate (k_q) constants of phenolic compounds combined with HSA.

	R^2	K_{SV} (M^{-1})	k_q (M^{-1} s^{-1})	Quenching Mechanism
Quercetin	0.96	3.84E+04 ± 5.18E+03[a]	5.41E+12 ± 7.30E+11[a]	static
Methyl gallate	0.97	9.97E+03 ± 7.01E+01[b]	1.40E+12 ± 9.89E+09[b]	static
Gallic acid	0.96	1.35E+03 ± 1.55E+01[c]	1.90E+11 ± 2.18E+09[c]	static
Protocatechuic acid	0.98	2.02E+03 ± 2.14E+02[c]	2.84E+11 ± 3.02E+10[c]	static
Epicatechin	0.97	5.77E+02 ± 1.10E+01[c]	8.12E+10 ± 1.54E+09[c]	dynamic
4-hydroxyphenyl acetic acid	0.93	2.62E+02 ± 2.09E+01[c]	3.69E+10 ± 2.95E+09[c]	dynamic
3-(3-hydroxyphenyl) propionic acid	0.25	5.91E+01 ± 2.16E+01[c]	8.33E+09 ± 3.04E+09[c]	dynamic

Data is expressed as mean ± std error for n=3 replicates. Different letters within a column indicate significant difference at $p<0.05$ by Tukey's multiple comparison test.

The fluorescence quenching tests of methyl gallate, gallic acid and protocatechuic acid demonstrated a structural trend in HSA-binding affinity. Each of these polyphenol metabolites demonstrated protein-ligand binding to HSA and also demonstrated complete conjugation in their carbon skeletons through delocalized electrons. Electron delocalization caused the hydrogen atoms in the hydroxyl and methoxyl groups to be more acidic and thus more stable in hydrogen bonding interactions with HSA (Figure 8). Accordingly, the acidity of the hydroxyl groups increased because the aromatic ring was linked to the hydroxyl or methyl group substitutions via a skeleton of unsaturated C-C double bounds. Furthermore, methyl gallate demonstrated significantly greater albumin-binding relative to gallic acid and protocatechuic acid possibly explained by its increased hydrophobic character. Increased binding from both carbon conjugation and methyl substitution has been reported in a previous studies on polyphenols with BSA (Poloni, Dangles, & Vinson, 2019) and HSA (Hui, Xiaojuan, Poklar Ulrih, Pradeep, & Jianbo, 2019). Moreover, the IIA subdomain of HSA consists of a hydrophobic pocket in which Trp-214 resides along with other aromatic and non-aromatic, non-polar amino acids which form stable binding interactions with other hydrophobic compounds (He & Carter, 1992). Overall, conjugation and increased hydrophobicity were the basis of the differential binding affinities demonstrated by fluorescence quenching tests.

The fluorescence quenching tests of 4-hydroxyphenyl acetic acid and 3-(3-hydroxyphenyl) propionic acid showed a dynamic quenching mechanism and were also explained by their structural differences from the other phenolics examined. Both 4-hydroxyphenyl acetic acid and 3-(3-hydroxyphenyl) propionic acid demonstrated C-C

saturation in their carbon skeleton which differed from the other compounds which demonstrated complete conjugation in their carbon skeletons (Figure 8). Moreover, 3-(3-hydroxyphenyl) propionic acid showed the poorest correlation to the Stern-Volmer quenching model ($R^2 = 0.25$) further supporting that its interaction with HSA was likely based upon the collision among molecules in solution (dynamic quenching) rather than an intermolecular binding interaction. Similarly, a previous study on quercetin and its derivatives concluded that there were three factors involved in quercetin-albumin binding, the C-ring 4-oxo moiety, C-ring C2-C3 unsaturation, and presence of B-ring hydroxyl groups (Claudine Manach et al., 1996). The former two factors, 4-oxo moiety and C2-C3 unsaturation, confer complete conjugation between all carbons in the flavonoid ring structure. The findings in this study are consistent with the proposed structural theory as quercetin demonstrated greater binding affinity to HSA ($K_{SV} = 3.84 \times 10^4\ M^{-1}$) compared to (-)-epicatechin ($K_{SV} = 5.77 \times 10^2\ M^{-1}$) possibly due to the presence of the 4-oxo moiety and C2-C3 unsaturation in quercetin and the absence of these structural features in (-)-epicatechin. Also, earlier studies on the structural basis for flavonoid antioxidant activity concluded that complete conjugation via electron delocalization is responsible for flavonoid radical-scavenging activity (Bors, Heller, Michel, & Saran, 1990). In this study, the same electron delocalization factor was present in the compound which demonstrated greater binding affinity (quercetin, methyl gallate, gallic acid and protocatechuic acid) but not in those which demonstrated lesser binding affinity ((-)-epicatechin, 3-(3-hydroxyphenyl) propionic acid and 4-hydroxyphenyl acetic acid). Thus, it is conceivable

that polyphenol metabolites with a conjugated carbon skeleton form stronger binding interaction in the IIA binding pocket of HSA.

3.3.2. Effect of Polyphenols on HSA Emission Maxima

Shifts in HSA emission maxima (λ_{max}) in the presence of polyphenols suggests that a conformational change was induced at the tryptophan residue (Trp-214) and may provide evidence of an intermolecular interaction. Tryptophan resides in the hydrophobic interior of proteins due to its aromatic moiety and either buries within its hydrophobic core or unfolds and becomes solvent- exposed when combined with other compounds. To show the effect of polyphenols on HSA emission maxima, the directional change of HSA emission maxima was monitored in response to increasing polyphenol concentration. HSA emission maxima was 340 ± 4 nm in all quenching tests and was in agreement with previous reports (Amiri et al., 2010; Dangles et al., 2001; Moriyama, Ohta, Hachiya, Mitsui, & Takeda, 1996). The fluorescence spectra showed that 4 of the 7 polyphenols tested induced a shift in HSA emission maxima (quercetin, methyl gallate, gallic acid and protocatechuic acid) while the other 3 did not show any effect on HSA emission maxima ((-)-epicatechin, 3-(3-hydroxyphenyl) propionic acid, and 4-hydroxyphenyl acetic acid) (Appendix A). Among the 4 compounds which caused a shift, HSA maxima demonstrated a bathochromic displacement (red shift) from 344 ±1 nm (mean ± std dev) to 398±89 nm and 343 ± 1 nm to 370 nm as quercetin and methyl gallate concentrations increased, respectively. The red shift in HSA emission max indicated that Trp-214 moved toward the hydrophobic core of HSA as a result of binding with quercetin and methyl gallate. However, HSA maxima demonstrated a hypsochromic displacement (blue shift) from 337

± 4 to 330 ± 1 nm and 341 ± 1 nm to 330 ± 1 nm when gallic acid and protocatechuic acid concentrations were increased, respectively. The blue shift indicated that HSA unfolded, exposing more of the fluorescent tryptophan residue (Trp-214) toward the solvent, as a result of binding with gallic acid and protocatechuic acid. No displacement was observed for (-)-epicatechin, 3-(3-hydroxyphenyl) propionic acid and 4-hydroxyphenyl acetic acid. The different effects on HSA emission max can be explained by the structural differences in the compounds tested. Quercetin and methyl gallate demonstrate greater hydrophobic character compared to gallic acid and protocatechuic acid, which demonstrate greater hydrophilic character. Thus, the blue shift in HSA emission max reflects protein fold toward the hydrophobic core of HSA in response to combination with the hydrophobic compounds quercetin and methyl gallate. The opposite effect, red shift in HSA emission max, reflects protein unfolding toward the aqueous solvent in response to combination with hydrophilic compounds gallic acid and protocatechuic acid. Though no previous fluorescence quenching studies on polyphenol metabolites and HSA were found, a previous study on quercetin reported a red shift in BSA emission maxima in response to combination with the polyphenol (Skrt et al., 2012) and this difference can be explained by the difference in the fluorophores of BSA and HSA as discussed above. The extra fluorescent tryptophan residue in BSA, Trp-134, resides in the IB subdomain which is more solvent exposed compared to Trp-213 which is buried in a hydrophobic pocket of the IIA subdomain while the single fluorescence tryptophan residue in HSA, Trp-214, also resides in the hydrophobic IIA subdomain (Labieniec & Gabryelak, 2006; Moriyama et al., 1996; Peters, 1996). Therefore, it is possible that interaction with an additional

fluorophore that is solvent-exposed may result in more quenching, explaining the disagreement between studies with HSA and studies with BSA.

3.4. Conclusions

In summary, this study showed that polyphenol metabolites bind to human serum albumin. HSA binding affinity was influenced by size and the presence of a conjugated carbon framework in the polyphenol. Also, HSA binding was accompanied by changes in HSA conformation. These results show that it is not just the larger, parent compounds that interact with HSA, but also the low molecular weight metabolic breakdown products. And, because it is believed to be the low molecular weight metabolites, rather than the parent compounds, that are responsible for polyphenol bioactivity and health benefits, it may be worthwhile to design studies that evaluate the role of HSA binding on the behavior of absorbed compounds in blood circulation.

4. COMBINATION OF GALLIC ACID AND ITS DERIVATIVE METHYL GALLATE WITH HSA MODULATES ANTIPROLIFERATIVE AND CYTOCHROME C ACTIVITY WITH THE METHYLATED DERIVATIVE SHOWING GREATER EFFECT

4.1. Introduction

Irregular apoptosis is a defining characteristic of cancer cell growth and strategies to induce apoptosis of abnormal cells is a key focus of cancer therapeutics. Naturally-occurring polyphenolic compounds found in fruits, seeds, and leaves of plants have demonstrated chemoprevention by their ability to modulate the mitochondrial release of cytochrome c and the activation of caspases responsible for initiation of the apoptotic process. For example, quercetin, a member of the flavonol polyphenols, demonstrated antiproliferative activity against HeLa cervical carcinoma cells by accumulation of reactive oxygen species (ROS) and disruption of mitochondrial function (Bishayee, Ghosh, Mukherjee, Sadhukhan, Mondal, & Khuda-Bukhsh, 2013). Gallic acid, a phenolic acid, induced cytochrome c release in a CK kinase-dependent activation of Bax and Bcl-2 proteins (M. L. Lin & Chen, 2017). Likewise, gallic acid and curcumin, a natural phenol with two aromatic *O*-methoxy-phenolic groups, demonstrated antiproliferative effect in MDA-MD-231 breast cancer cells by depletion of the endogenous antioxidant glutathione and cleavage of caspase-3 (Moghtaderi, Sepehri, Delphi, & Attari, 2018). Methyl gallate, a derivative of gallic acid, also prevented growth of tumor cells in by caspase activation and modulation of Bcl-2 gene expression in glioblastoma cells (Chaudhuri, Ghate, Singh,

& Mandal, 2015). While various polyphenols show chemopreventive potential *in vitro*, clinical efficacy in cancer patients has been limited due to poor translation of results. Investigation of human metabolism, pharmacology and analytical tools are required to improve efficacy of polyphenols in humans.

Human serum albumin (HSA) is the most abundant transport protein in circulation and albumin-binding plays a role in the therapeutic efficacy of chemopreventive agents. HSA transports a variety of ligands including xenobiotics from pharmaceutical or dietary intake through non-covalent interactions stabilized by hydrogen-bonding and hydrophobic interactions (Wani, Bakheit, Zargar, Alanazi, & Al-Majed, 2021). The multiple ligand-binding sites of HSA and its natural abundance at 35-50 g/L (Kaiser, Lacheta, Passon, & Schieber, 2019; Larsen et al., 2016) allows a variety of compounds to associate with the carrier, improving their stability and solubility profiles (Parodi, Miao, Soond, Rudzinska, & Zamyatnin, 2019). Plasma protein binding modulates the unbound concentration of an active compound thereby affecting the dosage necessary for therapeutic action (Du et al., 2014). In addition, an active compound's equilibrium ratio of albumin-bound to albumin-unbound fraction modulates half-life, clearance rate and toxicity, as only unbound compound is available for metabolic transformation and elimination (Larsen et al., 2016). Thus, albumin interacts with every compound that enters the body as a conveyor to sites of action, metabolism and elimination. Consequently, albumin binding is a determining factor in the safety and efficacy of biologically active compounds.

Although absorbed compounds circulate while associated to human serum albumin, the majority of *in vitro* bioactivity screening studies do not include albumin in

the model design and thus the effect of the albumin-phenolic interaction on the activity of phenolics is unknown. Information gathered from *in vitro* screening assays characterize mechanisms of action and intracellular activities of bioactive compounds; however, these results depend on simplifications made in the *in vitro* model, namely the assumption that bioactivity is independent of albumin. Therefore, the objective of this study was to investigate the effect of albumin binding on phenolic bioactivity by measuring antiproliferative activity of phenolics combined with HSA. The effect of albumin binding on the antiproliferative activity of phenolic acids gallic acid (GA) and methyl gallate (MG), with and without HSA, was measured by MTT assay in HepG2 human liver cancer cells and RT-qPCR and ELISA were used to assess the effect of HSA binding on Cytochrome C (Cyto C) gene and protein expression.

