Characterizing the Chemoprevention Potential of Amenity Grass Phenolic Extracts \textit{In Vitro} and the Corresponding Nutraceutical Targets within HepG2 Carcinoma Cells

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CHARACTERIZING THE CHEMOPREVENTION POTENTIAL OF AMENITY
GRASS PHENOLIC EXTRACTS IN VITRO AND THE CORRESPONDING
NUTRACEUTICAL TARGETS WITHIN HEPG2 CARCINOMA CELLS

by

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CHARACTERIZING THE CHEMOPREVENTIVE POTENTIAL OF AMENITY GRASS PHENOLIC EXTRACTS \textit{IN VITRO} AND THE CORRESPONDING NUTRACEUTICAL TARGETS WITHIN HEPG2 CARCINOMA CELLS

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This research has revealed significant chemopreventive potential belonging to the planet’s most renewable and abundant plant source: amenity grasses. The characterization results from Chapter 1 demonstrated the potential chemoprevention attributes of supina bluegrass and bermudagrass crude extracts linked to the extensive phenolic acid profiles of these amenity grasses. In contrast to cereals, amenity grass phenolic extracts (AGPE) offer appreciable amounts of unbound/free ferulic acid that would be available for rapid \textit{in vivo} absorption. The literature has shown ferulic acid to be a highly available and health benefitting phenolic compound. Chapter 1 \textit{in vitro} studies demonstrated AGPE-induced antiproliferation and apoptotic induction capacities in HepG2 and Caco-2 carcinoma cell lines. The \textit{in vitro} chemopreventive influences were shown to be dependent upon AGPE species, extract fraction, dose, and cell line. As with most natural systems, a significant gap in knowledge exists as to how such health promoting components modulate metabolism to maintain health. Thus, the objective of Chapter 2 was to monitor the metabolic alterations attributed to supina bluegrass and bermudagrass phenolic treatments in human hepatocellular liver carcinoma cells, HepG2, using Fourier Transform mid infrared (FT-mIR) spectroscopy. The FT-mIR
metabolomic method was used to assess temporal changes in the biochemical fingerprint of treated and untreated HepG2 cells compared to resveratrol positive control treatments. The results from this study illustrate amenity grass nutraceutical targets within the HepG2 metabolome compared to the documented biochemical influences of resveratrol. As an outcome to this study, the impacts of nutraceutical stimuli on the biochemical fingerprint were further elucidated by analyzing the ability of phenolics to downregulate the oxidative nature of cancer.
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Being an individual with a tendency to focus on antiknowledge, or rather what is not known, I was becoming increasingly frustrated with a way of life that rewarded information over understanding. Graduate school provided me with an unexpected change-of-course that rewarded this type of thinking. I was introduced to countless opportunities, abilities, and fulfillment that I was lacking prior to my enrollment in graduate studies. For this, these are the people that I am in debt to for facilitating the experiences and knowledge I gained while earning my MS degree.

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Chapter 1

Characterizing the phenolic extracts of two natural products with extensive phenolic acid profiles (*Cynodon dactylon* and *Poa supina*) and their corresponding chemoprevention potential *in vitro*
LITERATURE REVIEW

Nutraceuticals

Stephen Defelice, the chairman of the Foundation for Innovation in Medicine (New York, NY), coined the term nutraceutical (nutrition-pharmaceutical) in 1989 (Kalra, 2003). Currently, there is not a generally accepted definition for the term nutraceutical, and it is often synonymous with the term ‘functional food.’ In a 1999 policy paper, Zeisel distinguished whole foods from natural bioactive food compounds of whole foods available in a non-food matrix by using the term ‘functional foods’ to describe the former and ‘nutraceuticals’ to describe the latter (Zeisel, 1999). Under this more recent definition (which will be used in this context for the entirety of this graduate thesis), nutraceuticals refer to extractable natural compounds that provide medical or health benefits, including the prevention and/or treatment of disease. The term nutraceutical does not currently have a regulatory definition. Therefore, the U.S. Food and Drug Administration do not recognize the term ‘nutraceutical’ (Ameye & Chee, 2006). Although, public health officials do agree that nutraceutical development is a viable means to maintain health and defend against nutritionally induced acute and chronic diseases (Andlauer & Furst, 2002). Nutraceuticals are generally recognized as functional ingredients sold as powders, pills, and other medicinal forms not generally associated with foods (Ameye & Chee, 2006).

Nutraceutical and dietary guideline development primarily focuses on reducing four related chronic degenerative diseases. Specifically, heart diseases, cancer, type II diabetes and obesity are metabolic disorders with interrelated impairments in both cellular processes and metabolism, yet each disease also retains unique pathological
qualities (Thompson & Thompson, 2010). For decades cardiovascular disease (CVD) has been the leading cause of morbidity and mortality worldwide (Massaro, Scoditti, & Annun, 2010). By the end of 2010 though, cancer is expected to surpass CVD to become the number one killer among the chronic diseases (Aggarwal et al., 2009). Moreover, it is now reported that 90-95% of cancers are attributable to lifestyles, while only the remaining 5-10% can be attributed to genetics (Anand et al., 2008).

**Reactive Oxygen Species (ROS) and Oxidative Stress**

In spite of the human body’s extensive cellular antioxidant systems, oxidative damage from the deleterious effects of reactive oxygen species (ROS) inevitably occurs (Martin et al., 2008). The clinical implications of severe cellular damage may lead to molecular injury causing aging, age-related degenerative diseases (Parkinson’s disease and arthritis), and all four primary chronic diseases (Valko et al., 2007). Molecular damage from ROS is termed oxidative stress. Causes of oxidative stress in humans include toxins, infections, hypoxia-ischemia, hyperglycemia, xenobiotics, hyperlipidemias, cancer, inflammation, immune reactions, and elevated metabolism (Mandelker, 2008). A delicate balance between biological ROS and antioxidants in biological systems needs to be maintained for sufficient signal transduction, promotion of apoptosis, and regulation of immune responses (Castro & Freeman, 2001). Normal levels of ROS allow optimal cell function and promote apoptosis (Martindale & Holbrook, 2002), the preferred mechanism of cellular death. However, elevated ROS levels may lead to oxidative stress that can result in numerous biological events (Mandelker, 2008), as illustrated in Figure 1.1. Notable ROS mutagens include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (·OH). As mentioned above, these ROS are
natural byproducts of metabolism, but they also represent the common mutagens produced by radiation (Ames, Shigenaga, & Hagen, 1999).

**Figure 1.1.** Cellular fates and impacts of ROS (Avramov, 2009).

#### Nutraceutical Antioxidants

Oxidative stress caused by ROS in humans is a well-established physiological process intrinsically controlled by antioxidant systems (Martin et al., 2008). Human cells are inherently provided with a formidable array of enzymatic antioxidants (Halliwell, 1994; Ames, Shigenaga, & Hagen, 1999) that work to maintain redox homeostasis in the host. Stress-induced oxidative imbalances can result from excessive generation of ROS or a deficiency in enzymatic antioxidants. At this point, the surplus of cellular free radicals can initiate damaging oxidative chain reactions within tissues and can potentially lead to numerous pathological conditions. Fortunately, these oxidative surpluses in the body can be modulated with dietary and/or supplementary nutraceutical antioxidants. In general, antioxidants are loosely defined as compounds characterized by an ability to be oxidized in place of another present compound (Seifried et al., 2007).
Knowledge of antioxidant effects on human health has increased dramatically in recent decades with research into mechanisms, molecular targets and molecular interactions (American Institute of Cancer Research/World Cancer Research Fund., 1997). As a result, use of antioxidants in various forms of nutraceutical supplements has become widespread among US adults (Radimer et al., 2004). Nutraceutical antioxidants include notable compounds such as vitamins C and E, β-carotene, and phenolic compounds, among other plant-derived compounds with documented biological protective roles (Sheerens, 2001). Dietary antioxidants are often proposed as therapeutic agents to counteract oxidative damage and combat degenerative diseases such as cancer (Vitaglione et al., 2004). Supplemental cellular antioxidants are intended to prevent ROS concentrations from reaching damaging cellular levels in high-risk individuals (Seifried et al., 2007).

**Phenolic Antioxidants**

Diets rich in plant-based foods are said to reduce incidence of degenerative diseases, largely due to the abundant phenolic concentrations that confer antioxidant potentials (Kitts, 2006). Oxidative stress is believed to be a major mechanism involved in chronic degenerative diseases such as cancer and heart disease (Zhang et al., 2008). Phenolic compounds are often viable combatants of degenerative diseases due to their multiple biological effects such as antioxidant activity, antimutagenic activity, anti-inflammatory action and antiallergic activity (Karakaya, 2004; Mandelker, 2008). Phenols exert antioxidant activity through various mechanisms of action including free radical scavenging and inhibition of ROS generation during normal cellular metabolism (Zhang et al., 2008; Seifried et al., 2007). These antioxidant activities help reduce the
oxidative damage of lipids, proteins, and nucleic acids, which may thereby prevent cell
damage and/or death (Steinmetz & Potter, 1991).

Thousands of natural phenolics have been identified in edible plants (Scalbert &
Williamson, 2000). It is therefore currently difficult, if not impossible, to characterize
the precise nature and fate of all ingested phenols, but it is feasible to determine the main
classes of phenols consumed and the rendering foods (Scalbert & Williamson, 2000).
The bioactivity of these compounds relies partially on the release of the phenols from the
plant matrix during digestion (Zafrilla et al., 2001). Additionally, phenolic glycosolation
in vivo can also affect the bioavailability and solubility of phenols during absorption
(Zafrilla et al., 2001). From a practical standpoint, it is conceivable that a combination of
nutraceutical phenolics may provide more beneficial health effects as compared to a
nutraceutical phenolic compound in isolation (Xu et al., 2009). A natural plant extract
may contain numerous phenols in balanced proportions, which may demonstrate superior
metabolic influences (Ray et al., 2004). Numerous studies have reported the synergetic
effects of phenolic compounds in concert (Olsson et al., 2004; Seeram et al., 2005; Ray et
al., 2004). This underlines the importance of the natural product itself for nutraceutical
development.