4.2. Materials and Methods

4.2.1. Evaluation of Antiproliferative Activity

HepG2 cells (ATCC) were used to evaluate the antiproliferation activity of phenolics and phenolic-HSA combinations. Cells were grown in 10 mL (55 cm^2) culture dishes (Corning) at 37 °C and 5% CO_2 in DMEM 1X (Gibco) D-glucose positive (4.5 g/L), sodium pyruvate negative, L-glutamine positive). DMEM was supplemented with 10% fetal bovine serum (FBS) and 5% penicillin-streptomycin. Routine cell passage was performed when cells were 80% confluent by harvesting with trypsin (0.25%). Cells were seeded into 96-well plates (100 µL/well) and allowed to attach and reach 80% confluency before administration of phenolic or phenolic-HSA treatments.

Preparation of Cell Treatments

HepG2 cells were treated with phenolics and phenolic-HSA combinations for 48 hours before evaluation of cell viability. Cells were treated with phenolic standards (100 µL/well) solubilized in DMSO then diluted 1000X in 2.5% FBS DMEM or 2.5% FBS DMEM supplemented with 600 µM HSA (in PBS, 0.22µM sterile-filtered) (Sigma). Final concentrations of phenolic treatments in 2.5% FBS DMEM or 2.5% FBS DMEM supplemented with 600 µM HSA were 0-5000 µM and final concentration of DMSO in treatments were <0.1%.

Cell Proliferation Assay

The MTT assay was used to test the antiproliferation activity of phenolics and phenolic-HSA combinations on HepG2 cells. Cells were seeded at a density of $5x10^4$ cells/well in a 96-well plate. After plated HepG2 cells were treated with phenolics or phenolic-HSA combinations, 100 µL of MTT solution (0.45 mg/mL in PBS) (Thiazolyl Blue Tetrazolium Bromide, Sigma) was added to each well of a 96-well plate. HepG2 cells were incubated with MTT solution for 1 hour. Subsequently, MTT solution was aspirated and 100 µL DMSO was used to solubilize formazan crystals formed from enzymatic activity of viable cells. Viable cells demonstrated a purple color from the accumulation of formazan crystals and were quantified by measurement of absorbance at 570 nm on a microplate reader (BMG Labtech ClarioStar Plus).

4.2.2. Evaluation of Biochemical Effects

Gene Expression Analyses

In order to evaluate the effect of HSA binding at the genetic level, mRNA expression of Cytochrome C (Cyto C) was measured in HepG2 cell lysates by RT-qPCR. Cells were seeded on 6 well plates and grown to 80% confluence. Cells were treated (2hrs) with C, or GA, MG and GA+HSA or MG + HSA at the IC_{50} determined by MTT assay. Total RNA was extracted with a Zymo Quick RNA Microprep kit (Zymo Research Corp, Irvine CA). Extracted RNA was quantified and used to synthesize cDNA using an iScript Reverse Transcription kit (Bio-Rad, Hercules, CA). mRNA expression was analyzed by qPCR using the comparative C(T) method described previously (Schmittgen & Livak, 2008).

Protein Expression Analyses

Effects of HSA-binding at the protein level were evaluated by ELISA of Cyto C levels in cell lysates. HepG2 cells were seeded onto 10 cm cell culture plates and were grown to 80% confluence before treatment with GA, MG (at IC_{50} levels) with and without HSA (600 µM). All treatments were solubilized in 2.5% FBS-supplemented DMEM culture medium unless otherwise specified; control treatment consisted of 2.5% FBS-supplemented DMEM only. After 40 hours, treatment was removed, and cells were harvested with a cell scraper in 5mL 1X PBS. After concentration by centrifugation (4000 RPM, 4 C, for 10 min), cells were re-suspended in 300 µL cell lysis buffer comprised of Pierce RIPA buffer (Thermo Scientific #8990), proteinase inhibitor (Halt #1861281) and phosphatase inhibitor (Sigma #P5726) at a ratio of 100:1:1 (v/v). Protein samples were

clarified by centrifugation (10,000 rpm, 4 °C, 30 min), aliquoted and stored at -80 °C until analysis.

Total protein concentration in HepG2 cell lysates were determined spectrophotometrically according to the Bradford assay. Briefly, protein cell lysates were diluted 20X in nano-pure H_2O and combined with Bradford reagent (Bio-Rad) on a 96-well plate before absorbance was red at 595 nm. BSA (2 mg/mL, Fisher BioReagents) was used as the standard. Protein measurement was based upon chemical reaction between the Coomassie Brilliant Blue G-250 dye in the Bradford reagent and protein in samples and standards. Concentration was determined using the BSA standard calibration curve.

Quantitative detection of Cyto C in HepG2 cell lysates was determined by a commercial sandwich ELISA kit (Invitrogen) according to the manufacturer's instructions. Briefly, samples were pre-diluted in assay buffer and combined with a biotin-conjugated detection antibody in microwells pre-coated with capture antibody. After 2-hour incubation, wells were emptied, washed and streptavidin-HRP was added for 1 hour before TMB substrate was added to react with HRP. The substrate-HRP signal was detected spectrophotometrically at 450 nm. Concentration of Cytochrome C was determined using the Cytochrome C standard calibration curve.

A limitation of the experimental design for evaluation of biochemical effects was the absence of a time course study identifying the time at which Cyto C levels were elevated before evaluation of the effect of phenolic treatment on Cyto C gene and protein expression.

4.2.3. Statistical Analyses

Antiproliferative activity was expressed in % cell proliferation and was calculated by normalizing the absorbance of each treatment to the abs of the control: Abs_t/Abs_c*100. The median inhibitory concentration (IC_{50}) was determined based upon the dose-response curve. Cyto C gene expression was normalized to B-actin and expressed as the mean fold change ± std error. Cyto C protein expression was normalized to mg total protein and was expressed mean ± std error. All statistical differences were determined with Tukey's Multiple Comparisons test at $p<0.05$.

4.3. Results and Discussion

4.3.1. Evaluation of Antiproliferative Activity

Post consumption, absorbed phenolics circulate in human plasma associated to serum albumin and consequently the albumin-phenolic interaction may alter the protective effects of phenolics. One of the protective effects of phenolics is modulation of mitochondrial function which is often demonstrated by uptake and reduction of MTT dye in the mitochondria of viable cells. In order to determine the effect of HSA binding on the antiproliferative activity of phenolic acids gallic acid (GA) and methyl gallate (MG), MTT assay was performed on HepG2 cells after 48hr treatment with phenolics alone and combined with HSA at its physiological concentration (600 µM). The IC_{50} results of GA (436 ± 25 µM) and MG (310 ± 52 µM) were consistent with those reported previously and show that both phenolics reduce cancer cell proliferation in a dose-dependent manner (Figure 16 and Figure 17) (Kamatham, Kumar, & Gudipalli, 2015 2015; Lima et al., 2016; Okamura, Hashimoto, Shimada, & Sakagami, 2004 & Sakagami, 2004; G. Sun, Zhang,

Xie, Zhang, & Zhao, 2016 Zhang, & Zhao, 2016). However, combination with HSA increased GA and MG IC_{50} values more than 5-fold to 2466 ± 340 μM and 5167 ± 289 μM, respectively (Table 2, Figure 18 and Figure 19), suggesting more GA, MG was required to produce the cytotoxic effect. Similar results were found in a study on the antiproliferative effect of phenolics combined with milk proteins in which casein-bound GA demonstrated 48.4% of its cytotoxicty in HepG2 cells compared to cytotoxicity of GA alone (Mehanna, Hassan, El-Din, Ali, Amarowicz, & El-Messery, 2014). A study on ginseng terpene ginsenoside Rh2 (G-Rh2) showed that HSA-bound G-Rh2 yielded 50% of free Gh2 antiproliferative activity (Y. Lin, Li, Song, Zhu, & Jin, 2017). Moreover, the antioxidant activity of albumin-bound catechin was reported to be 80% of free catechin (Arts et al., 2002). Thus, the IC_{50} increases demonstrated in this study are consistent with previous studies that link protein-polyphenol binding to altered polyphenol bioactivity. Furthermore, to demonstrate that the increase in IC_{50} was due to albumin-phenolic binding, rather than utilization of HSA as a nutrient source, the response of control cells was compared to control cells with HSA by student's t test Error! Reference source not found.). When HSA was added to the culture medium, no significant differences ($p<0.05$) were found in HepG2 cells grown in either 10% or 2.5% FBS culture medium. Thus, the larger concentration needed to induce the antiproliferative effect demonstrated by the increase in IC_{50} was due to phenolics binding to HSA, an additional compound in the natural environment of blood circulation to organs and tissues. In addition, the albumin in the FBS of the culture medium was considered negligible based upon a previous report of albumin in 10% FBS at 6.2 μM (0.41 g/L) (J. Xiao & Högger, 2015), nearly 2 orders of

magnitude lower than the 600 µM HSA used in this study. Overall, investigation of GA, MG antiproliferative activity in the presence of HSA at physiological levels demonstrated the need to have controlled conditions mimic the natural environment of an absorbed compound as closely as possible.

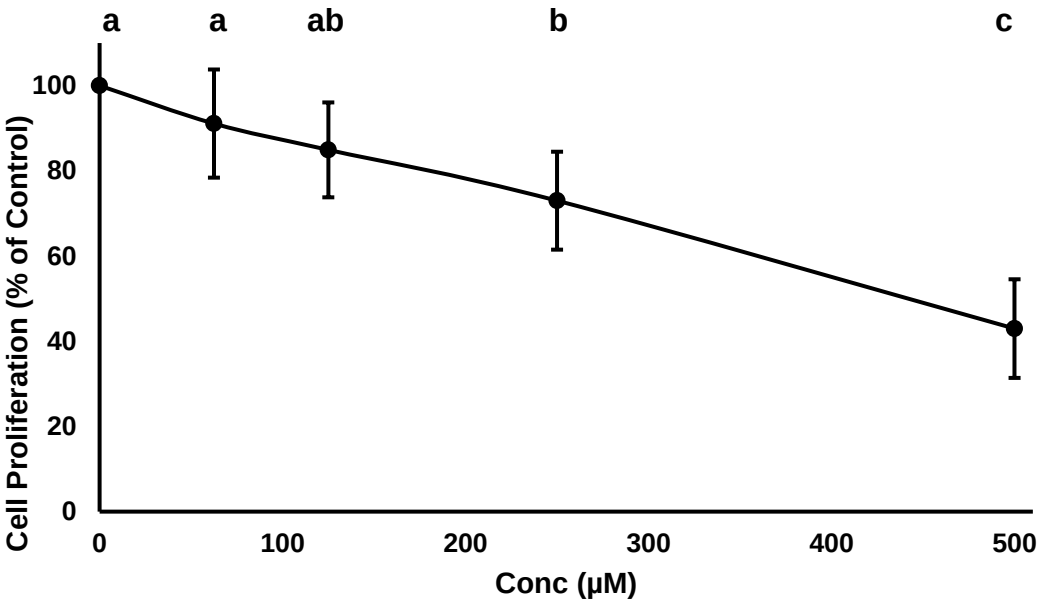

Figure 16. Representative dose-response effect of GA (0-500 µM) on HepG2 cell proliferation. Data is expressed as mean ± CV% for at least 3 replicates. Data points with different letters are significantly different at $p<0.05$ according to Tukey's multiple comparisons test.