Phenolic compounds primarily consist of phenolic acids, flavonoids, stilbenes,
and lignans (Karakaya, 2004). The chemical base for this diverse family of molecules is
the phenol structure: a hydroxyl substitution on an aromatic ring (Wildman, 2001). From
this basic structure, larger and more complex phenolic compounds are synthesized to
service a wide variety of plant processes. Classification of phenolic compounds typically
reflects the nature of a compound’s carbon skeleton and its corresponding oxidation state
(Beecher, 1999). Phenolic monomers, dimers, oligomers and polymers are all widely found in nature, foods and drinks (Beecher, 1999).

Phenolic acids (Figure 1.2) are low molecular weight compounds ubiquitously found in fruits, vegetables and cereals (Clifford, 1999). The growing interest in phenolic acids is related to their reported antioxidant activities in vivo (Rice-Evans, Miller, & Paganga, 1996). A review by Lafay & Gil-Izquierdo (2008) outlined the extensive bioavailability of phenolic acids. Numerous dietary phenolic acid aglycones including gallic, caffeic, ferulic, coumaric and chlorogenic acids can be actively absorbed by the stomach (Konishi, Zhao, & Shimizu, 2006). Caffeic and ferulic acid aglycones also reportedly have absorption potentials within the small intestine (Lafay & Gil-Izquierdo, 2008). Esterfication of phenolic acids has been shown to considerably decrease their bioavailability (Adam et al., 2002). However, flavonoid cleavage by gut microflora to bioavailable phenolic acid products has also been documented in vivo (Manach, Mazur, & Scalbert, 2005).

Despite being the most consumed polyphenolic compounds in the human diet, phenolic acids have not received a fraction of the attention and research as that expended on the flavonoid phenolic class (Mateos, Goya, & Bravo, 2006; Scalbert & Williamson, 2000). Increased phenolic acid intake is primarily driven by the global consumption of coffee, which is rich in chlorogenic acids (Karakaya, 2004). The two main subclasses of phenolic acids are benzoic acid derivatives and cinnamic acid derivatives (Lafay & Gil-Izquierdo, 2008). Nutritionally notable benzoic acid derivatives include gallic, ellagic, protocatechuic, and 4-hydrobenzoic acid (Lafay & Gil-Izquierdo, 2008). These benzoic-
derived phenolic acids show limited distribution in foods, yet gallic acid is reported to have excellent bioavailability.

Cinnamic acid derivatives make a significantly larger contribution to daily polyphenol intake than do benzoic acids or flavonoids. Notable cinnamic acids include \( p \)-coumaric, caffeic, ferulic, and sinapinic acids (Solecka, 1997). These derivatives are rarely free and are mainly bound or esterified to organic acids, sugars, lignin or lipids (Clifford, 1999). Caffeic and quinic acids combine to form chlorogenic acid (5-caffeoylquinic acid). Chlorogenic acid is found in high concentrations within coffee and many fruits (Mateos, Goya, & Bravo, 2006). Ferulic acid can be found free within tomatoes and beer, but is most abundant in cereal grains where it becomes cross-linked to cell wall lignins (Palmer et al., 2008; Mateos, Goya, & Bravo, 2006; Grabber & Lu, 2007).

**Figure 1.2.** Phenolic acid structures (Chrzanowski, Sempruch, & Spraw 2007).
Flavonoids

Flavonoids are a class of polyphenolic compounds ubiquitous in photosynthesizing cells and widely found throughout the plant kingdom (Havsteen, 1983). Dietary flavonoids are regarded as potent antioxidants (Cook & Samman, 1996) and facilitate numerous biological events in humans such as free radical scavenging, metal chelation, modulation of enzymatic activity, inhibition of cellular proliferation, and alteration of signal transduction pathways (Bravo, 1998; Cushnie & Lamb, 2005). Diets rich in polyphenolics have been shown to reduce oxidative damage to DNA, thus preventing mutagenesis-linked carcinogenesis (Meyskens & Szabo, 2005; Zhang et al., 2008). An overwhelming body of evidence suggests that flavonoids may be health promoting dietary compounds (Middleton, 1996) that reduce the risk of chronic human diseases (Zhang et al., 2008; Martin et al., 2008; Beecher, 1999). Furthermore, global interest in the antimicrobial properties of flavonoids has occurred in view of the recent string of drug resistant bacterial infections, such as MRSA (Cushnie & Lamb, 2005).

Thousands of flavonoids have been identified in nature and hundreds of these have been reported in average human diets (Loke et al., 2008). Flavonoids can be found in fruit, vegetables, herbs, grasses, grains, nuts, seeds, stems, leaves, roots, and flowers, as well as, tea, coffee, cola, cocoa, wine, beer, spices, honey, bacteria and algae (Cushnie & Lamb, 2005; Fan et al., 2011; Kulkarni et al., 2006; Aggarwal et al., 2009). In the United States, it is estimated that average daily flavonoid intake ranges from 500-1000 mg per day, but this estimate can be much higher for people with herbal rich diets (Skibola & Smith, 2000).
For such a vast group of compounds with relatively homogenous structure, flavonoids display an extensive range of biological activities (Cushnie & Lamb, 2005). While simple phenolic acid precursors result from the shikimic pathway, synthesis of polyphenolic compounds requires participation from the malonic acid pathway (Wildman, 2001). It is well accepted that slight differences in flavonoid structural properties affect their bioavailability for intestinal absorption, as well as their formation and occurrence in blood plasma (Harbaum et al., 2008). Among the major subclasses of dietary flavonoids (Figure 1.3) are the flavonols, proanthocyanidins (including catechins), isoflavonoids, flavones, and flavanones (Skibola & Smith, 2000; Wildman, 2001; Harborne, 1988). The basic flavonoid structure consists of the diphenylpropane skeleton (C6-C3-C6) with various hydroxylation, alkylation and methylation substitutions (Karakaya, 2004). The presence and arrangements of these substitutions are important indicators for health-benefitting phytoactivities and are also used to distinguish between flavonoid subclasses (Figure 1.3).

**Figure 1.3.** Family of major dietary flavonoid groups (Skibola & Smith, 2000)
**Lignin-bound Phenolics**

Lignin is a polymer of cross-linked phenolics that is an integral component of secondary cell walls in plants (Boerjan & Baucher, 2003; Raes et al., 2003; Sarath et al., 2006). After cellulose, lignin is the most abundant organic molecule in plants and often classified as a fiber because it is not readily bioavailable to humans (Wildman, 2001). Phenolic compounds are present in free, as well as lignin-bound cell wall compounds (Harbaum et al., 2008). Plants have the capacity to incorporate a range of bound phenolic compounds into their lignin matrix (Palmer et al., 2008; Clifford, 1999). In particular, grasses can contain significant amounts of phenolic acids cross-linked to ester and ether linkages in the lignin matrix (Palmer et al., 2008; Clifford, 1999). This is most likely due to the extensive lignification in grass secondary and primary cell walls. Many of these lignin-bound phenolics can become bioavailable after cleavage by intestinal bacteria (Milder et al., 2005; Manach, Mazur, & Scalbert, 2005). These previously bound phenolics reportedly have increased intestinal absorption potentials and have been implicated in risk reduction of cardiovascular diseases and specific cancers (Kuijsten., 2005; Milder et al., 2005). Several studies have reported the enhanced antioxidant potentials of bound phenolic compounds compared to their free phenolic constituents (Harbaum et al. 2008).

The growing interest surrounding the health benefits of lignin-bound phenolic acids is supported by a number of recent studies. Bound phenolic acids from beer have been linked to a decreased risk of specific degenerative diseases (Szwajgier, Pielecki, & Targonski, 2005b). Harbaum *et al.* (2008) reported the occurrence and benefits of bound phenolic acids from chinese cabbage. Grains, such as soft wheat (Moore et al., 2005), are a prime host of bound phenolics. Moore *et al.* (2005) reported significant amounts of
bound ferulic acid in wheat grains that displayed potential for release by the gut microbiota *in vivo*. However, only limited data exists regarding bound phenolic investigations within various plant species. More research is needed to better understand the antioxidant potential of these bound phenolic compounds. Naturally, plants and grains with extensive cell wall lignification should prove to be viable options for future bound phenolic research.

**Resveratrol**

Stilbenes constitute a relatively small class of phenolic compounds and their structures consist of two aromatic rings connected by a two carbon methylene bridge (Pezzuto, Kondratyuk, & Shalaev, 2006). Resveratrol, a natural phenolic phytochemical most notably derived from grapes and wine, is the most extensively studied stilbene. Epidemiological studies have demonstrated that red wine consumption is linked to lower incidences of cardiovascular diseases (CVD) (Kraft et al., 2009). This phenomena is recognized as the *French Paradox*, which describes how the French suffer less from CVD compared to other industrialized nations despite diets rich in saturated fats (Richard, 1987). The biological activity of resveratrol was first recognized by Siemann and Creasy (1992). The chemopreventive properties of resveratrol have since been studied extensively and were first reported by Jang *et al.*, 1997. Resveratrol has displayed profound chemopreventive properties within all three of the general stages of carcinogenesis (Surh, 1999; Jang *et al.*, 1997; Lee, Zhang, & Sanderson, 2008). Additionally, resveratrol has been shown to induce apoptosis at higher concentrations, yet distinct metabolic influences have been reported at lower concentrations (Lee, Zhang, & Sanderson, 2008; Kraft et al., 2009). Resveratrol chemoprevention has been studied
extensively in an array of *in vitro* models including breast, prostate, leukemic, melanoma, lung, liver, and colonic cell lines [reviewed in: (Aggarwal et al., 2004; De la Lastra & Villegas, 2005)].

**Cancer and Chemoprevention**

Cancer is a complicated disease that manifests itself over a period of years. According to Seifried *et al.*, (2007), “Cancer can be viewed as a number of distinct diseases, each defined by different genetic lesions, with certain characteristics shared by most cancers and cancer typers.” Generally, cancer progression begins when a normal cell is transformed through the activation of proto-oncogenes and the down-regulation of tumor suppression genes such as p53 (Aggarwal et al., 2009). Transformed cells may then begin uncontrolled proliferation and eventually become insensitive to anti-growth signals (Aggarwal et al., 2009). At this point, cells are able to evade apoptosis, which results in tumor formation and the self-sufficiency of disease progression (Aggarwal et al., 2009; McDougall et al., 2008). Furthermore, as tumor progression continues, growth is aided by the formation of new blood vessels that provide nutrients to the developing tumor. This event fuels tumor growth and supports tumor invasion into other tissues, resulting in metastasis (Aggarwal et al., 2009). Unregulated and prolonged production of cellular ROS has been linked to the DNA mutations and modifications in gene expression leading to carcinogenesis (Mandelker, 2008). Particularly, activation of signal transduction pathways AP-1 and NF-κB have been accredited to ROS, which leads to the transcription of genes involved in cell growth regulatory pathways (Mandelker, 2008).