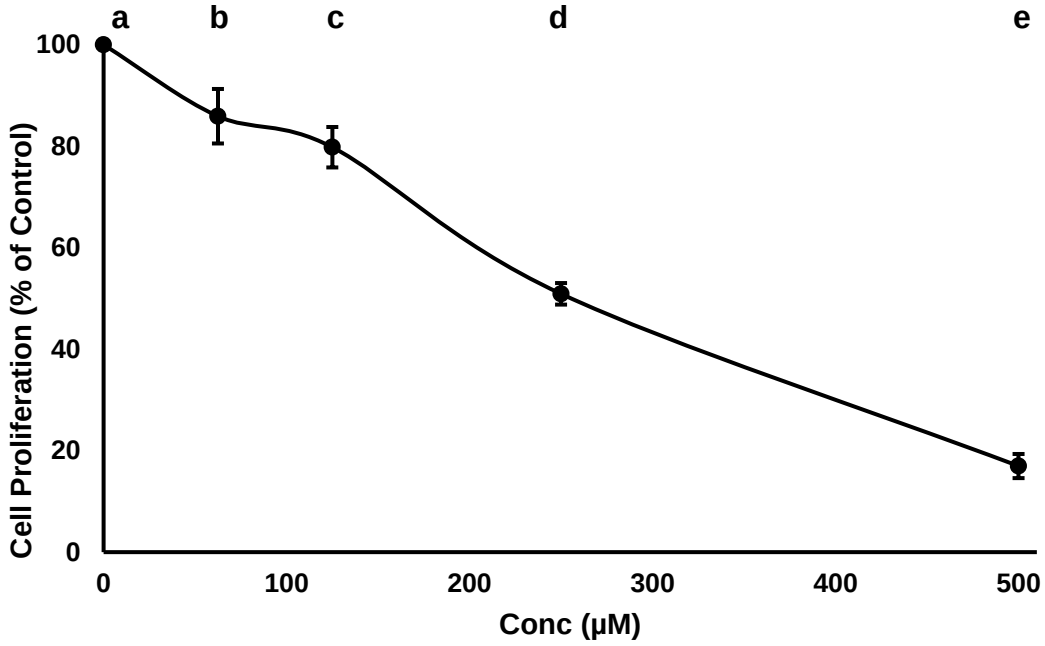

Figure 17. Representative dose-response effect of MG (0-500 µM) on HepG2 cell proliferation. Data is expressed as mean ± CV% for at least 3 replicates. Data points with different letters are significantly different at p<0.05 according to Tukey's multiple comparisons test.

GA and MG showed significantly different antiproliferative activities when studied in combination with HSA. When MG was combined with HSA its IC_{50} increased more than GA, suggesting that MG was less potent than GA when in the in the environment of HSA (Table 2). A possible explanation for the difference between IC_{50}s is a difference in HSA binding affinity of GA and MG. In a previous study on protein-ligand binding between low molecular weight phenolics and HSA, the Stern-Volmer quenching constant of MG (9.97E+03 ± 7.01E+01) was nearly 10X greater ($p<0.05$) than GA (1.35E+03 ± 1.55E+01) [unpublished manuscript] (Tillman & Talcott, 2021). This

difference was attributed to the methyl substitution contributing greater hydrophobicity and stronger hydrogen bonding to the Trp-214 residue in HSA as well as the other non-polar and aromatic residues in the HSA binding pocket. The methyl substitution was also shown to increase the plasma protein binding affinity of MG compared to GA in patients with type II diabetes (J. Xiao, Zhao, et al., 2011). Thus, it is conceivable that the increased binding affinity to HSA is responsible for the higher concentration of MG required to achieve cell death induction in HepG2 cells. Overall, HSA binding modulated the antiproliferative activity of GA, MG in agreement with previous studies on structure-affinity relationships.

Table 2. Gallic acid (GA) and Methyl gallate (MG) antiproliferative activity (IC_{50}) with and without HSA (600µM) in Hep G2 cells.

Compound	IC_{50} (µM) ± Std Dev
GA	436 ± 25^{a}
MG	310 ± 52^{a}
GA+HSA	2466 ± 340^{b}
MG+HSA	5167 ± 289^{c}

IC_{50}= concentration (µM) at which 50% of cell proliferation was inhibited. Data are expressed as mean ± std dev of at least 3 replicates. Different letters within rows indicate a significant difference at $p< 0.05$ Tukey's multiple comparisons test.

Table 3. Independent t tests of control cells and control cells supplemented with HSA (600 µM) in MTT assay.

		Group	Mean	Std Error	DF	*t*	*p*
10% FBS media	Test 1	C	2.413	0.187	4	0.1284	0.904
		C+HSA	2.456	0.272			
	Test 2	C	2.402	0.103	4	2.006	0.1153
		C+HSA	2.138	0.082			
	Test 3	C	0.761	0.016	6	0.4711	0.6542
		C+HSA	0.773	0.020			
2.5% FBS media	Test 1	C	0.659	0.023			
		C+HSA	0.705	0.027	5	1.248	0.2671
	Test 2	C	0.662	0.054			
		C+HSA	0.577	0.007	5	1.848	0.1238
	Test 3	C	1.289	0.032			
		C+HSA	1.409	0.071	5	1.71	0.1479

Data is expressed as mean of $n \geq 3$ independent experiments. Significance level was $p<0.05$.

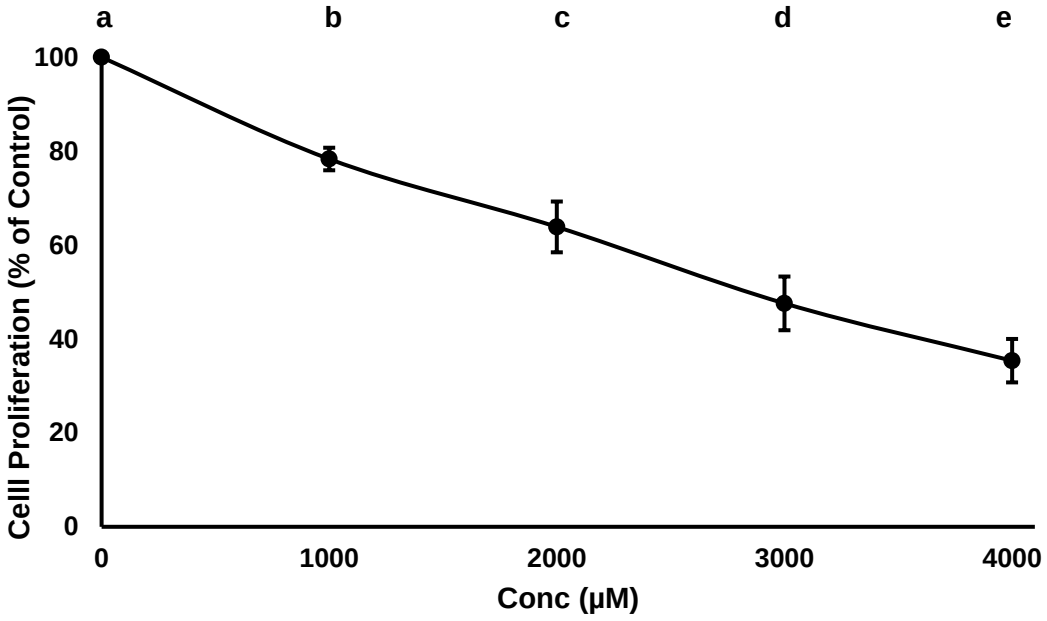

Figure 18. Representative dose-response effect of GA (0-4000 µM) on proliferation of HepG2 cells supplemented with 600 µM HSA. Data is expressed as mean ± CV% for at least 3 replicates. Data points with different letters are significantly different at $p<0.05$ according to Tukey's multiple comparisons test.

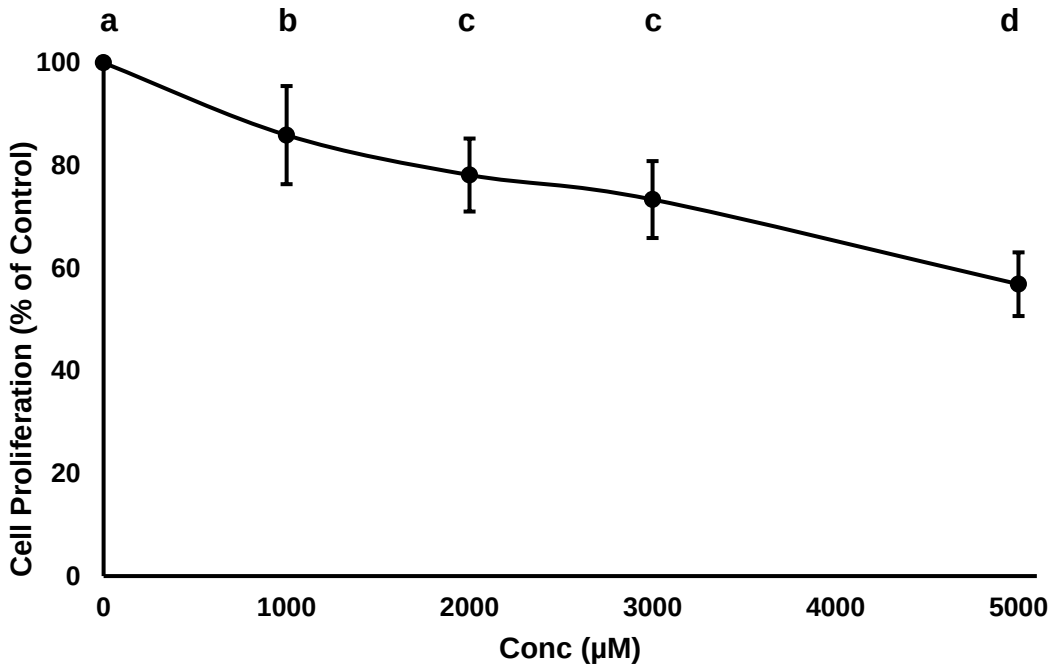

Figure 19. Representative dose-response effect of MG (0-5000 µM) on proliferation of HepG2 cells supplemented with 600 µM HSA. Data is expressed as mean ± CV% for at least 3 replicates. Data points with different letters are significantly different at least p<0.001 according to Tukey's multiple comparisons test.

4.3.2. Evaluation of Biochemical Effects

The effects of albumin-phenolic binding on mitochondrial activity were further investigated. Because cytochrome C (Cyto C) is a biomarker central to metabolic diseases of the mitochondria (Eleftheriadis, Pissas, Liakopoulos, & Stefanidis, 2016), gene and protein expression were measured in Hep G2 cells treated with GA, MG in the presence and absence of HSA. RT-qPCR results indicated GA-HSA binding suppressed Cyto C mRNA levels relative to untreated control cells (Figure 20). After 2 hr treatment, Cyto C mRNA levels in cells treated with GA were no different than untreated control cells

suggesting that Cyto C was not involved in the cellular response to GA treatment. However, when GA was combined with HSA, RT-qPCR results showed a significant reduction ($p<0.05$) in Cyto C mRNA levels relative to the untreated control cells suggesting that Cyto C was involved cell death induction when cells were treated with GA combined with HSA. In order to determine if this change in Cyto C expression was due to GA-HSA binding or utilization of HSA, mRNA levels of untreated cells were compared to untreated cells supplemented with HSA. When HSA was added to the culture medium, no significant differences ($p<0.05$) were found in Cyto C mRNA levels between control cells or control cells supplemented with HSA. Thus, the RT-qPCR results indicated that Cyto C was involved in the response to GA only when GA was combined with HSA and that the response was not due to HSA supplementation. The different results observed when GA was assayed with and without HSA demonstrate the potential to observe different outcomes when the controlled conditions of *in vitro* experiments mimic physiological conditions. However, this trend was not observed in Cyto C mRNA levels of cells treated with MG and MG+HSA (Figure 20) possibly because the sharp decrease ($p<0.05$) caused by MG alone reduced Cyto C mRNA levels to the detection limit such that decreases beyond this amplification cycle were not detectable by the qPCR assay. Consequently, Cyto C protein expression was run as a confirmatory test to evaluate the effects of HAS binding on GA, MG bioactivity. When Cyto C protein levels were measured by ELISA after treatment with GA, MG with and without HSA, similar results were found as with mRNA levels: GA-HSA binding significantly modulated Cyto C protein levels whereas treatment with GA did not; and MG-HSA binding did not induce

any further changes in Cyto C levels beyond the change induced by MG alone (Figure 21 The effect of HSA binding at the protein level was also observed in the immunoblotting results of the study on ginsenoside Rh2 (G-Rh2) in which the apoptosis-inducing cleavage of PARP by caspase-3 was absent when G-Rh2 was combined with HSA, effectively reducing Gh2 antiproliferative activity (Y. Lin et al., 2017). Overall, the results from the investigation of gene and protein expression indicated HSA-phenolic binding alters the biochemical targets of phenolics in mechanistic studies.

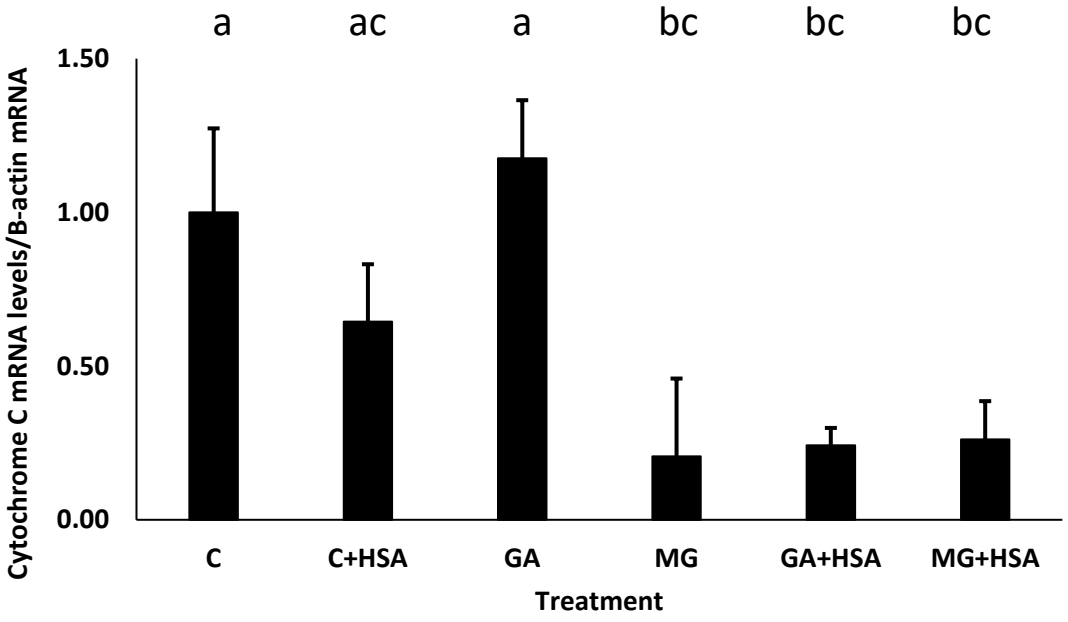

Figure 20. Cytochrome C mRNA levels in Hep G2 cell lysates. Hep G2 cells were treated with GA, MG at IC_{50} levels (with and without HSA (600 µM)) for 2hrs and analyzed by real time RT-qPCR as a ratio to B-actin mRNA, values are means ± SE (n≥3 replicates). Columns with different letters are significantly different at p<0.05 according to Tukey's multiple comparisons test.

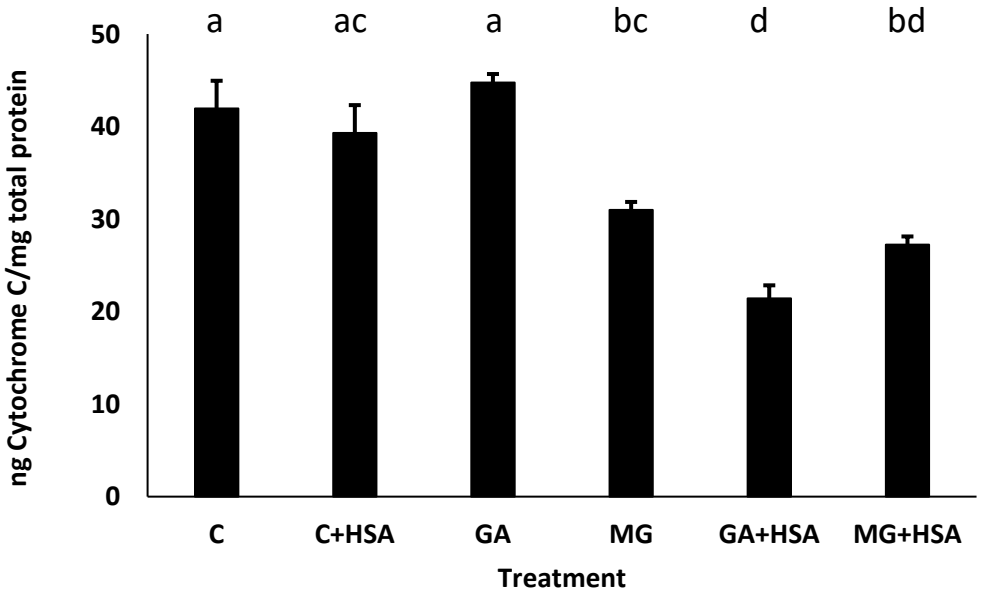

Figure 21. Cytochrome C protein levels in Hep G2 cell lysates. Cytochrome C levels were quantitatively detected by ELISA after treatment with GA, MG (with and without HSA (600 µM)) as detailed in Materials & Methods. Data are expressed as mean ± std error. Columns with different letters are significantly different ($p<0.05$) according to Tukey's multiple comparisons test.

4.4. Conclusions

In summary, this study demonstrated altered antiproliferative activity, gene and protein expression after treatment of phenolics under conditions inclusive of HSA at physiological levels. Antiproliferation tests showed increased IC_{50} values when phenolics were combined with HSA, with MG demonstrating a greater increase in IC_{50} than GA. Also, mRNA expression tests showed differential regulation of Cytochrome C when GA, MG were combined with HSA and corresponding results at the protein level. An

association between structural substitutions and effects with HSA and bioactivity may exist, as MG demonstrated antiproliferative potency and greater effect on Cyto C at the mRNA and protein level relative to GA. Inclusion of HSA at levels that most closely mimic the *in vivo* physiological environment may lead to more reliable toxicity screening results for compounds with disease prevention capability.

5. ADDITION OF TANNIN ACYL HYDROLASE INCREASES *Rhus coriaria* GALLOTANNIN BIOACCESSIBILITY IN A CLINICAL STUDY

5.1. Introduction

Sumac, *Rhus coriaria*, is a fruit with a natural acidic pH and high astringency. It's consumed as spice or condiment after drying and milling processes and but is also brewed into a lemonade-like beverage or herbal tea. Gallotannins are polymers of β-glucogallin, monogalloyl glucose, connected by *m*-depside ester linkages (**Figure** 22) (Niemetz & Gross, 2001), are responsible for the astringency of sumac. While gallotannins have historically been used to tan leather, they have also been used as medicinal compounds in Mediterranean and Middle Eastern cultures and several reports demonstrate their bioactivities (Banerjee, Kim, Krenek, Talcott, & Talcott, 2012; Fang, Kim, Noratto, Sun, Talcott, & Mertens-Talcott, 2018; Serrano et al., 2009; Zargham & Zargham, 2008). The health benefits are suggested to come from gallic acid (Ghaznavi et al., 2018; Kosuru, Roy, Das, & Bera, 2018; J. Sun et al., 2014; Velderrain-Rodriguez et al., 2018), the hydrolytic product of gallotannin digestion. Within the human colon, tannase-expressing microbiota digest and metabolize gallotannins which are too large to be absorbed across the barrier of small intestine cells. However, the composition of host gut microbiota varies by diet and disease status (Williamson & Clifford, 2017),and as a result, populations with GI diseases may not be exposed to such therapeutic compounds if they do not possess tannase-expressing gut microbiota.

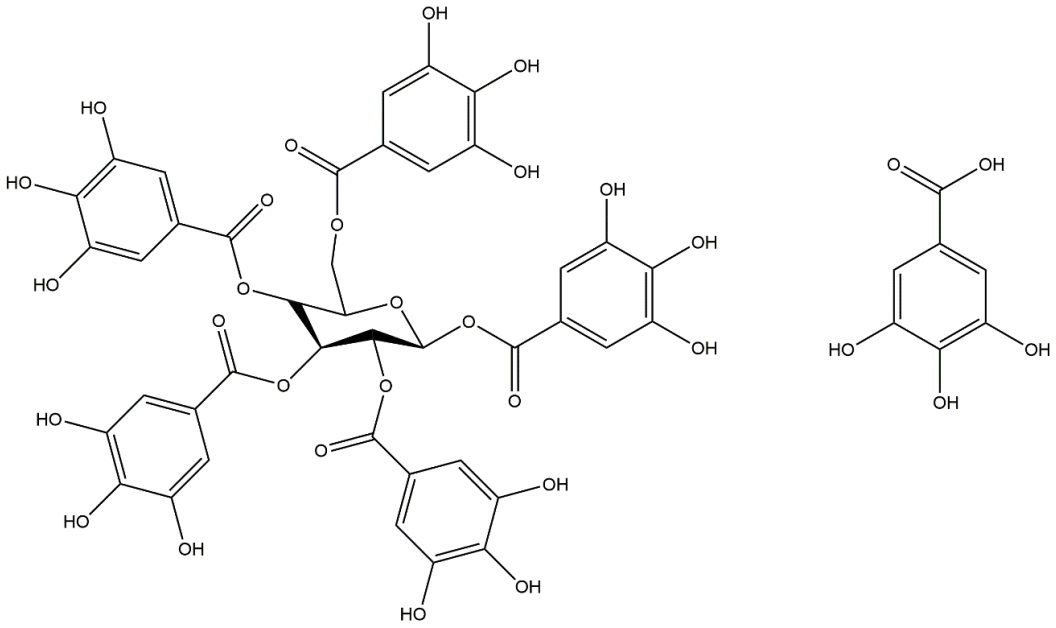

Figure 22. Structure of a gallotannin, 1,2,3,4,6-penat-O-galloyl-β-D-glucose (a), and its hydrolytic product gallic acid (b).

Food processing with tannin acyl hydrolases (tannase), a commercial esterase, has been used to improve the organoleptic properties of tea, *Camellia sinensis*. Tannase clarifies tea by hydrolyzing the ester bonds of gallated catechins and gallotannins to liberate units of gallic acid, resulting in a product with fewer insoluble solids and better sensory characteristics (C. S. Kumar, Subramanian, & Rao, 2013). Tannases have also demonstrated therapeutic application to enhance the biological activity of foods compared to unhydrolyzed controls. For example, *in vitro* models of obesity have shown that tannase treatment of green tea reduced lipid accumulation in adipocytes (H. S. Kim et al., 2020; Roberto et al., 2016). Similar effects were observed in studies on antioxidant activity (Macedo, Battestin, Ribeiro, & Macedo, 2011; Martins, Macedo, & Macedo, 2020). In

addition, we have reported that pre-processing of mango juice with commercial tannase increased free gallic acid by cleaving galloyl groups from gallotannins (Sirven, Negrete, & Talcott, 2018). Thus, food processing with commercial enzymes like tannase may be a strategy to avoid the digestion required by colon microbiota and improve absorption in populations with chronic disease.

While there are several reports on the use of tannase to improve black tea characteristics, and emerging *in vitro* literature on the therapeutic application of tannases, there is a lack of information on the clinical efficacy of tannase pretreatment. The objective of this study was to evaluate the efficacy of tannase on sumac gallotannin bioaccessibility in a crossover pilot clinical trial. The concept of gallotannin bioaccessibility improvement was demonstrated in an *in vitro* hydrolysis model and then evaluated in a human clinical trial of sumac tea pre-hydrolyzed with tannase.

5.2. Materials and Methods

5.2.1. Chemicals

Gallic acid, methyl gallate, ethyl gallate, rhodanine, citric acid and methanol (MeOH) with 0.1% formic acid (HPLC grade ≥ 99.9%) were purchased from Sigma Aldrich. 88% formic acid was purchased from Fischer chemicals. Nanopure water was prepared from a Thermo Scientific Barnstead Nanopure filter system. Purified drinking water was purchased from a local market. Tannin acyl hydrolase (5000 U/g) was purchased from Enzyme Development Corporation (EDC S#31648). Ground sumac powder (Cerez Pazari) was sourced from Turkey and imported by Nakal Tasimacilik Gida

Ins (Istanbul, Turkey). Sumac was shipped at ambient temperature, and stored in a cool, dry place away from direct light.

5.2.2. Gallic Acid Release from Tannase-Treated Sumac Tea

Sumac tea was hydrolyzed by combining 200 µL sumac tea at 0.04 g/mL (final concentration) and 200 µL tannase solution at 10 U/mL or 0.002 g/mL, pH 5.4 (final concentration) over 48 hours. The reaction was stopped by addition of 1600 µL of MeOH (0.1% formic acid) followed by centrifugation to concentrate sample. An unhydrolyzed control consisted of 200 µL sumac tea at 0.04 g/mL (final concentration) and 200 µL nanopure water acidified with 1M citric acid to pH 5.4 in place of tannase. Gallic acid standards (0-22 µM) were used for quantitative determination of gallic acid release. Release of gallic acid from tannase-treated sumac tea was determined by the Rhodanine assay as previously described (Inoue & Hagerman, 1988). For the assay, 400 µL of the tannase-treated sumac tea and a standard curve was mixed with 600 µL of 0.667% rhodanine in 100% MeOH. After 10 min, 400 µL 0.5 M NaOH was added to develop a rhodanine-gallic acid chromogen. After 20 min, sample was diluted with 8.6 mL nanopure H_2O and absorbance was measured at 520 nm.

Study Design and Study Procedures

The institutional review board at Texas A&M University approved the clinical study protocol IRB No 2019-1143F. The study design was a randomized, crossover study with 3 treatments. Healthy volunteers (n=11, 10 female, 1 male; ages 20-36 yrs) were recruited and screened for the following criteria: no history of chronic diseases, intestinal disorders, acute cardiac events, seizure, stroke, cancer, liver or renal dysfunction; no

recurrent hospitalizations; no antibiotics or prescription medications other than hormonal treatments; no pregnancy or lactation; no alcohol or substance abuse; no smoking more than 1 pack per week and no exercise greater than 60 minutes, five times per week. No study participant had an allergy to fruits, nuts, spices, herbs or botanicals.