Cancer chemoprevention uses agents that impede progression, reverse, or inhibit carcinogenesis in healthy subjects, thus reducing their risk of developing an invasive or
clinically significant disease (Hail Jr, Cortes, Drake, & Spallholz, 2008). Subsequently, an effective chemopreventive agent should intervene early in the carcinogenesis process to eliminate premalignant cells before they become malignant, or protect normal cells from mutations (Hail Jr, Cortes, Drake, & Spallholz, 2008). There has been significant success within chemoprevention development in the last fifteen years that has correlated with decreases in certain cancer types (Kellog, Sigman, & Greenwald, 1999). Yet, development of chemopreventive agents is still inefficient and slow (Zou et al., 2005). Therefore, the search for more bioactive and less toxic chemopreventive agents such as natural products has been an extremely active scientific pursuit (Wang et al., 2009; Zou et al., 2005). Development of non-invasive cancer treatments is needed and often required because traditional cancer treatments are toxic to normal cells and often cause serious side effects (Wasser, 2002). Desirable characteristics of chemopreventive agents include the following (Aziz, Kumar, & Ahmad, 2003):

1. Little or no toxic effect in normal, healthy cells
2. High efficacy against multiple tumor sites
3. Suitable form for oral consumption
4. Known mechanism of action
5. Low cost to the consumer
6. Acceptance by human population

Natural bioactive products from plants, microorganisms, and marines have been deemed a desirable source of chemopreventive agents due to their low toxicity and increased biocompatibility (Wang et al., 2009; Zhu & Wu, 2009). The documented bioactivities of phenolic compounds have been shown to prevent damage to lipids,
proteins, and nucleic acids, thereby protecting against cell damage and death (Zhang et al., 2008; Steinmetz & Potter, 1991). It is well documented that phenolic-rich natural products demonstrate suppressive effects against carcinogenesis and mutagenesis (Karakaya, 2004; Kitts, 2006; Thompson et al., 2008), as well as the reduction of oxidative damage to DNA, thus preventing the onset of carcinogenesis (Murugan et al., 2010; Lee, Zhang, & Sanderson, 2008; Zhang et al., 2008). Phenolic antioxidants may also confer chemopreventive activities in vivo by terminating free radical chain reactions (Yu et al., 2002). Free radical chain reaction damage can contribute to cell injury and death, accelerate the aging process, and further promote tumorigenesis (Wong, Li, & Stadlin, 1999; Yu et al., 2002). Cancer and other degenerative diseases/conditions may be prevented or improved through phenolic antioxidant treatments (Merken & Beecher, 2000; Neff, 1997; Yu et al., 2002).

**Apoptosis and Cancer**

The hallmark cellular indicators of carcinogenesis include genetic mutations, unregulated cell proliferation, and the down-regulation of apoptosis. Apoptotic dysfunction is also a key component in numerous other diseases, so scientific attempts to manipulate this dysfunction has proved widely appealing (Mandelker, 2008; Murugan et al., 2010). The entire mechanism determining cellular life and death is controlled through a convoluted regulatory process heavily impacted by oxidative stress (Mandelker, 2008). The literature demonstrates that stress-induced changes to specific biological modulators can switch the cell death mechanism from apoptosis to necrosis (Susin, Zamzami, & Kroemer, 1998). Hence, sustained oxidative stress often leads to inflammation and/or necrosis by means of genetic alterations (Haddad, 2002).
Carcinogenesis-linked DNA damage can arise from direct ROS interaction with DNA units or can be caused by reactive compounds generated by oxidized lipids (Seifried et al., 2007).

Cell death inevitably occurs through either apoptosis or necrosis, with apoptosis being the preferred cellular death mechanism. Although necrosis and apoptosis can result from the same oxidative stress, their death mechanisms are different (Halliwell, 1994). During apoptosis, cell death occurs voluntarily as a function of internal signaling, thus cascading apoptotic cells into a programmed dismantling. This process externalizes phosphatidylserine (Mates & Sanchez-Jimenez, 2000), a plasma membrane constituent, which provokes cell removal by phagocytes to ensure minimal collateral damage to surrounding tissues (Logue & Martin, 2008).

Apoptosis is marked by cell shrinkage, nuclear condensation, and DNA fragmentation that occurs without the release of inflammatory cytokines involved in necrotic inflammation (Murugan et al., 2010; Seifried et al., 2007; Mandelker, 2008). The activation of caspase proteases, members of the cysteine family, is believed to initiate the apoptotic cascade (Seifried et al., 2007; Murugan et al., 2010; Logue & Martin, 2008). Proteins within the Bcl-2 and NF-\(\kappa\)B families regulate the activation of these caspase proteins (Logue & Martin, 2008; Murugan et al., 2010; Mandelker, 2008). The Bcl-2 proto-oncogene is unique in the way that it can inhibit apoptosis when expressed (Mates & Sanchez-Jimenez, 2000). Bcl-2 proteins protect against apoptosis by blocking cytochrome c release, an essential apoptotic signaling event (Mates & Sanchez-Jimenez, 2000). The prosurvival NF-\(\kappa\)B transcription factor also plays a decisive role in tumorigenesis, given its ability to control various genes involved in apoptosis (Sethi,
Sung, & Aggarwal, 2008; Murugan et al., 2010). Activation of NF-κB enables the nuclear factor to translocate the nucleus and regulate anti-apoptotic gene expression (Aggarwal et al., 2009; Murugan et al., 2010). Chemopreventive strategies that target apoptosis and the related signaling molecules are central in designing effective disease interventions (Murugan, et al., 2010; Shen & Tergaonkar, 2009). Phenolic compounds such as resveratrol and the polyphenols from grapes and tea have been shown to selectively suppress cancer proliferation by inducing apoptosis through NF-κB regulation (Lee, Zhang, & Sanderson, 2008; Xu et al., 2009; Murugan, et al., 2010).

**Liver and Colon Cancer Models**

Human liver carcinoma is the fifth most common cancer worldwide and most liver cancer patients die within a year of diagnosis (Llovet, 2003; Tan et al., 2009). At present, the search for antitumor liver cancer drugs remains an active area of research (Tan et al., 2009). Many liver cancer studies use HepG2 cell lines as *in vitro* models to investigate cytotoxic treatment effects and alterations in cellular redox balances (Xu et al., 2009). **Figure 1.4** illustrates the relationship between oxidative stress and chronic liver damage. HepG2 cells retain many hepatocyte characteristics such as Phase I and II enzyme induction and metabolism of xenobiotics (Cai et al., 2009).

Colorectal cancer is the second leading cause of cancer death in North America and Europe, as well as the fourth most common form of cancer worldwide (Boyle & Langman, 2000). Many researchers have demonstrated the protective effects of phytochemicals in relation to colon cancer (Yi, Fischer, & Akoh, 2005). Oxidative stress has been linked to a number of intestinal pathological conditions, thus it is important to assess novel protective mechanisms concerning the intestinal epithelium (Wijeratne,
Cuppett, & Schlegel, 2005). One of the most widely accepted *in vitro* models for the intestinal lining is the human colon adenocarcinoma cell line, Caco-2. Caco-2 cell lines display many of the same morphological and functional properties of *in vivo* intestinal epithelial cells and mucosa (Meunier et al., 1995). Caco-2 cells in monolayer exhibit similar characteristics of the intestinal epithelium such as brush border microvilli, tight junctions, and dome formation (Wijeratne, Cuppett, & Schlegel, 2005).
**Phenolic Literature Summation**

As I began reviewing the nutraceutical literature, I quickly became aware of the disparity between public perception and scientific evidence regarding phenolic antioxidants. The scientific knowledge gained over the past 20-30 years concerning the role of oxidative stress in disease has most likely lead to public fear mongering and the corresponding demand for antioxidant supplements. To compensate, it appears science
took an overtly reductionist approach to phenolic antioxidant research, thereby neglecting the possible secondary effects of phenolics in vivo.

Recent research has illustrated that the primary health benefits achieved from phenolic antioxidant consumption are most likely derived from the modulating role phenolics play in disease signaling, rather than their cellular protective effects, per se. Thus, as many oxidative stress pundits are advocating, the public and researchers alike are often misguided in their rationale that ‘antioxidants’ inhibit the processes associated with ageing and disease (Hail Jr, Cortes, Drake, & Spallholz, 2008). To summarize, phenolic antioxidants’ role in chemoprevention is not, in fact, driven by a direct antioxidant mode of action, but rather by their modulating impacts on health and disease.

**Nutraceutical Grasses**

Staple food crops corn, rice, wheat, beans and potatoes are globally consumed in large amounts on a daily basis. As such, it should be recognized that these foods are the primary vehicle by which health-promoting chemicals are delivered to the human body (Thompson & Thompson, 2010). Typical Western diets present a monopoly of the family Poaceae in the staple foods consumed (Thompson & Thompson, 2010). The Poaceae, or grass family, is the world’s largest plant family and the most important to humans. “Were it not for the grasses, civilization as we know it today would not exist” (Moore, 1960). Of the staple food crops listed above, the cereal species corn, rice, and wheat are all members of the Poaceae family. Yet, a comprehensive nutraceutical evaluation of the Poaceae family does not exist outside of the cereal grasses.