Three days (72 hrs) before consumption of each study treatment, the study participants agreed to avoid foods and beverages with tannins including sumac, coffee, tea, wine, beer, tree nuts, sorghum, barley, and several fruits including mangoes, berries, grapes and pomegranates. Study participants also agreed to fast for 12 hours before each treatment. Study participants consumed the 3 treatments on 3 different days separated by a washout period of at least 3 days. (Diet recalls and baseline (t=0) plasma levels were reviewed during analysis to evaluate participants' adherence to dietary restrictions and baseline subtractions of GA, MG were made if necessary.) On the day of study treatment consumption, participants submitted a meal log listing the foods and beverages consumed during the washout before a baseline (t=0 hr) blood sample (20 mL) was collected. The study treatment was administered after the baseline blood draw. Administration of the study treatment was chosen in a random order known to study personnel but blind to study participants. A final blood sample was collected 90 min post-prandial. Blood samples were collected in 10 mL EDTA tubes and immediately processed by centrifugation (4000 x *g*, 10 minutes, 4 °C) to separate plasma. Blood plasma (8 mL) was acidified to 5% with 88% formic acid and extracted with 4 sample volumes of MeOH (0.1% formic acid). The MeOH solution was spiked with an internal standard of ethyl gallate at 1 µM for in-study validation. Plasma extracts were stored at -80 °C until LC-MS analysis.

Study Treatment Preparation

Sumac tea treatments (200 mL, 0.04 g/mL) were prepared according to Good Manufacturing Practices (GMPs) at Texas A&M University's Department of Food Science and Technology. Three study treatments were prepared: the first was sumac tea pre-hydrolyzed with tannase for 48 hours before serving to study participants (Pre-Treated), the second was sumac tea served with tannase at time of consumption (Co-Consumption) and the third was an unhydrolyzed sumac tea (Control). The Pre-Treated study treatment was prepared by adding sumac powder (8 g) to 100 mL boiling water and steeping for 30 minutes to simulate an herbal-infused tea. Pre-Treated tea was filtered through a commercial coffee filter, cooled to room temperature, diluted with an equal volume of tannase solution (100 mL, 20 U/mL or 0.004 g/mL) made with potable water and hydrolyzed for 48 hours at room temperature. The Co-Consumed study treatment was prepared exactly as the Pre-Treated study treatment with one exception: the tannase solution (100 mL) was added to the sumac tea 1 minute before serving to study participants. Likewise, the Control study treatment was prepared by diluting the stock sumac tea (0.08 g/mL) with an equal volume of water without tannase.

5.2.3. LC-MS Analysis

Gallic acid levels in sumac tea study treatment as well as levels of gallic acid and methyl gallate derived from the consumption of sumac tea were evaluated by LC-MS. A C_{18} 4µm 2 x 150 mm column (Synergi Fusion RP, Phenomenex) was used for gradient separation and an ESI triple quadrupole mass spectrometer (Thermo TSQ Quantiva) equipped with an HPG 3400 RS pump was used for identification. The LC gradient

program began with 90% H_2O (0.1% FA) and 10% MeOH (0.1% FA) at 450 µL/min. After 5 min, MeOH was increased to 40% and then to 95% after 7 min before returning to initial conditions. Ion chromatograms were acquired in multiple reaction monitoring (MRM) mode and samples were scanned for 1 quantifying ion and 2 confirmation ion**Table** 4). The MS system ran XCalibur 4.1 Method Editor in negative ionization mode with ion source voltage of 2300V, ion transfer tube at 350 °C, vaporizer temperature at 400 °C, gas flows of 50 (arbitrary) units sheath gas, 15 units auxiliary gas and 1 unit of sweep gas, and collision induced dissociation (CID) gas pressure of 1.5 mTorr. Product ion collision energy (CE) was tuned to the precursor and product ions of each targeted compound.

Table 4. List of Multiple Reaction Monitoring (MRM) transitions in LC-MS analysis.

Compound	$[M-H]^-$ (m/z)	Quantifying ion (m/z)	Confirming ion 1 (m/z)	Confirming ion 2 (m/z)
GA	169	125	79	81
MG	183	124	168	78
EG (IS)	197	124	169	125

Abbreviations: GA, gallic acid, MG, methyl gallate, EG, ethyl gallate, IS, internal standard.

Quality Control (QC) and Calibration Solutions

A quantitative analysis of the sample preparation and LC-MS method was conducted with calibration standards and quality control (QC) samples using the external

standard method. Assay sensitivity, selectivity, instrument accuracy, precision and stability were evaluated in spike-and-extract studies. Stock solutions of GA, MG, and EG were prepared at 6.25 µM in a mobile phase (solvent blank) comprised of 80% acidified MeOH (0.1% formic acid) and 20% acidified H_2O. In addition, QC samples were prepared with calibration solutions spiked in to plasma matrix at 3 concentration levels. Plasma matrix consisted of baseline plasma pooled from fasted, healthy volunteers (n=11), extracted with 4 sample volumes of acidified MeOH, vortexed and centrifuged to separate from precipitated proteins leaving the plasma supernatant composed of 80% acidified MeOH. Blank samples (mobile phase only) and matrix blanks were also assessed in between injection batches to evaluate carry over.

Sensitivity

Sensitivity was determined by assay linearity as well as by evaluation of limit of quantification (LOQ) and limit of detection (LOD). To evaluate linearity, a serial dilution of GA and MG calibration standard (0.0015 – 6.25 µM) was spiked into a pool of plasma matrix from fasted, healthy volunteers (n=11) and the linear regression coefficient of peak areas versus concentration was measured. Limits of quantification (LOQ) and detection (LOD) were identified as the lowest concentration with a peak area signal-to-noise (S/N) ratio of 5 for LOQ and S/N of 3 for LOD.

Selectivity

Selectivity was determined by extraction efficiency and matrix effect. Extraction efficiency was measured by comparison of peak areas in pre-spiked and post-spiked plasma matrix and was expressed as recovery percent (% Recovery = [Pre-spike

Area/Post-spike Area] *100). Matrix effect was measured by comparison of peak areas in post-spiked plasma matrix and solvent blank (ME = [1 – Post-spike Area/Solvent Blank] *100). Briefly, pre-spiked plasma referred to addition of calibration solution before extraction with 4 sample volumes MeOH; post-spiked plasma referred to addition of calibration solution after extraction; and, the solvent blank referred to an 80% acidified MeOH and 20% acidified H_2O solution. Both extraction efficiency and matrix effect were determined at 3 QC levels at 0.391, 0.781 and 3.12 µM.

Accuracy and Precision

Instrument accuracy and precision was determined by spiking a calibration solution of GA and MG at 1.56, 3.12 and 12.5 µM into a pool of plasma matrix from fasted, healthy volunteers (n=13). CV% of spiked plasma measured in triplicate measurements within a day (intraday) and across three consecutive days (interday) was measured.

Stability

Stability of GA, MG under conditions of the autosampler (4°C) was measured over 24 hours the same manner as intraday precision.

5.2.4. Statistical Analysis

Gallic acid levels in the hydrolysis models of sumac tea combined with tannase were tested with n=6 replicates (Rhodanine assay) and were reported as mean % of control ± CV%. Means were analyzed with 1-way ANOVA and Tukey's Multiple Comparisons test or the two-tailed, unpaired t-test ($\alpha = 0.05$). For the clinical trial, the sumac tea study treatment and each study participant's plasma were analyzed in triplicate (n=3) and was

reported as mean concentration ± standard error. The means were analyzed with the nonparametric 1-way ANOVA (Kruskal-Wallis) and Dunn's Multiple Comparisons Tests. GraphPad Prism 5 was used to calculate all ANOVA statistics.

5.3. Results and Discussion

5.3.1. Gallic acid release in hydrolysis model

The high molecular weight of gallotannins prevents digestion and absorption in the upper GI tract of humans making the colon the primary site for digestion by bacterial-induced hydrolysis. However, the hydrolysable *m*-depside linkages in gallotannins present an opportunity to improve gallotannin bioaccessibility earlier in the digestion process with the pre-hydrolysis or concomitant consumption of food-grade tannases. Tannase has long been used as a food processing aid to improve organoleptic quality of gallotannin-rich foodstuffs like juice and tea, but has not been used in sumac or to improve gallic acid bioaccessibility. To model the principle of gallotannin bioaccessibility improvement, sumac tea was hydrolyzed with tannase from *Aspergillus oryzae* and gallic acid released measured by the Rhodanine assay. After incubation with tannase, gallic acid levels in sumac increased over 24hrs at pH 2.9 (Figure 23) indicating that tannase was active at the low pH of sumac tea. Furthermore, in this study, tannase demonstrated rapid activity in sumac tea as gallic acid release increased to 221 ± 7% ($p<0.05$) of the control within 1 minute of processing (Figure 23). Gallic acid release from sumac tea remained constant between 1 min and 24 hours, where it showed a slight increase ($p<0.05$) at 24 hours and no further increases were observed between 24 and 48 hours. These results are consistent with our previous findings in which tannase (0.5 U/mL) hydrolyzed mango juice

gallotannins in a pH 2 gastric digesta matrix and free gallic acid levels remained higher ($p<0.05$) than untreated controls during the pH 7 small intestine digestion phase as well (Sirven et al., 2018). Taken together, the *in vitro* evidence of tannase activity across physiological pH levels and the demonstration of tannase activity in the acidic medium of sumac tea suggest that tannase-treated sumac tea may increase absorption of gallotannin hydrolytic product gallic acid in humans. Overall, the hydrolysis model illustrated the concept of gallotannin bioaccessibility improvement by pre-processing with tannase and suggests that tannase may be an effective therapy to improve gallotannin digestion.

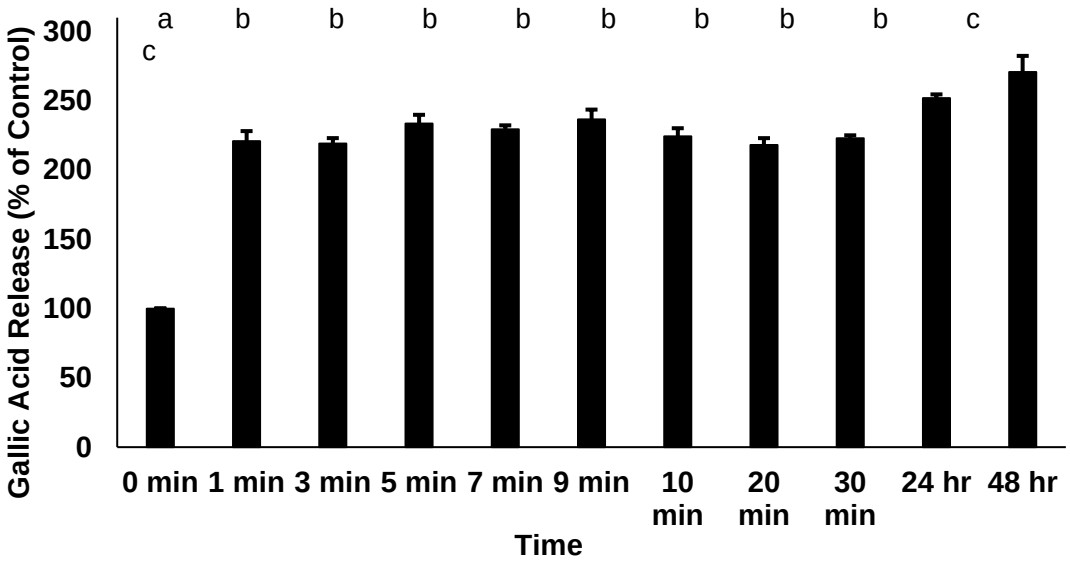

Figure 23. Gallic acid release from sumac gallotannin in a hydrolysis model. Sumac tea (200 µL, 0.04 g/mL was incubated with 200 µL tannase (10 U/mL, 0.002 g/mL) and gallic acid was determined by spectrophotometric detection of gallic acid-rhodanine complex. Data is expressed as mean ± CV% for at least 4 replicates. Columns with different letters are significantly different ($p<0.05$) according to Tukey's multiple comparisons test.

5.3.2. Gallic acid absorption in clinical study

A quantitative analysis of the analytical method was performed before the clinical assessment. Ethyl gallate was chosen as the internal standard (IS) for its superior signal-to-noise ratio and its structural similarity to the gallotannin metabolites of interest. The IS recovery showed significant difference between human plasma and mobile phase suggesting matrix suppression by plasma proteins as a possible interference in assay performance (Figure 24). As a result, the extraction method and LC-MS conditions were validated for sensitivity, selectivity, instrument accuracy, precision and stability. The response linearity was determined between 0.0015 and 6.25 µM in plasma matrix and the linear correlation coefficient (R^2) was greater than 0.99. The limits of quantification (LOQ) for GA, MG and EG were 0.0970, 0.0030 and 0.0060 µM respectively; and the limits of detection (LOD) were 0.0490, 0.0015, and 0.0030 µM, respectively. Extraction recoveries of GA, MG and EG were determined at 3 QC levels, 0.391, 0.781 and 3.12 µM, and ranged between 45 and 92% for GA, 70 and 100% for MG and 72 and 96% for EG Table 5). An evaluation of the matrix effect was performed to assess the effect of matrix suppression by plasma proteins on quantification of GA, MG, and EG and ranged between 0.4 and 73% for GA, 36 and 88% for MG and 36 and 64% for EG (Table 6). The repeatability of the method was evaluated by intraday precision (Table 7) and the reproducibility of the method was evaluated by an interday precision (Table 9). Precision at each QC level demonstrated an error rate below 10% and instrument accuracy was within 15% of the nominal QC calibrator concentrations, in accordance with the acceptability criteria of FDA's Bioanalytical Method Validation guidelines (FDA, 2018).

Sample stability during analysis was determined by intraday precision of samples under conditions of the autosampler (4 °C) and was within acceptability criteria as well (Table 10) indicating little sample degradation during LC-MS analysis.

The results in the quantitative analysis of the LC-MS method highlighted two surprising results concerning the effect of protein-ligand binding on bioactivity and bioaccessibility of phenolic compounds. First, MG demonstrated greater sensitivity with a LOQ of 3 nM compared to GA with a LOQ of 49 nM, despite MG showing a greater interaction with albumin in fluorescence quenching studies Error! Reference source not found.). Second, MG demonstrated more effect on Cyto C gene and protein expression levels relative to GA relative to untreated controls (Figure 20 and Figure 21). These differences may be explained by the greater hydrophobic character in MG which allows it to interact favorably with organic solvents as described in previous chapters.

Table 5. Extraction efficiencies of calibration solutions in plasma matrix.

QC Level (µM)	EG (%)	GA (%)	MG (%)
0.391	72.3	45.1	70.4
0.781	83.0	73.2	83.6
3.125	96.2	92.2	100.5

EG, ethyl gallate. GA, gallic acid. MG, methyl gallate. Data is expressed as recovery percent as defined in Materials and Methods.

Table 6. Matrix suppression of calibrator standards in plasma matrix.

QC Level (μM)	EG (%)	GA (%)	MG (%)
0.391	64.5	73.9	88.6
0.781	49.6	65.2	52.5
3.125	36.5	0.4	36.8

EG, ethyl gallate. GA, gallic acid. MG, methyl gallate. Data is expressed as defined in Materials and Methods.

Table 7. Repeatability (intraday precision) of internal standard (EG), GA and MG spiked into plasma matrix.

QC Level	EG	GA	MG
1.56	4.74	8.81	6.23
3.12	1.43	2.30	1.98
12.5	1.8	6.09	0.72

EG, ethyl gallate. GA, gallic acid. MG, methyl gallate. Data is expressed as CV% of spiked plasma measured in triplicate 3 times within 1 day.

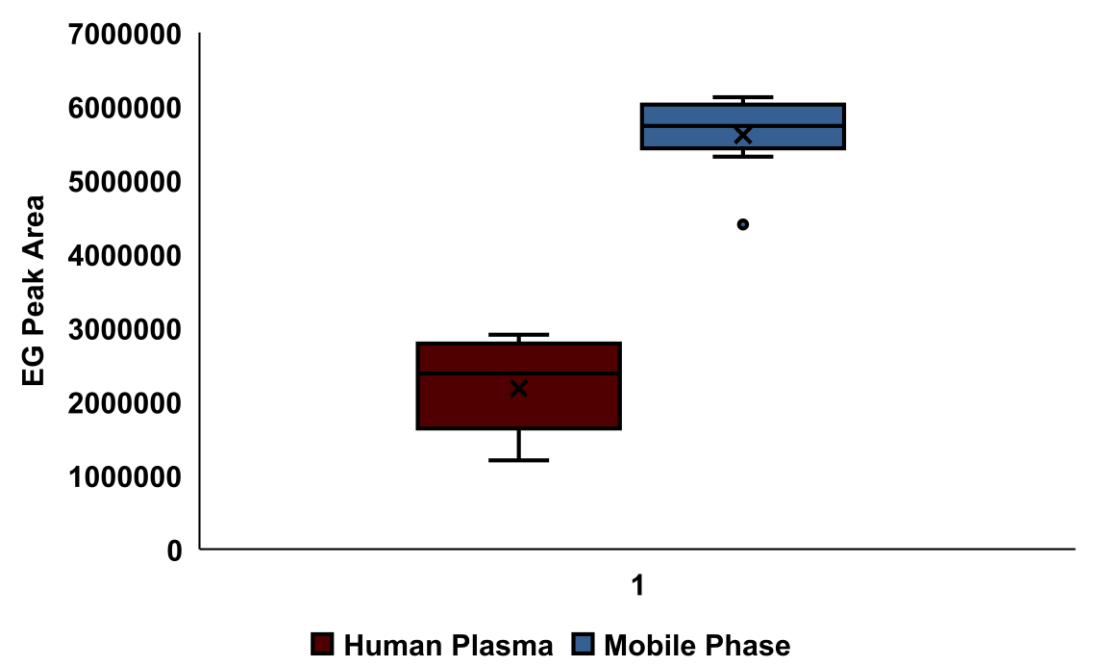

Figure 24. Matrix differences of internal standard (IS) ethyl gallate (EG) in extracted QC samples (n=9). Data shown is response (peak area) of EG in plasma and mobile phase samples spiked with standard solutions of GA, MG at 0.19, 0.39, and 0.78 µM in triplicate and extracted with MeOH. The MeOH solution was spiked with EG at 1 µM.

Table 8. Repeatability (intraday precision) of internal standard (EG), GA and MG spiked into plasma matrix.

QC Level	EG	GA	MG
1.56	4.74	8.81	6.23
3.12	1.43	2.30	1.98
12.5	1.8	6.09	0.72

EG, ethyl gallate. GA, gallic acid. MG, methyl gallate. Data is expressed as CV% of spiked plasma measured in triplicate 3 times within 1 day.

Table 9. Reproducibility (interday precision) of internal standard (EG), GA and MG spiked into plasma matrix.

QC Level	EG	GA	MG
1.56	2.24	1.07	0.77
3.12	0.06	1.00	0.21
12.5	1.78	2.4	2.07

EG, ethyl gallate. GA, gallic acid. MG, methyl gallate. Data is expressed as CV% of spiked plasma measured in triplicate 3 times across 3 consecutive days.

Table 10. Sample degradation rate (stability) over 24 hours under conditions of autosampler (4 °C) for internal standard (EG), GA and MG spiked into plasma matrix.

QC Level	EG	GA	MG
1.56	4.93	4.99	5.76
3.12	4.70	1.39	5.68
12.5	3.73	1.65	3.92

EG, ethyl gallate. GA, gallic acid. MG, methyl gallate. Data is expressed as CV% of spiked plasma measured in triplicate 3 times within 1 day.

The study treatment in the clinical trial consisted of sumac powder infused in boiling water for 30 minutes to a final concentration of 0.04 g/mL. GA levels in the study treatment were 449 ± 1.8 µM (Co-Consumed), 534 ± 1.7 µM (Pre-Treated) and 171 ± 1.0 µM (Control). Hydrolysis of sumac tea increased gallic acid levels to 263 ± 2% (mean ± CV%) and 313 ± 2% of the control for the Co-Consumed and Pre-Treated teas respectively (**Figure** 25). GA release determined by LC-MS was in agreement with GA release determined spectrophotometrically by Rhodanine assay in which GA increased to 221 ±

7 % and 271 ± 12% of the control after 1 minute and 48 hours of hydrolysis, respectively. Furthermore, gallic acid levels in the unprocessed control sumac tea, 171 µM (29.0 ppm), were comparable to a previous studies on tea in which oolong tea demonstrated 135 µM (22.95 ppm) GA in a 0.027 g/mL sample (Pinto et al., 2020) and Assam black tea demonstrated 187.5 µM gallic acid in a 0.03 g/mL sample (Shahrzad, Aoyagi, Winter, Koyama, & Bitsch, 2001). In this study no methyl gallate was detected in sumac tea study treatments, the result in this study agreed with a previous study on sumac extracts in which only trace levels of methyl gallate were detected (Dalar, Dogan, Bengu, Mukemre, & Celik, 2018) and the previous study on Assam black tea (Shahrzad et al., 2001) in which no methyl gallate was found.

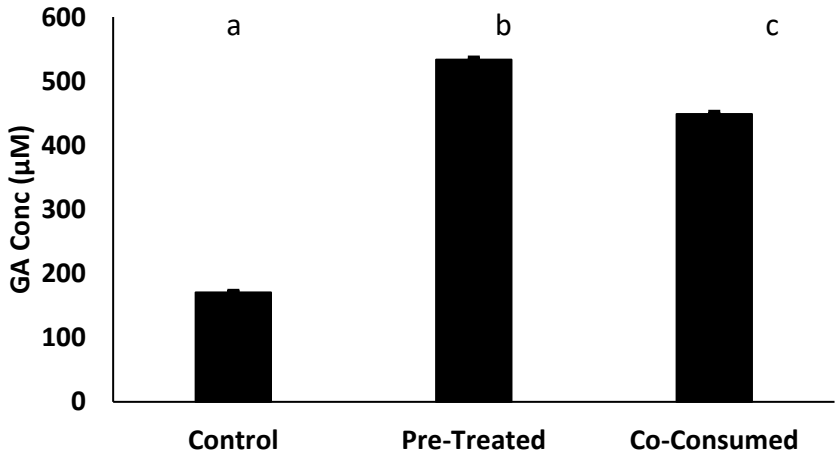

Figure 25. Concentration of gallic acid levels in the sumac tea (0.04 g/mL) study treatments. Data is expressed as mean ± std error. Columns with different letters are significantly different ($p<0.05$) by ANOVA and Tukey's multiple comparisons test. GA = gallic acid. Control = unaltered sumac tea. Pre-Treated = sumac tea hydrolyzed for 48 hours. Co-Consumed = sumac tea hydrolyzed for 1 minute.

Plasma levels of gallic acid and one of its liver metabolites, methyl gallate, were evaluated in 11 human participants (10 female, 1 male; ages 20-36) after consumption of 2 tannase-treated sumac teas and an unprocessed control in a crossover pilot human clinical trial. All analyses included an internal standard (IS) for in-study validation of the sample preparation and LC-MS method. The relative standard error (RSE) of the IS was 4%, demonstrating that the extraction and LC-MS method was reproducible in the detection of compounds absorbed after consumption of sumac tea. GA, 2.41 ± 0.26 µM, was detected in all participants 90 min after consumption of the unaltered sumac tea and was in agreement with previous studies which found 2.09 + 0.22 µM GA absorbed into plasma 1.4 hrs after consumption of Assam black tea (Shahrzad et al., 2001). No MG was detected in any participant, possibly because the early time at which samples were taken, 90 minutes, only allowed for gastric digestion and small intestine absorption, preceding metabolism by methylating enzymes in the liver. A similar study on human plasma metabolites from berry fruit puree showed plasma 2hrs post prandial and found GA levels below LOQ values, but found methylated GA derivatives at their peak level at that time (Pimpao, Ventura, Ferreira, Williamson, & Santos, 2015). However, for the first time, an increase ($p<0.05$) in GA absorption was found in plasma after consumption tannase-treated sumac tea treatments, 3.54 ± 0.32 µM (Pre-Treated) and 3.42 ± 0.23 µM (Co-Consumed), relative to the untreated control, 2.41 ± 0.26 µM (Figure 26). These results suggest that pre-hydrolysis of gallotannin into gallic acid subunits may be an effective strategy for delivery of gallotannin metabolites to human circulation. Also, GA plasma levels after consumption of sumac tea hydrolyzed for 1 minute prior to consumption (Co-

Consumed) were equivalent (p=0.77) to GA plasma levels after consumption of sumac tea hydrolyzed for 48 hours (Pre-Treated). These results provided clinical evidence supporting the use of tannase supplementation in addition to use as a processing aid in the production of RTD pre-hydrolyzed beverages. Pre-hydrolysis of sumac tea increased gallic acid absorption and may lead to an increase in therapeutic effects of gallic acid.