Additional nutraceutical research on amenity grasses within the Poaceae family is limited to select epidemiologically related studies. Bermudagrass (Cynodon dactylon)
has been used as an anti-diabetic agent in traditional Indian medicine (Kirtikar & Basu, 1980). More recent assessments of bermudagrass in modern medicine have further acknowledged anti-diabetic potential, as well as advantageous affects on high-density lipoproteins (HDL) levels for lessening coronary risks (Singh et al., 2007). Aqueous bermudagrass extracts have been reported as anti-inflammatory, diuretic and anti-emetic agents (Ahmad et al., 1994). Antiulcer properties have also been attributed to bermudagrass extracts used in rat studies (Patil et al., 2005). Epidemiological insights have lead to the elucidation of anti-cancer properties possessed by *Cymbopogon citratus* or lemon grass (Suayeyun et al., 1997) and *Sasa Senanensis* or bamboo grass (Seki et al., 2008). Wheatgrass (*Triticum aestivum*) seedlings demonstrate various antioxidant qualities (Kulkarni et al., 2006) such as superoxide scavenging abilities (Peryt et al., 1992) and inhibition of DNA oxidation (Falcioni et al., 2002). Nonetheless, despite significant epidemiological indicators and research, the notion of researching grasses for human health has remained bottlenecked and erratic at best. This lends the possibility that one of Earth’s most abundant resources for human benefit has been underutilized in modern medicine.

The specific aim of this chapter was to characterize the antioxidant activities and *in vitro* cancer antiproliferation effects on HepG2 and Caco-2 cell lines in response to phenolic extracts of two amenity grass species. Compositional analyses of amenity grass phenolic extracts were also included to speculate on the possible phenolic agents responsible for eliciting nutraceutical benefits. The initial hypothesis for this research stated that select amenity grasses would display enhanced phenolic antioxidant capacities that could be of nutraceutical benefit. The rationale behind this hypothesis stemmed from
the elevated phenolic biosynthesis abilities attributed to grasses as a function of satisfying the phenolic demands needed for extensive lignification to occur.
MATERIALS AND METHODS

Seed Sources

Supina bluegrass [Poa supina Schrad. ‘Supranova’] seed was generously provided by United Seed (Omaha, NE) and bermudagrass [Cynodon dactylon (L.) Pers. var. dactylon ‘Sovereign’] seed was purchased from Seeds West (Yuma, AZ).

Grass Samples

A preliminary screening of amenity grasses was conducted using a multitude of seeded grass species. This screening lead to the selection of two grass species (a C3 and a C4 species) as a result of elevated antioxidant activities (ORAC assay). The two grass species chosen included supina bluegrass [Poa supina Schrad. ‘Supranova’] and bermudagrass [Cynodon dactylon (L.) Pers. var. dactylon ‘Sovereign’]. Grasses were planted in May of 2010 and grown from seed in greenhouses at the University of Nebraska-Lincoln’s East Campus. Supina bluegrass samples were maintained under 15 hr ~ 30 °C max day / 9 hr ~ 17 °C min night growth conditions. Bermudagrass samples were maintained under 17 hr ~ 34 °C max day / 8 hr ~ 20 °C min night growth conditions in a separate warm season greenhouse. Relative humidity in the cool and warm season greenhouses ranged daily from 30-80% and 45-90% respectively. Supplemental lighting was supplied with halide lamps (200 mol photons m$^{-2}$ s$^{-1}$) and soil mixtures consisted of 34% peat, 31% perlite, 31% vermiculite and 4% screened topsoil, amended with 2 kg dolomite m$^{-2}$. Additionally, grasses were watered, fertilized (20-20-20 nutrient solution containing 200 ppm N) and trimmed as needed.
**Collection and Extraction**

Amenity grass sampling consisted of trimming and collecting the apical 1/3 fraction of above ground tissue growth. Preliminary data indicated no significant differences in phenolic concentrations or antioxidant activities between tissue fractions within the upper 2/3 of growth. Grass samples were collected between 75-100 days of post-germination growth and sampling occurred at the same time of day to ensure metabolic and photoperiod consistency. Sample collections were transferred directly to the lab for fresh weight (fw) extractions. Additionally, blueberry samples (*Michigan Summer Blueberries*, S/N MSB136760) were purchased from the local market in September of 2010. Blueberry samples were refrigerated for one day after purchase before extraction. Both fresh grass and blueberry samples were briefly ground with a coffee grinder before phenolic extractions. Blueberry samples for collection consisted of equal parts skin and pulp to replicate a dietary positive control.

Cell wall phenolic extractions were carried out according to a modified procedure from Sarath et al. (2006). Soluble free cell wall phenolics (Figure 1.5) were extracted twice (1:25 w/v) with 50% aqueous methanol (acidified to pH 5) followed by 50% aqueous acetone. Each extraction was facilitated with 1 hr of mixing/vortexing and supernatants were pooled after centrifuging at 3000 g for 10 min. Sample pellets (residues) were vacuum-dried for 24 hr at 50 °C to remove residual solvents. Pooled supernatants were stored at -20 °C until drying or further testing.

Soluble bound cell wall phenolics were extracted from the vacuum-dried residues (Figure 1.5). Residues were hydrolyzed with 2 M NaOH (1:10 w/v) for 4 hr at room temperature to release the soluble bound phenolic compounds. The saponified solution
was acidified to a pH ≤ 2 with 12 M HCl and the released phenolics were extracted thrice with ethyl acetate (1:1 v/v). The organic phases were pooled and stored at -20 °C until drying or further testing. A detailed flow chart for the phenolic extraction process is illustrated in Figure 1.5. Cell wall bound phenolics for blueberry extracts were not determined because a comparison to grasses would not be relevant considering the differences in cell wall compositions.
**Figure 1.5.** Phenolic extraction procedure

**Grass Sample**
*Fresh weight (fw)*

**Extract Sample 2x; 1:25 (w/v)**
- 1<sup>st</sup> extract: 50% aqueous MeOH acidified with 2M HCl to pH 5
- 2<sup>nd</sup> extract: 50% aqueous Acetone
- 1 hr mixing and 10 min centrifuging at 3000 g for each extract

**Collect Supernatants**

**Pooled Supernatants**

**Evaporate/Dry**
*Evaporate organic solvents in oven @ 50° C and remaining aqueous solution under vacuum @ 50° C*

**Redissolve**
*Acidified aqueous MeOH*

**Soluble free**

**Sample Residue**

**Dry**
*Evaporate residual solvents*

**Hydrolysis**
*2M NaOH; 1:10 (w/v) for 2 hr @ room temperature*

**Acidify**
*12M HCl to pH ≤ 2*

**Extract 3x**
*Ethyl acetate; 1:1 (v/v) vortex for 1 min and allow separation*

**Collect Organic Phases**
*Pool 3 organic phases*

**Evaporate/Dry**
*Evaporate organic solvents in oven @ 50° C and remaining aqueous solution under vacuum @ 50° C*

**Redissolve**
*Acidified aqueous MeOH*

**Soluble bound**
**Determination of Total Phenolics**

The Folin-Ciocalteu method was used to determine total phenolic levels of the crude extracts as described by Singleton & Rossi (1965). Sample extract aliquots (100 µL) were reacted with 100 µL of Folin-Ciocalteu reagent (Sigma, St. Louis, MO, USA) and 4.5 mL of nanopure water. After 3 minutes of mixing, 0.3 mL of 2% (w/v) sodium carbonate was added and samples were subsequently incubated at 25 °C for 2 hr with intermittent shaking and venting. Detection of the phenolic compounds was achieved using a Beckman Coulter DU 800 Spectrophotometer (Fullerton, CA) set at wavelength 760 nm. Gallic acid in methanol (MeOH) served as the standard and total phenolics were expressed as mg gallic acid equivalents (GAE) g⁻¹ fresh weight (fw) tissue.

**Determination of Total Flavonoids**

To quantify total flavonoids, 125 µL sample extract aliquots were added to 37.5 µL of 5% (w/v) sodium nitrite and 0.625 mL of nanopure water according to Adom & Lui, (2002). After 4-6 min of incubation, 75 µL of 10% (w/v) aluminum chloride was added to the sample. Following an additional 5-7 minute incubation, 0.25 mL of 1.0 M sodium hydroxide and 0.4 mL nanopure water were added to the sample. The sample mixtures were vortexed to remove any precipitate and total flavonoids were detected at 510 nm. Catechin hydrate served as the standard and total flavonoids were expressed as mg catechin equivalents (CE) g⁻¹ fw tissue.

**HPLC Analysis**

Vacuum dried amenity grass phenolic extracts (AGPE) were dissolved in 100% MeOH and filtered through a 0.2 µm nylon membrane before analysis. A Vydak reversed phase, 5 µm C-18 column was used for separation. Phenolic acid and flavonoid detection
was achieved using a Waters HPLC system (Franklin, MA, USA) with a photodiode array detector (2996), autosamplers (717plus), and Millenium\textsuperscript{32} monitoring software (version 4.0). The analytical conditions were as follows: autosampler temperature, 8 °C; injection volume, 25 µL; detection, 200-400 nm; monitoring wavelength, 254 nm (flavonoids) and 340 nm (phenolic acids); flow rate, 1 mL min\textsuperscript{-1}. The mobile phases consisted of (A) 1% formic acid in deionized water and (B) 25% acetonitrile and 1% formic acid in deionized water. A duel pumping system was used to form mobile phase gradients by varying the proportions of mobile phase A to B. A 0-60 min mobile phase B elution (0-100%) and 60-65 min mobile phase A elution (0-100%) was used for detection and was followed by 65-70 min of isocratic mobile phase A. The phenolic acids and flavonoids were identified by matching their retention time to those of standards. Phenolic content percentages were expressed on the basis of a specific weight of crude/hydrolyzed extract.

**Oxygen Radical Absorbance Capacity (ORAC)**

The ORAC method was completed as described by Ou, Hampsch-Woodill, & Prior, (2001) to measure antioxidant activities of the sample extracts. A stock solution of standard was prepared by dissolving 0.010 g of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) in 10 mL of 75 mM potassium phosphate buffer, pH 7.4. A 50-fold standard serial dilution and extract samples (dissolved in 50% aqueous MeOH) were prepared in 75 mM potassium phosphate buffer solution (pH 7.4), which also served as the blank. A 25 µL aliquot of prepared extracts, blanks, and standard serial dilutions were added to their respective wells within a 96-well plate.
The fluorescent probe fluorescein (150 µL of 8.16 x 10⁻⁵ mM) in buffer solution (pH 7.4) was incubated with standards, samples and blanks for 10 min and shaking occurred for 3 min of incubation time. Briefly, the reaction was activated by adding 25 µL of 153 mM 2, 2’-azobis (2-amidinopropane) hydrochloride (AAPH) after incubation. Specifically, AAPH generated peroxyl radicals oxidize fluoresceine, causing a decrease in fluorescence (excitation and emission wavelength of 485 nm and 520 nm respectively) that was measured every 1.5 min at 37 °C with a BMG Labtech FLUOstar Optima microplate reader (Durham, NC) until values plateau. ORAC results were calculated using the area under the curve (AUC) differences between blanks and samples and expressed as µM trolox equivalents (TE) g⁻¹ fw sample. Higher ORAC values correlate with greater preventative peroxyl quenching activities.