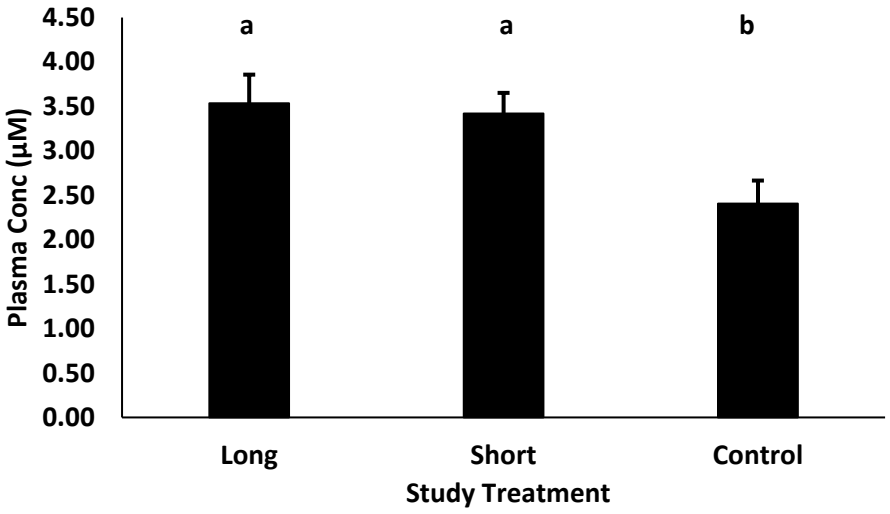

Figure 26. Plasma concentration of gallic acid after consumption of tannase-treated sumac tea (200 mL). n=11 study participants consumed tannase-treated (10 U/mL or 0.002 g/mL) sumac tea (0.04 g/mL) after 72-hour gallotannin diet restriction in a crossover pilot trial on gallotannin bioaccessibility improvement. Pre-Treated = 48hrs; Co-Consumed = 1 min. Data is expressed as mean ± std error of pooled triplicates from n=11 participants. Columns with different letters are significantly different ($p<0.05$) according to Dunn's multiple comparisons test.

5.4. Conclusions

In these studies, the clinical efficacy of tannase for the purpose of gallotannin bioaccessibility improvement was demonstrated for the first time. Sumac tea with pre-digested gallotannins yielded higher plasma concentration levels than sumac with intact gallotannins. Furthermore, tannase demonstrated rapid hydrolytic activity as there was no difference between sumac tea processed 48-hr before consumption (Pre-Treated) or minutes before consumption (Co-Consumed). A limiting factor in gallotannin metabolism is the presence of tannase-expressing colon microbiota in the host and these results demonstrated that the dependence upon a host's colon microbiota can be circumvented by pre-processing with commercial tannase enzymes. Tannase hydrolysis of the *m*-depside bonds in gallotannin released free gallic acid molecules and transformed gallotannin into a more accessible form. Therefore, gallotannin hydrolysis before or during consumption may be an effective strategy to increase the likelihood of obtaining the health benefits of gallic acid by delivering gallotannin metabolites into the circulation.

6. SUMMARY AND CONCLUSIONS

The aim of the present research was to examine the role of phenolic-albumin binding in the bioaccessibility and bioactivity of the phenolics gallic acid (GA) and its metabolite methyl gallate (MG). The three main findings were: (1) protein-ligand binding between albumin and phenolics demonstrated a structural dependence based upon substitutions about the phenolic ring, (2) albumin-binding influenced antiproliferative activity by modulation of IC_{50} in *in vitro* cancer cell models and (3) albumin-binding influenced the interpretation of clinical endpoints specifically measurements of systemic absorption into plasma by LC-MS methodologies.

First, fluorescence spectroscopic methods demonstrated significantly greater binding affinity ($p<0.05$) in MG (K_{SV}= 9.97E+03 ± 7.01E+01) compared to GA (K_{SV}= 1.35E+03 ± 1.55E+01). These findings indicated that methyl substitution results in a more stable interaction with albumin and is supported by structure-activity relationships (SARs) described in the literature. Second, regarding the role of albumin-binding in *in vitro* cell culture approaches, the IC_{50} significantly increased (>500 % increase for GA, >2000 % increase for MG) when phenolic treatments were combined with HSA and administered to liver cancer cells as a chemopreventive, indicating that albumin-binding modulated GA, MG chemopreventive potency. Third, the LC-MS method performance parameters in the clinical trial of sumac gallotannin bioaccessibility improvement showed significant internal standard recovery differences ($p<0.05$) between human plasma and mobile phase (organic solvent). Internal standard recovery differences reflected signal suppression by the plasma matrix, affecting the interpretation of the systemic absorption of sumac

gallotannin hydrolytic products and metabolites into the bloodstream. Thus, ion suppression by albumin-binding in the plasma matrix was a remarkable factor in evaluation of the success rate of the gallotannin bioaccessibility improvement methodology. Taken together, the results indicated that phenolic bioavailability and bioactivity are not exclusively independent of albumin binding. This conclusion was underscored by the difference in GA, MG response to albumin measured in fluorescence spectroscopic studies and *in vitro* cell culture studies. MG demonstrated greater binding affinity to albumin and demonstrated greater reduction in chemopreventive potency when combined with albumin in *in vitro* cancer cell models.

To my knowledge, this investigation has been the first attempt to examine the effects of phenolic interaction with human serum albumin (HSA) on phenolic bioaccessibility and bioactivities. Prior to this study, it was difficult to predict how low-molecular weight phenolics would be affected by interaction with albumin, the carrier protein to which absorbed compounds are bound in circulation. Considering the findings of this investigation, there is now more information on the role of albumin binding in preclinical and clinical studies. The *in vitro* study demonstrated the importance of using albumin at physiological levels in preclinical models of safety and efficacy. Translation of *in vitro* results may be improved when the model mimics physiological conditions more closely. Also, human clinical studies may be better served by precise monitoring of matrix suppression due to plasma proteins. Because albumin is at the center of systemic circulation to sites of metabolism, elimination or therapeutic action, matrix effects from albumin-binding interference in LC-MS analyses potentially alters the outcomes of

phenolic ADME studies. Future phenolic bioactivity studies with albumin are needed to characterize the *in vitro* cellular activities of albumin-mediated transport and *in vivo* systemic effects of albumin gene knockout. In conclusion, a greater focus on phenolic-albumin interactions in physiological processes could contribute to the translation of the dose-response relationship between consumption of phenolics and therapeutic effects.

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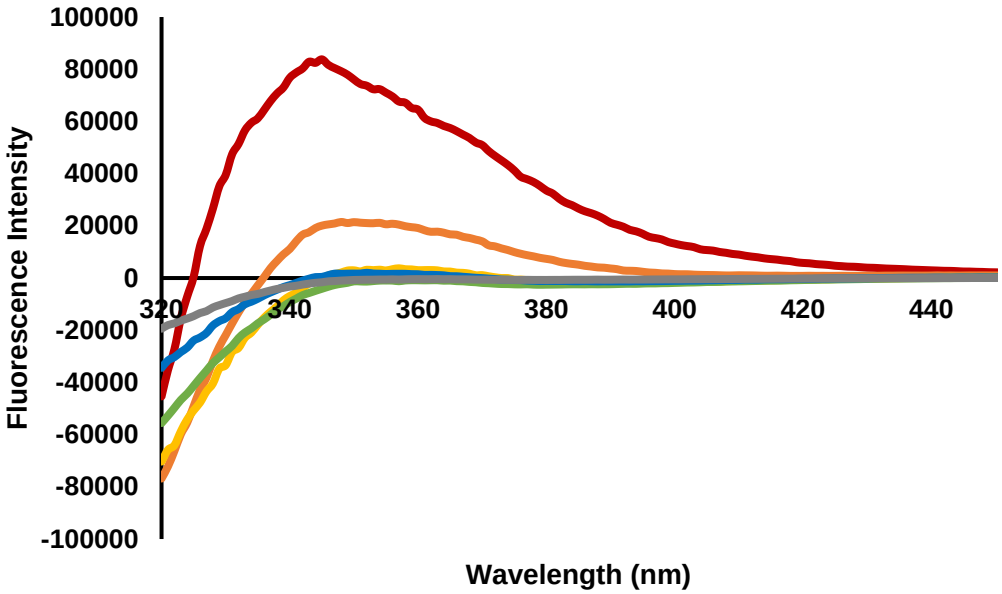
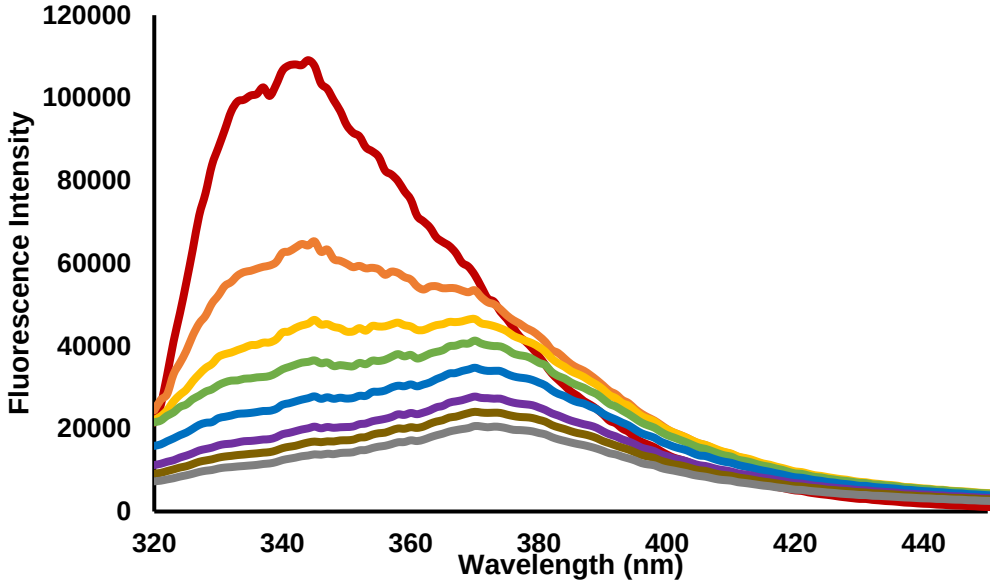
APPENDIX A

Quenching of HSA emission intensity in response to increasing concentration of phenolic compounds a) quercetin, b) methyl gallate, c) gallic acid d) protocatechuic acid, e) (-)-epicatechin, f) 4-hydroxyphenyl acetic acid, and g) 3-(3-hydroxyphenyl) propionic acid. HSA (175 µL, 22 µM) and increasing concentrations of phenolics (25 µL, 0-700 µM) were combined, excited at 295 nm and emission spectra recorded from 320 - 450 nm. Inner-filter effects of phenolics acid have been removed by subtraction. Concentrations of phenolics (µM) are color coded: Red = 0, Orange = 100, Yellow = 200, Green = 300, Blue = 400, Purple, 500, Brown = 600, Gray = 700.