**Cell Culture**

Human hepatocellular liver carcinoma (HepG2) and human colon carcinoma (Caco-2) cell lines were chosen as model cancer lines. These cell lines have been shown to be model mammalian systems for monitoring oxidative stress (Martin et al., 2008; Wijeratne et al., 2005). Caco-2 cells provided by the American Type Culture Collection (ATCC) (Rockville, MD) were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1.5 g L⁻¹ sodium bicarbonate, 1% nonessential amino acid (NEAA), 50 units mL⁻¹ penicillin with 50 µg mL⁻¹ streptomycin, 1% L-glutamine and 20% fetal bovine serum (FBS) according to Wijeratne and Cuppett, (2006).

HepG2 cells were cultured in Modified Eagle Medium Eagle (MEME) (Sigma, St. Louis, MO, USA) supplemented with 2.2 g L⁻¹ sodium bicarbonate, 11 mL L⁻¹ penicillin/streptomycin (50 units mL⁻¹ penicillin with 50 µg mL⁻¹ streptomycin), and 10%
FBS. Both cell lines were grown in 75 cm$^2$ culture flasks and maintained at 5% CO$_2$, 95% air at 37 °C using a CO$_2$ water-jacketed incubator (Thermo Fisher Scientific Inc., Forma Series II 3110, Waltham, MA). Cell populations were subcultured every 2-3 days after washing with 10x phosphate buffer saline (PBS) and trypsinization (EDTA).

**MTT Cell Viability Assay**

Cell viability was determined through metabolism of MTT tetrazolium salt (3-(4,5-dimethylthiazole-2yl)-2,5-diphenyltetrazolium bromide) by a mitochondrial enzyme (succinate dehydrogenase) of living cells into a colorimetric end product and evaluated using the MTT kit provided by ATCC (Manassas, VA). After reaching ~80% confluence, 100 µL cultured cell aliquots in DMEM or MEME (3.5 x 10$^6$ cells mL$^{-1}$) were briefly distributed to 96 well plates and incubated at 37 °C and 5% CO$_2$ to form a partial monolayer. After 24 hr, the medium was removed and refreshed with growth medium [0.1% dimethylsulphoxide (DMSO) according to Zhang *et al.* (2008)] containing phenolic treatments (500, 250, 125, 62, 31, or 15 µg/mL). After 18 hr of treatment incubation, 10 µL of MTT reagent (0.1 mg/mL) was added to each well. Cells were incubated with the MTT reagent for 2 hr before adding 100 µL of lysing buffer to each well and incubating for another 4 hr. The colorimetric end product (crystallized purple formazan) was estimated by measuring the absorbance at 570 nm with a BMG Labtech FLUOstar Optima microplate reader (Durham, NC). The cell viability percentage was assessed by comparing the average absorbance of treated cells with the corresponding absorbance of untreated cells.
**Annexin V-FITC Apoptosis Induction Assay**

The Annexin V-FITC Apoptosis Detection Kit (Sigma, St. Louis, MO, USA) was used to evaluate phospholipid externalization in the plasma membrane (Murugan et al., 2010), an event typically associated with apoptosis. When HepG2 cells reached ~80% confluence, 0.7 mL cultured cell aliquots in MEME (3.5 x 10⁶ cells mL⁻¹) were briefly distributed to 24 well plates and incubated at 37 °C and 5% CO₂ to form a partial monolayer. After 24 hr of incubation, the medium was removed and refreshed with growth medium [0.1% dimethylsulphoxide (DMSO) according to Zhang et al. (2008)] containing amenity grass phenolic treatments (500 µg/mL GAE) or resveratrol (125 µg/mL). After 18 hr of treatment incubation, the adherent cells were harvested after washing with ice-cold 10x PBS and trypsinization. MEME was then added to harvested cells to deactivate the tryspin and the cell solutions were briefly centrifuged at 2000 rpm for 2 min (4 °C). Finally, cells were washed with 10x PBS, centrifuged (2000 rpm, 2 min, 4 °C), and resuspended in 1x binding buffer (500 µL aliquots). For analysis with flow cytometry, cells were stained with annexin V-FITC (fluorescein isothiocyanate) and PI (propidium iodide) according to the manufacturer’s instructions. The cells were then incubated in the dark for 15 min and analyzed using flow cytometry (BD FACSCalibur, Franklin Lakes, NJ) at an excitation of 488 nm and an emission wavelength of 525 nm for the annexin V-FITC and 575 nm for PI. A special thanks to Danielle Shea and UNL’s Virology department for the flow cytometry instrumentation.

**Statistical Analysis**

All experiments were performed in triplicate using three subsamples within each of three total machine reps. Cell culture assays followed a randomized complete block
design blocking for replicates. Results were analyzed using the Statistical Analysis Software (SAS) package (Cary, NC, USA). UV-Vis, ORAC, and MTT results were interpreted using the one-way analysis of variance (ANOVA) and Tukey mean separation techniques to determine significant differences (p > 0.05).
RESULTS

Total Phenolic Concentration (TPC)

TPC results, as determined by UV-Vis absorbance, reflect amenity grass phenolic extracts (AGPE) sampled between 75-100 days of post-germination growth. The soluble free phenolic and flavonoid concentrations did not significantly differ between the grasses, but AGPE did surpass blueberry extracts in both soluble free phenolic and flavonoid concentration (Table 1.1). A significantly greater concentration of cell wall bound phenolics (hydrolyzed fraction) was obtained for bermudagrass extracts, yet bound AGPE fractions exhibited similar total flavonoid levels.

<table>
<thead>
<tr>
<th></th>
<th>Soluble free (mg/100 g)</th>
<th>Soluble bound (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phenolic Composition</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supina bluegrass</td>
<td>442 ± 49&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>89 ± 13&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bermudagrass</td>
<td>589 ± 62&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>442 ± 84&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blueberry</td>
<td>495 ± 39&lt;sup&gt;c&lt;/sup&gt;</td>
<td>---</td>
</tr>
<tr>
<td><strong>Flavonoid Composition</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supina bluegrass</td>
<td>236 ± 24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90 ± 9.4&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bermudagrass</td>
<td>255 ± 23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>123 ± 27&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blueberry</td>
<td>149 ± 9.6&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>---</td>
</tr>
</tbody>
</table>

Table 1.1. Total phenolic concentrations of crude extracts; <sup>a</sup>Results expressed as milligrams of gallic acid equivalents (GAE) 100 grams⁻¹ of fw sample. Data represented as mean ± SD (n=3). Means with the same letter are not significantly different (P ≥ 0.05) according to Tukey’s HSD test. <sup>b</sup>Results expressed as milligrams of catechin equivalents (CE) 100 grams⁻¹ of fw sample. Data represented as mean ± SD (n=3). Means with the same letter are not significantly different (P ≥ 0.05) according to Tukey’s HSD test. Moisture content of grass tissues was determined to be ≈.84 for supina bluegrass and ≈.61 for bermudagrass samples.

HPLC Analysis

The phenolic profiles (primarily phenolic acids) of crude AGPE were detected and reported at 340 nm using HPLC (Figure 1.6). The phenolic acid compositions of AGPE were distinct among species and extract types, yet each of the soluble AGPE (A
and B) did display some notable similarities. Vitamin C (more visible at lower wavelengths) and ferulic acid were apparent in significant quantities within each soluble AGPE. Additionally, appreciable amounts of resveratrol were detected in soluble bermudagrass AGPE (B), which represents a unique amenity grass constituent. The HPLC analysis of bound bermudagrass extracts (C) revealed a phenolic profile dominated solely by two phenolic acid compounds. Interestingly, neither of these specific conjugates was detected in the soluble bermudagrass extract.

**Figure 1.6.** HPLC analysis of crude AGPE at 340 nm: (A) Supina bluegrass soluble extract; (B) Bermudagrass soluble extract; (C) Bermudagrass bound/hydrolyzed extract.
Table 1.2. Peak identification and compound percentages of AGPE at 340 nm. Results represent means of triplicate analyses ± SD. Percent results expressed on the basis of a specific weight of crude/hydrolyzed extract; retention time (t<sub>r</sub>).

<table>
<thead>
<tr>
<th>Peak</th>
<th>t&lt;sub&gt;r&lt;/sub&gt; (min)</th>
<th>Compound</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Supina bluegrass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.48</td>
<td>Vitamin C</td>
<td>3.69 ± 0.51</td>
</tr>
<tr>
<td>2a</td>
<td>31.6</td>
<td></td>
<td>3.32 ± 0.45</td>
</tr>
<tr>
<td>2</td>
<td>32.9</td>
<td></td>
<td>23.6 ± 1.01</td>
</tr>
<tr>
<td>3</td>
<td>38.8</td>
<td>Ferulic acid</td>
<td>30.9 ± 1.99</td>
</tr>
<tr>
<td>3a</td>
<td>39.4</td>
<td></td>
<td>11.3 ± 0.27</td>
</tr>
<tr>
<td>4</td>
<td>42.7</td>
<td></td>
<td>12.5 ± 0.13</td>
</tr>
<tr>
<td>5</td>
<td>47.0</td>
<td></td>
<td>2.31 ± 0.17</td>
</tr>
<tr>
<td>(B) Bermudagrass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.48</td>
<td>Vitamin C</td>
<td>1.85 ± 0.28</td>
</tr>
<tr>
<td>3</td>
<td>38.8</td>
<td>Ferulic Acid</td>
<td>6.25 ± 0.99</td>
</tr>
<tr>
<td>3a</td>
<td>39.4</td>
<td></td>
<td>7.32 ± 1.12</td>
</tr>
<tr>
<td>6</td>
<td>41.5</td>
<td></td>
<td>5.03 ± 0.61</td>
</tr>
<tr>
<td>7</td>
<td>42.1</td>
<td></td>
<td>4.70 ± 0.06</td>
</tr>
<tr>
<td>8</td>
<td>45.1</td>
<td></td>
<td>11.8 ± 0.73</td>
</tr>
<tr>
<td>9</td>
<td>48.2</td>
<td></td>
<td>9.43 ± 0.21</td>
</tr>
<tr>
<td>10</td>
<td>49.7</td>
<td></td>
<td>3.61 ± 0.11</td>
</tr>
<tr>
<td>11</td>
<td>54.7</td>
<td>Resveratrol</td>
<td>3.95 ± 0.45</td>
</tr>
<tr>
<td>12</td>
<td>56.3</td>
<td></td>
<td>9.29 ± 0.43</td>
</tr>
<tr>
<td>12a</td>
<td>56.7</td>
<td></td>
<td>12.5 ± 0.36</td>
</tr>
<tr>
<td>(C) Bermudagrass bound</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>32.9</td>
<td></td>
<td>26.6 ± 0.20</td>
</tr>
<tr>
<td>13</td>
<td>37.3</td>
<td></td>
<td>59.8 ± 0.55</td>
</tr>
</tbody>
</table>

**Antioxidant Activity (ORAC)**

The ORAC antioxidant activity assay measured the ability of crude phenolic extracts to scavenge peroxyl radicals. The highest ORAC values (**Figure 1.7**) were produced by the free and bound bermudagrass phenolic extracts (103.52 and 91.37 µM TE gr<sup>−1</sup> fw, respectively), which differed significantly from the free and bound supina bluegrass extracts (51.55 and 36.08 µM TE gr<sup>−1</sup> fw, respectively), as well as the blueberry phenolic extracts (24.12 µM TE gr<sup>−1</sup> fw). With the exception of bound supina bluegrass extracts, all of the AGPE results for the ORAC assay were significantly higher than the blueberry phenolic extracts.
Figure 1.7. Oxygen Radical Absorbance Capacity (ORAC); Results expressed as μM of Trolox Equivalents (TE) per gram of fresh weight (fw) sample. Data represented as mean ± SE (n=3). Means with the same letter are not significantly different (P ≤ 0.05) according to Tukey’s HSD test.

**MTT Cell Viability Assay**

The mitochondrial activity of Caco-2 and HepG2 cancer cells demonstrated dose-dependent responses to the AGPE and control treatments (Figure 1.8), although bioactivities significantly differed among treatments and cell lines. The most significant suppression of Caco-2 mitochondrial activity (A) was exhibited by supina bluegrass treatments (EC$_{50}$~85 μg ml$^{-1}$ GAE) followed by bermudagrass treatments (EC$_{50}$~230 μg ml$^{-1}$ GAE). EC$_{50}$ values refer to the concentration of extract that causes 50% inhibition of mitochondrial activity. Both AGPE treatments demonstrated lower Caco-2 EC$_{50}$ values than the blueberry phenolic treatments. In contrast to the Caco-2 assays, bermudagrass treatments demonstrated the greatest HepG2 suppression (C). Significant suppression of
HepG2 growth by bermudagrass extracts resulted for 250 and 500 µg ml\(^{-1}\) GAE treatments, whereas no significant mitochondrial suppression occurred for the supina bluegrass treatments at the same concentrations. In general, the observed EC\(_{50}\) values were lower among Caco-2 cell populations compared to HepG2 for AGPE treatments.

**Figure 1.8B** represents Caco-2 mitochondrial inhibition by bermudagrass bound (hydrolyzed) fractions. Bound bermudagrass phenolic extracts displayed inhibitory influences on mitochondrial activity at higher concentrations, but pro-growth influences were apparent at lower treatment concentrations. It should also be noted that bound bermudagrass results (Caco-2) displayed the greatest variability among all treatments. **Figure 1.8D** displays the impacts of resveratrol, ascorbic acid (Vitamin C), and bound bermudagrass phenolic extracts on HepG2 growth. A significant dose-dependent pro-growth effect was produced by the ascorbic acid treatments, while both hydrolyzed bermudagrass and resveratrol treatments had negligible impacts on HepG2 activity.
A  

**Caco-2 MTT Assay**

- Supina bluegrass
- Bermudagrass
- Blueberry (+ Control)

![Graph showing relative Caco-2 cell viability vs. sample concentration (μg mL⁻¹) across different plant species for Caco-2 MTT Assay A.](image)

B  

**Caco-2 MTT Assay**

- Bound Bermudagrass

![Graph showing relative Caco-2 cell viability vs. sample concentration (μg mL⁻¹) for Bound Bermudagrass for Caco-2 MTT Assay B.](image)
Figure 1.8. Inhibition of Caco-2 and HepG2 cancer cell population growth by amenity grass phenolic extracts compared to phenolic and dietary positive controls. The MTT assay was used to estimate viable cell populations after cell incubation with treatments for 18 hr. Data expressed as mean ± SE (n=3) and data points displaying (*) are significantly different than the untreated control cells (100% viability) according to Tukey’s HSD test (α=.05).
**Annexin V-FITC Apoptosis Assay**

Untreated and treated HepG2 cell populations were stained with annexin V (AV) and propidium iodide (PI) and analyzed using flow cytometry (1.4 x 10^4 gated events). Stained cells were assigned to FACS display quadrants using a fluorescence-activated cell sorting histogram that corresponded with apoptotic expression (Figure 1.9).

Untreated HepG2 cell populations (A) displayed high viability (89.5%) among harvested cells. The dominant viability among untreated controls corresponded with an apoptotic shift among phenolic treatment populations. None of the concentrated phenolic treatments significantly induced necrosis at the given time point, and the most drastic shift from viability amongst AGPE occurred in the 500 μg mL^-1 supina bluegrass treated cells (C). The largest percentage of late apoptotic cells (29.8%) resulted from the 500 μg mL^-1 bermudagrass treatments (D) and HepG2 viability (39.1% viable cells) was best maintained with 125 μg mL^-1 resveratrol treatments.
Figure 1.9. Flow cytometry analysis of apoptotic induction in HepG2 cells. Untreated cells (A) and cells treated with 125 µg mL\(^{-1}\) resveratrol (B), 500 µg mL\(^{-1}\) supina bluegrass (C), and 500 µg mL\(^{-1}\) bermudagrass (D) were stained with annexin V and PI and analyzed using FACS fluorescent detection. Phenolic treatments were applied for 18 hr and quadrant results (gated percentages) are representative of three independent trials.
Figure 1.10. Apoptosis assay quadrant assignments
DISCUSSION

The scientific literature has recently been inundated with publications that describe the phenolic antioxidant attributes within an array of potential nutraceutical foods. This compilation of research has revealed a number of scientific facts concerning phenolic compounds for human health:

1. Natural plant matrices and extracts contain numerous phenols in balanced proportions. These phenolic combinations have been shown to elicit superior metabolic influences compared to phenolic compounds in isolation (Liu, 2004; Olsson et al., 2004; Seeram et al., 2005; Ray et al., 2004).

2. The plant matrix has a considerable impact on phenolic metabolism and absorption (Adam et al., 2002; Harbaum et al., 2008).

3. The ability of phenols to defend against disease is primarily a result of modulating influences on disease processes (Murugan et al., 2010; Xu et al., 2009; Crozier, Jaganath, & Clifford, 2009). Only fringe medicines still popularize the notion that antioxidants block or inhibit the processes associated with ageing and disease (Hail Jr et al., 2008).

4. Natural product production limitations and the effects of physiologically unrealistic phenolic dosage recommendations should be taken into account before advocating the nutraceutical development of a natural product (Duthie, Gardner, & Kyle, 2003; Crozier, Jaganath, & Clifford, 2009).

Future generations of phenolic research efforts must build off of these realities and incorporate progressive logic into their focus. This study attempted to meet the terms of this statement by focusing on the chemoprevention attributes and practicality of the
natural product itself. Using the understanding that amenity grasses are the most renewable and abundant plant resource on Earth, the objective of this project was to characterize the phenolic chemoprevention potential of two grass species rich in phenols, particularly phenolic acids.

Despite the fact that phenolic acids are the most widely consumed phenolic compounds in the human diet, phenolic acids have received only a fraction of the research expended on flavonoids (Mateos, Goya, & Bravo, 2006; Scalbert & Williamson, 2000). In reality, flavonoid studies have displayed a number of uncertainties in terms of flavonoid consumption and health benefits, including pro-oxidant potentials (Dickancaite et al., 1998; Hail Jr et al., 2008; Skibola & Smith, 2000), erratic *in vivo* metabolism (Hollman et al., 1999; Scalbert & Williamson, 2000; Duthie, Gardner, & Kyle, 2003), and low levels of absorption (Williamson & Manach, 2005; Crespy et al., 1999). For these reasons, this study emphasized the chemoprevention potential belonging to a phenolic acid-dominated plant matrix.

The results showed that the phenolic concentrations and antioxidant activities of supina bluegrass and bermudagrass are comparable to other highly regarded natural products. While TPC and ORAC results may be arbitrary to *in vivo* modes of action (corresponding to health benefits), these tests provide a phenolic signature or point of reference applicable to their nutraceutical potential. HPLC analyses in this study showed that both AGPE species contained extensive phenolic acids, yet the compositions were distinct for each species. Similarities between AGPE profiles included significant amounts of ferulic acid and vitamin C (which was much more apparent at 240 nm). Clinical studies investigating vitamin C and E benefits in isolation have often reported
inconclusive results (Appel, van Garderen-Hoetmer, & Woutersen, 1996). This phenomenon may be explained by a lack of complementary phenols that provide synergistic effects (Xu et al., 2009). The presence of appreciable amounts of vitamin C and phenolic acids in AGPE may provide evidence for nutraceutical attributes belonging to the synergistic effects of vitamins and phenols.