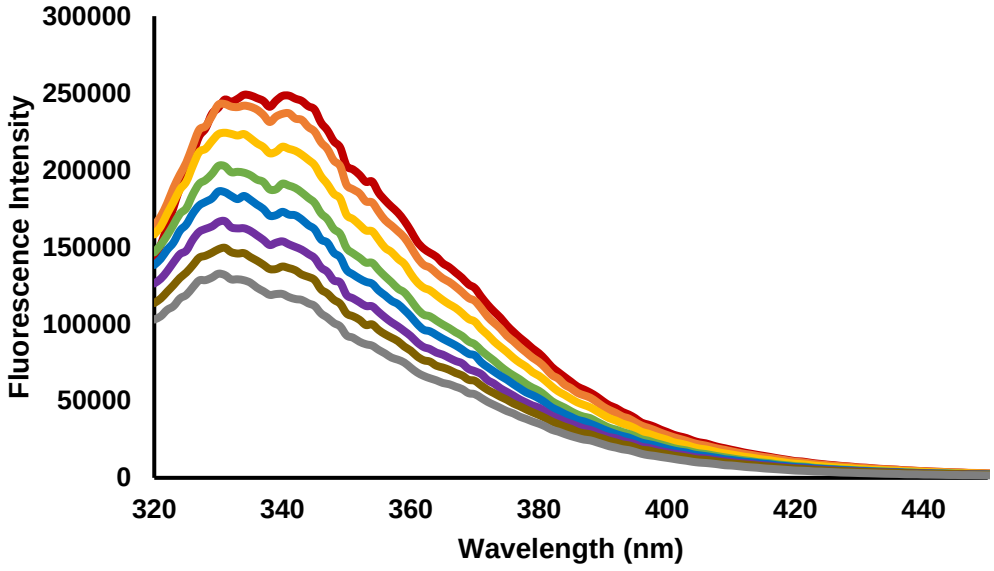
a) Quercetin



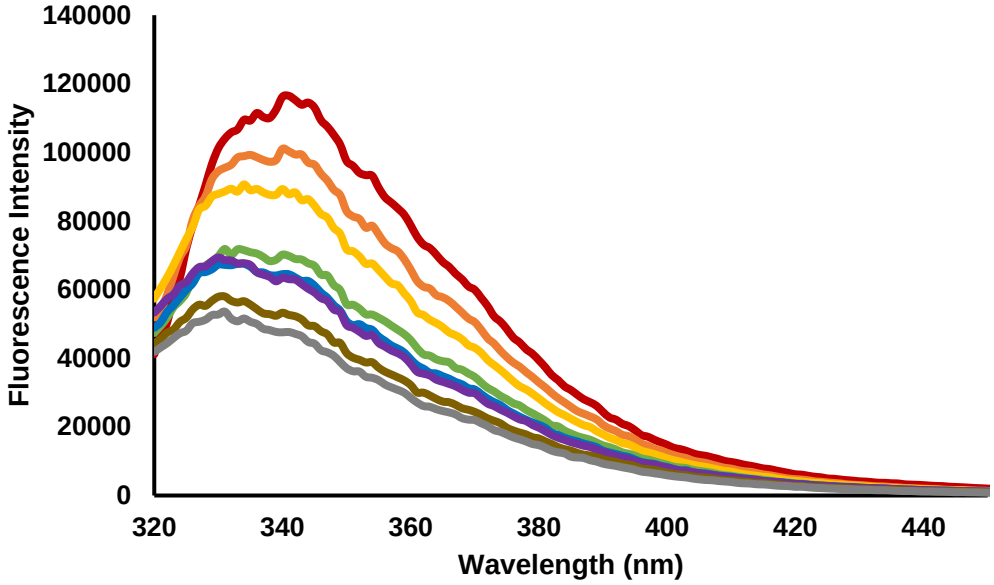
b) Methyl gallate



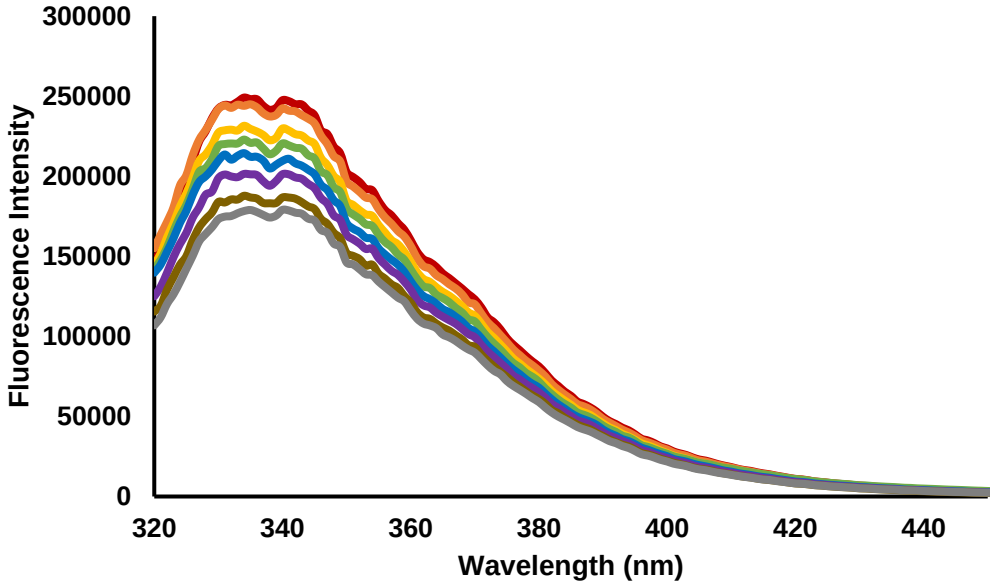
c) Gallic acid



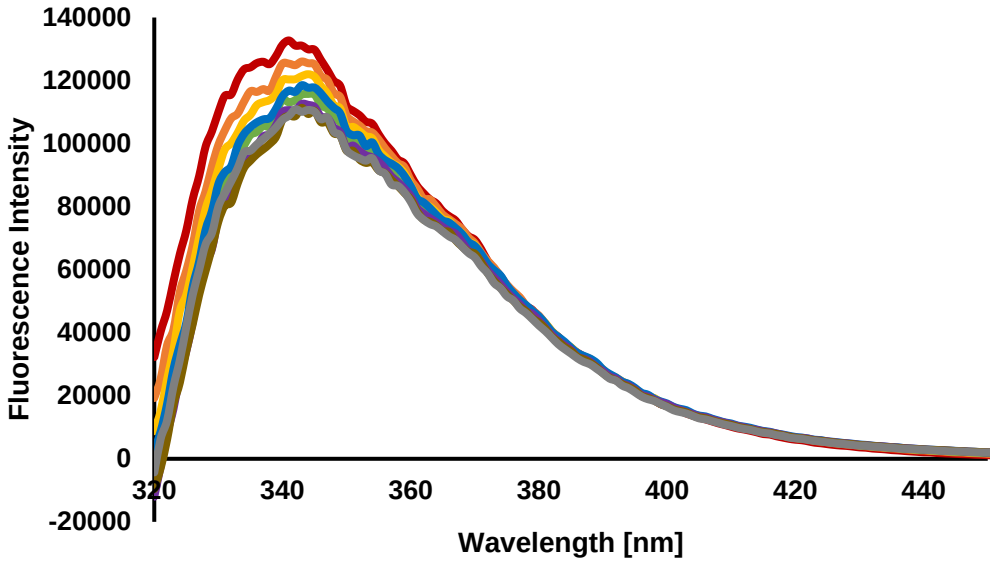
d) Protocatechuic acid



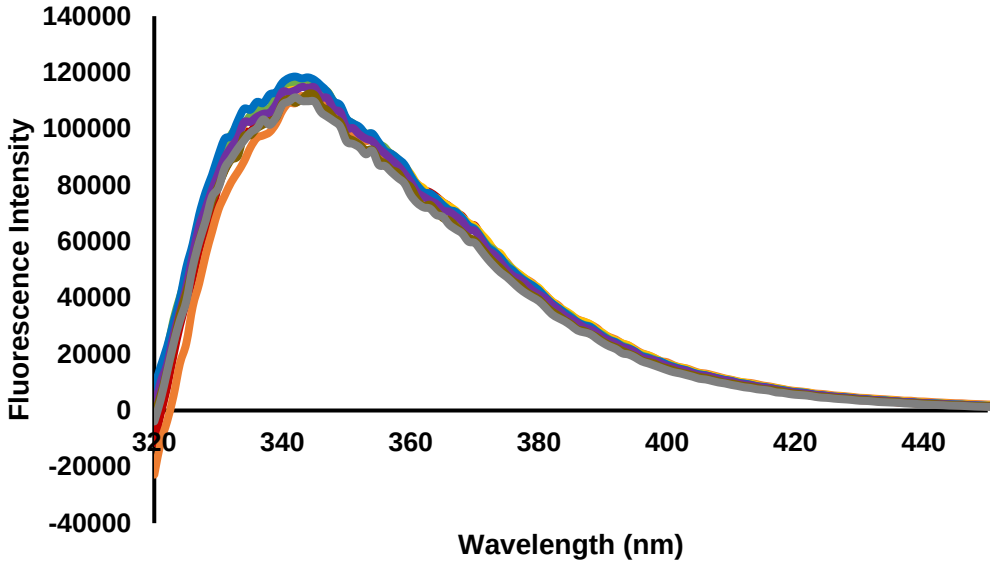
e) (-)-epicatechin



f) 4-hydroxyphenyl acetic acid

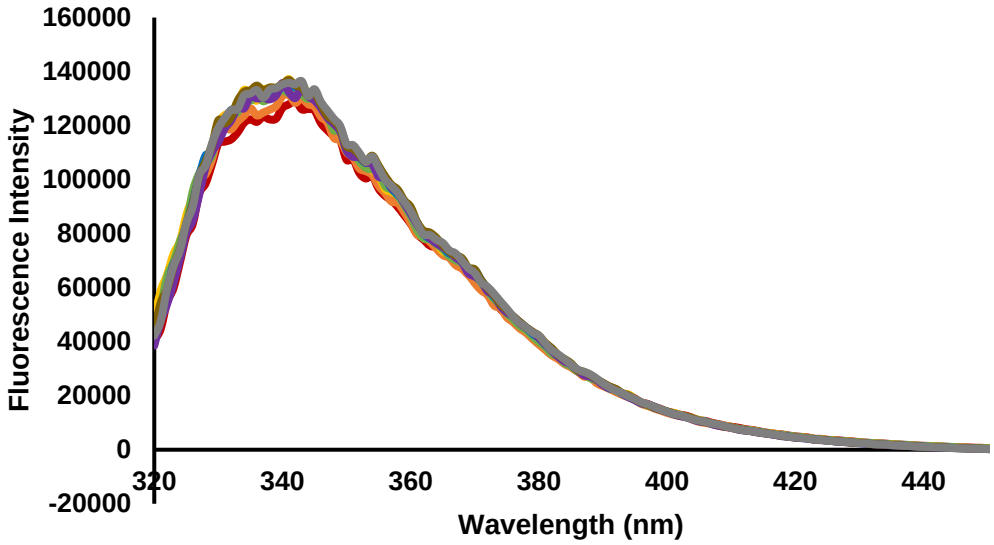


g) 3-(3-hydroxyphenyl) propionic acid

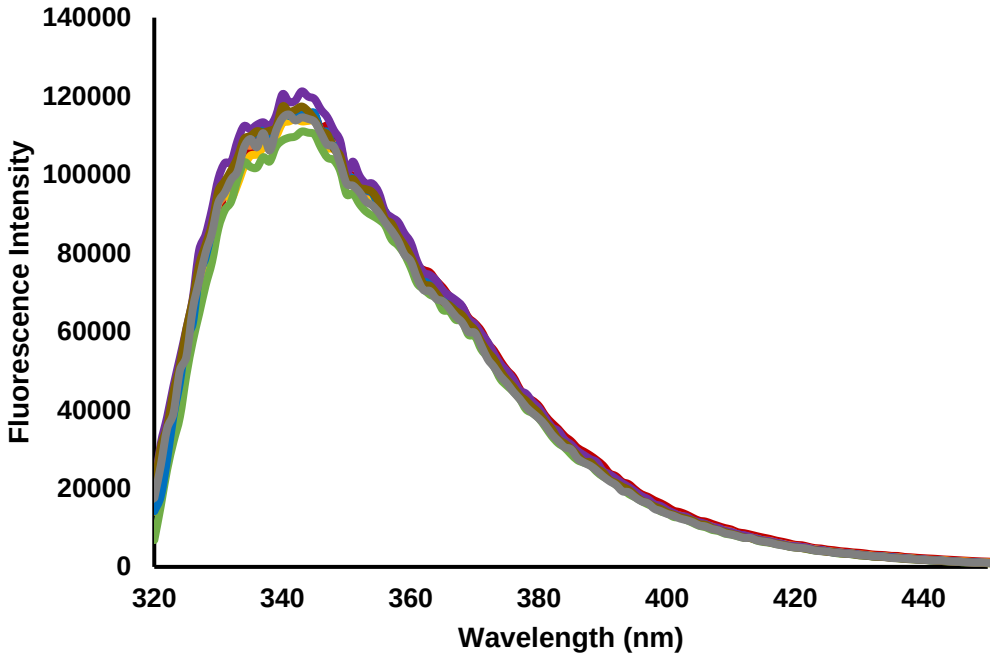


APPENDIX B

Quenching of HSA emission intensity in response to increasing concentration of methanol. HSA (175 µL, 22 µM) and increasing concentrations of methanol (25 µL, 0-0.7%) were combined, excited at 295 nm and emission spectra recorded from 320 - 450 nm. Concentrations of methanol (µM) are color coded: Red = 0, Orange = 0.1%, Yellow = 0.2%, Green = 0.3%, Blue = 0.4%, Purple, 0.5%, Brown = 0.6%, Gray = 0.7%.



Quenching of HSA emission intensity in response to increasing concentration of methanol. HSA (175 µL, 22 µM) and increasing concentrations of methanol (25 µL, 0-2.1%) were combined, excited at 295 nm and emission spectra recorded from 320 - 450 nm. Concentrations of methanol (µM) are color coded: Red = 0, Orange = 0.3%, Yellow = 0.6%, Green = 0.9%, Blue = 1.2%, Purple, 1.5%, Brown = 1.8%, Gray = 2.1%.


140000
120000
100000
80000
60000
40000
20000
0
Fluorescence Intensity
320
340
360
380
400
420
440
Wavelength (nm)