Free ferulic acid (FA) has been shown to be readily absorbed in vivo and its bioavailability is not limited by intestinal and hepatic metabolism (Adam et al., 2002). The source and forms of FA ingested determine the overall FA bioavailability, however it has been demonstrated that other phenolic acids, such as caffeic and chlorogenic acid, are often metabolized into ferulic and isoferulic acid in the intestine (Lafay et al., 2006). Paquet, Clifford, & Williamson, (2008) reported that colonic absorption of FA can occur through a combination of mechanisms: passive transcellular diffusion and facilitated transport. These absorption processes leave free FA and a small percentage of conjugated FA available for the liver and other tissues (Paquet, Clifford, & Williamson, 2008). Furthermore, the fractional availability of FA for tissues has been reported to be ~50% of the absorbed dose (Adam, et al., 2002), which is quite substantial considering that quercetin was only reported to have an available dose absorbance of ~9% (Crespy et al., 1999).

Additionally, Adam et al, (2002) reported that the release of esterfied FA from a complex matrix is most effective when FA is bound in the primary cell wall- this supports the nutraceutical potential of amenity grasses, which display extensive primary cell wall lignification. This present study’s HPLC analysis of hydrolyzed bermudagrass extracts revealed a bound phenolic profile consisting solely of two phenolic acid
compounds. These bound compounds were also shown to possess appreciable antioxidant capacities according to ORAC results- an attribute that should be emphasized considering the reported enhanced absorption potentials of bound phenols (Harbaum et al. 2008; McDougall et al., 2008).

In view of the absorption potential for FA-rich natural products, this study focused on the chemoprevention attributes of AGPE using HepG2 and Caco-2 cancer lines. Two of the essential alterations in cell physiology necessary for carcinogenesis and tumorigenesis are deregulated cell growth and suppression of apoptosis. Because these two processes are central to tumor development, they present obvious targets for chemopreventive or therapeutic intervention (Evan & Vousden, 2001). The MTT and annexin-V assays are two widely used methods that evaluate cell proliferation and apoptosis induction, respectively.

Mcdougall et al, (2008) reported Caco-2 cell lines to be a physiologically relevant model for first-pass screening of chemoprevention compounds. The MTT results for Caco-2 cell populations in this study monitored the inhibition of mitochondrial activity by AGPE and compared it to that of blueberry extracts (Figure 7). Inhibition of Caco-2 proliferation by berries is well-documented in the literature (Jensen et al., 2002; McDougall et al., 2008). Our cell viability study demonstrated dose-dependent Caco-2 mitochondrial activity suppression among each of the phenolic treatments. The most prevalent and statistically significant suppression of mitochondrial activity occurred with crude supina bluegrass extracts. It should also be noted that bound bermudagrass AGPE exhibited Caco-2 antiproliferation at higher concentrations, but a pronounced low-dose pro-growth effect was apparent at lower concentrations. Physiologically relevant doses
of hydrolyzed phenolics would most likely fall into these lower concentration ranges.

This report has outlined the *in vitro* antiproliferative influences of AGPE on Caco-2 cancer cells according to the MTT assay. Considering the global prevalence of colorectal cancer, preliminary evidence supporting potentially novel colorectal protective mechanisms should not be ignored.

The HepG2 cell line is widely used as a model system for the liver. Characterization of phase I and phase II enzymes, as well as xenobiotic transporters have confirmed HepG2 cells as a suitable model for metabolic studies (Brandon et al., 2006). While intestinal absorption would invariably present the liver with metabolized AGPE products *in vivo*, some AGPE compound consistencies would be maintained *in vitro* considering free FA’s rapid absorption in the stomach and the intestinal absorption of free and glucuronidated FA (Olthof, Hollman, & Katan, 2001; Mateos, Goya, & Bravo, 2006). The HepG2 MTT results for this study revealed AGPE effects that contrasted the Caco-2 study. In this study, HepG2 mitochondrial activity was significantly inhibited by bermudagrass phenolics, but not by supina bluegrass. Additionally, hydrolyzed bermudagrass extracts had a null effect on HepG2 mitochondrial activity.

Resveratrol and vitamin C were used as positive and negative controls for the HepG2 study, respectively. Control treatment additions and omissions between cell lines were a reflection of dietary points of reference according to documented treatment impacts. Additionally, adequate concentrations of bound supina bluegrass fractions were unable to be obtained for MTT assays. Resveratrol was chosen as the positive HepG2 control due to its highly-regarded chemopreventive potential and its extensive use in bioassay tumor models (Lee, Zhang, & Sanderson, 2008). Conversely, vitamin C
(ascorbic acid) was used as a negative control for validation purposes. Vitamin C in isolation has been shown to provide a protective effect to cells in vitro, thus allowing hepatic cells to further proliferation by regulating excessive oxidation (Yin, Kim, Moon, & Lee, 2005). Our results were consistent with the literature, which demonstrated resveratrol’s negligible impact on HepG2 mitochondrial (Marcsek, Kocsis, Szende, & Tompa, 2007) and vitamin C’s pro-growth effects at elevated concentrations in vitro (Chen & Cederbaum, 1998).

Cell cycle disruption is a prerequisite for apoptotic death (Hickman, 1992). Considering bermudagrass’ impact on HepG2 mitochondrial activity, bermudagrass extracts demonstrated the greatest percentage of late apoptotic induction (29.8%) according to the annexin V analysis. In contrast, supina bluegrass and resveratrol treatments showed limited influences on mitochondrial activity, but considerable apoptotic shifts resulted. Supina bluegrass extracts displayed the greatest percentage of early apoptotic induction (72.9%), while cells treated with resveratrol maintained the greatest percentage of viability (39.1%) among treatments. The lesser extent of observed HepG2 apoptosis with resveratrol could be attributable to the physiological unrealistic resveratrol treatment concentration (~ 547 µM). The elevated treatment concentration was intended to serve as a positive control for this apoptotic study, but it could have triggered a distinct xenobiotic response in HepG2 cells- thus, reducing the apoptotic response. It has been reported in the literature that effective doses of resveratrol to induce tumor apoptosis may only range from 40 to 200 µM (Aggarwal & Shishodia, 2006).
The lack of correlation between annexin V and MTT results for supina bluegrass and resveratrol could be explained by a number of occurrences. Cell viability assays display some notable limitations and oversights in terms of extrapolating conclusive chemopreventive results. For example, the MTT assay can be misleading if treatments affect intracellular activities other than mitochondrial respiration (Weyermann, Lochmann, & Zimmer, 2005), or if treatments are able to revert precancerous cells back to a healthy state without significantly affecting viability. Additionally, apoptosis is an energy-dependent process that requires functioning mitochondria. Thus, cell viability assays do not take into account the fact that necrosis would ensue once intracellular energy stores had been exhausted (Bradbury, Simmons, Slater, & Crouch, 2000). While apoptosis assays complement the cell viability studies, the annexin V assay only measures whole cells and cannot be used to draw conclusions about the subsequent fate of the cells. When drawing conclusions from the annexin V assay, it is important to remember that dying cells are engaged in a cascade of molecular events that are reversible until a first irreversible process takes place (Galuzzi & Kroemer, 2009).

In conclusion, characterization results demonstrated the potential chemoprevention attributes of AGPE, which were confirmed in our preliminary in vitro studies. The in vitro chemopreventive effects of AGPE were shown to be dependent upon AGPE species, extract fractions, doses, and cell lines. Characterization results illustrated AGPE’s extensive phenolic acid profiles, which displayed major contributions from ferulic acid compounds. The literature has shown ferulic acid to be a highly available and health benefitting compound. In contrast to cereals, AGPE offer
appreciable amounts of unbound/free ferulic acid that would be available for rapid absorption.

This research has revealed significant chemopreventive potential belonging to the planet’s most renewable and abundant plant source. Future studies should focus on \textit{in vivo} bioavailability and metabolism, influences on healthy tissues, and determine physiological relevant doses of AGPE.
Chapter 2

Application of mid IR spectroscopy to monitor phenotypic responses in HepG2 cells treated with resveratrol and phenolic acid-rich amenity grass extracts
LITERATURE REVIEW

Nutritional Genomics and Metabolomics

Nutritional genomics is a comprehensive approach for investigating the influence of diet and genetic variation as risk factors for chronic disease (Kaput et al., 2005). It is well documented that genomic expressions can be regulated by, and dependent upon, nutrients, micronutrients, and phytochemicals found in foods and supplements (Kaput, et al., 2005). In the past, the central dogma of molecular biology supported a unidirectional flow of information from gene to transcript to protein (Hollywood, Brison, & Goodacre, 2006). However, this concept has been proven false through elucidation of the dynamics involved in expression of genetic information. Hollywood, Brison, & Goodacre (2006) adequately summarized the historical landscape of molecular biology in a recent review: “It is fair to comment that molecular biology has generally been bogged down by hypothetico-reductionist thinking where small parts of the jigsaw have been studied in isolation.”

Nutritional genomics has embraced the need for molecular reform by generating a resurgence of systems biology approaches to understand the complex interplay of genes and the environment. This paradigm shift in the post-genomic era has primarily been facilitated by the integrative analyses of the ‘-omic’ disciplines (Hanash et al., 2002; Westerhoff & Palsson, 2004; Hollywood, Brison, & Goodacre, 2006). While functional genomic approaches couple the analysis of gene products such as mRNA [transcriptomics (Baldwin, Crane, & Rice, 1999)] and proteins [proteomics (Santoni et al., 1998)], these methods do not adequately describe how these upstream processes correlate to biological function or phenotype (Fiehn et al., 2000). Because it is known that
metabolic fluxes are not solely regulated by gene expression (ter Kuile & Westerhoff, 2001), the study of the metabolome (metabolomics) provides information most closely related to the phenotype. The metabolome has been generalized as the collection of low molecular weight molecules present in cells that participate in general metabolic reactions and are required for maintenance, growth, and normal cell function (Oliver et al., 2003). The investigation of intracellular metabolites is a powerful asset for understanding complex biological systems, but an inclusive analysis of the complete set of intracellular metabolites is not yet achievable (Kim, et al., 2010).

**Metabolic Fingerprinting**

One strategy for metabolomic analysis is metabolic fingerprinting, or rather a non-targeted approach to metabolite analysis. Metabolic fingerprinting is the rapid classification of samples through high-throughput analyses, which provides a snapshot of the metabolic composition at a given time (Johnson et al., 2003). The advantage of fingerprinting techniques is their rapidity, yet these techniques do not generally allow for specific metabolite information (Hollywood, Brison, & Goodacre, 2006). For this reason, fingerprinting strategies do not involve generating quantitative data on a specific metabolite, or even a group of metabolites with similar chemistries or biological origin, as this requires more time consuming and invasive methodologies (Kim, et al., 2010). In general, metabolic fingerprinting analyses utilize physiochemical techniques to measure alterations in biochemical pathways, provide screening for integrative nutrigenomic studies, and can serve as diagnostic tools (Fiehn, 2002).
**FTIR Spectroscopy**

Fourier transform infrared spectroscopy (FTIR) is an ideal technique for high-throughput metabolic fingerprinting (Johnson et al., 2003). FTIR resolves the chemical profile of a sample by measuring the entire chemical spectrum through means of absorbance of specific wavenumbers of IR light (Baker et al., 2008). These absorbance values relate to IR-induced vibrations of specific chemical bonds or functional groups. Specifically, IR absorbance depends on the atoms involved in the bond and the strength of the intermolecular interactions; thus, the combined chemical spectrum from molecules in a sample essentially creates an IR fingerprint for a specimen (Baker et al., 2008).

The primary region of interest for FTIR analysis of biological samples is in the mid-IR, which is defined as 4000-600 cm\(^{-1}\) (Dunn & Ellis, 2005). Cellular constituents including carbohydrates, lipids, amino acids, and fatty acids, as well as proteins and polysaccharides can all be analyzed simultaneously in the mid-IR (Harrigan & Goodacre, 2003). FTIR band assignments for specific cellular constituents have been tentatively assigned to spectral windows of interest (Table 2.1) according integrative assessments of the literature (Walsh et al., 2007; Fabian et al., 2006; Naumann, 2001). Shifts in the ratios and/or conformations of the biomolecules responsible for a FTIR spectral region gives rise to subtle changes in the corresponding peaks (i.e. shifts, shapes, and/or intensity) and are indicative of intracellular alterations (German et al., 2006; Gasparri & Muzio, 2003).

In general, FTIR spectroscopy is an established, yet constantly evolving technique that allows for rapid, non-invasive, highly reproducible, and reagentless analyses (Dunn & Ellis, 2005). The availability of computerized FTIR instrumentation has improved the
signal-to-noise ratio and allowed for extensive data manipulation (Kong & Yu, 2007). Additionally, band narrowing methods, Fourier self-deconvolution, and second derivative manipulations have enriched IR spectra interpretations and provided the foundation for qualitative and quantitative assessments (Dong et al., 2002). Second derivative spectral analyses enhance the resolution of overlapping IR bands in a spectrum and can allow for elucidation of secondary structures (Susi & Byler, 1983). Popular statistical assessments of FTIR data include cluster analyses, such as principal component analysis (PCA) and hierarchical cluster analysis (HCA). Multivariate techniques such as these are able to cluster analyze FTIR data according to co-expressed unique biochemical fingerprint constituents (Hollywood, Brison, & Goodacre, 2006). Equipped with mathematical resolution enhancement techniques and various options for data analysis (Kong & Yu, 2007), FTIR has become extremely useful for metabolic fingerprinting applications within the life sciences. Specifically, FTIR applications for pathology include considerable options for disease detection, diagnosis, and treatment (Kim et al., 2010).

Table 2.1. Tentative FTIR band assignments (adapted from Kim et al., 2010)

<table>
<thead>
<tr>
<th>Wavenumber/cm⁻¹</th>
<th>Assignment</th>
<th>FT-IR vibrational modes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>~3400 to ~3300</td>
<td>Amide A</td>
<td>Str. mode of N–H</td>
</tr>
<tr>
<td>~1690 to ~1620</td>
<td>Amide I</td>
<td>ν(C=O)</td>
</tr>
<tr>
<td>~1590 to ~1530</td>
<td>Amide II</td>
<td>δ(N–H) and ν(C–N)</td>
</tr>
<tr>
<td>~1340 to ~1240</td>
<td>Amide III</td>
<td></td>
</tr>
<tr>
<td>Nucleotide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>~1225</td>
<td></td>
<td>PO₂⁻ (asymmetric phosphate)</td>
</tr>
<tr>
<td>~1080</td>
<td></td>
<td>PO₂⁺ (symmetric phosphate)</td>
</tr>
<tr>
<td>Lipid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>~2924 and ~2850</td>
<td>Membrane lipids</td>
<td>Symmetric CH₂ str. mode of CH₃ chains</td>
</tr>
<tr>
<td>~2958 and ~2873</td>
<td>Membrane lipids</td>
<td>Asymmetric CH₃ str. mode of CH₃ end groups</td>
</tr>
<tr>
<td>~1746</td>
<td></td>
<td>C=O</td>
</tr>
<tr>
<td>~1463</td>
<td></td>
<td>CH₂ bending</td>
</tr>
<tr>
<td>~1165</td>
<td></td>
<td>Ester CO–O–C str.</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>~1200 to ~900</td>
<td>Sugar (glycogen)</td>
<td>νₛ(C–O) coupled to the δ(C–O–H)</td>
</tr>
<tr>
<td>~1030</td>
<td></td>
<td>νₛ(C–O–C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>νₕ(C–O)</td>
</tr>
</tbody>
</table>
FTIR and Cancer

As many diseases are an outcome of metabolic disorders (including cancer), metabolomic approaches are being developed as a means to identify biomarkers for disease (Hollywood, Brison, & Goodacre, 2006). In particular, FTIR spectroscopy allows researchers to monitor the different phases of conformational and functional changes (biomarkers) in complex biological systems including both tissue and cell cultures (Giambattista et al., 2010). Therefore, the use of FTIR for the identification of biomarkers within disease processes can serve as a logical starting point for therapeutic intervention and drug discovery (Hollywood, Brison, & Goodacre, 2006).

The detection of cancer biomarkers with FTIR has been recognized for its ability to provide various types of information on small structural changes of biologically important constituents during cancer development and progression (Takahashi et al., 1999). This stems from the fact that FTIR techniques not only possess the sensitivity to discriminate between normal and cancerous cells/tissues (Argov et al., 2004; Kim et al., 2010), but also are able to monitor basic cancer processes such as changes in metabolite concentrations (Liu et al., 1999) and shifts in the secondary structures of biomolecules (Malins et al., 2004). For example, the amide I band (1700-1600 cm\(^{-1}\)) is almost entirely comprised of C=O stretch vibrations from peptide linkages, while the amide II band (1575-1480 cm\(^{-1}\)) is derived mainly from in-plane N-H bending and C-N stretch vibrations (Kong & Yu, 2007). Shifts in amide I or II peaks are indicative of alterations in protein secondary structures of intracellular proteins (Mantsch & Chapman, 1996). Although, it has been shown that the amide II band demonstrates much less protein conformational sensitivity than its amide I counterpart (Krimm & Bandekar, 1986).
Baker et al., (2008) suggested that shifts in the amide I region represent biomarkers for cancer aggressiveness that correlate to protein conformational changes. Additionally, Fabian et al., (2006) used the amide II region to differentiate colorectal adenocarcinoma from non-cancerous tissue and Giambattista et al., (2010) related amide I shifts ($\alpha$-helix) to increased molecular disorder and apoptosis induction.

Phosphate bands are due to $PO_2^-$ vibrational stretching and asymmetric stretching, which may be indicative of phosphorylated DNA components (Tobin et al., 2004; Walsh et al., 2007). The phosphate bonds of interest for cancer researchers occur primarily in the nucleic acid region ($1300-900 \text{ cm}^{-1}$). Lasch, Pacifico, & Diem (2002) suggested that increases in cell division and metabolic activity correlate to more pronounced $PO_2^-$ bands of DNA, RNA, and phospholipids. This occurrence is significant for the study of cancer considering cancerous cells exhibit higher divisional rates than non-cancerous precursor cells (Lasch et al., 2004). Argov et al., (2002) confirmed this hypothesis by analyzing the phosphate band at $\sim 1080 \text{ cm}^{-1}$. Diem, Boydston-White, & Chiriboga, (1999) proposed that the ‘observability’ of the DNA phosphate backbone band at $\sim 970 \text{ cm}^{-1}$ was a result the decondensation of nuclear DNA- an event consistent with cell division. Furthermore, Giambattista et al., (2010) showed that UVB radiation-induced $PO_2^-$ shifts at $\sim 1244 \text{ cm}^{-1}$ correlated to apoptotic events in Jurkat cells.

FTIR analyses have also been able to monitor alterations in DNA absorbance linked to cancer progression and the opposed apoptosis phenomena. Liu et al., (2001) reported an absorption decrease in the nucleic acid spectral region following apoptosis in leukaemic cells. Using ATR-FTIR spectroscopy, Gasparri & Muzio, (2003) reported similar findings and postulated that the condensation of chromatin during apoptosis may
lead to significant increases in DNA visibility, thus decreasing the intensity of nucleic acid bands. In contrast, evidence from the literature demonstrates FTIR’s ability to detect cancer associated DNA lesions. Malins et al., (2004) reported substantial FTIR-detectable modifications in the DNA base and backbone structures as normal tissues progress to a cancerous state. DNA spectral regions of interest included ~1720 cm\(^{-1}\) (C\(_4\)=O stretching; thymine residues) and ~1606 cm\(^{-1}\) (NH\(_2\) bending and C=\(\equiv\)N stretching; adenine residues). Additionally, the spectral vibrations from ~1700-1350 cm\(^{-1}\) and ~1275-750 may be respectfully attributed to alterations in nucleotide base and backbone structures, which may relate to DNA conformational changes (Malins et al., 2003; Tsuboi, 1969). Considering that conformational changes in DNA structure are expected to alter gene expression and the fidelity of transcription and replication (Turner, 2001), these shifts in the FTIR spectra may be strongly correlated to the onset of cancer.

Tissue specific glycogen concentration shifts during the onset of carcinogenesis are well documented in the literature. In many organs such as the colon (Rigas et al., 1990) and lung (Yano et al., 1996), glycogen levels in cancerous cells are higher than compared to normal cells. Yet, in organs such as the liver and cervix, glycogen levels are lower in cancer cells compared to the levels in corresponding normal cells (Sahu & Mordechai, 2005). In many cancer tissues, the increase in energy demands equates to the accumulation of glycogen in cancerous tissue (Rigas et al., 1990). However, it has been hypothesized that in organs such as the liver, where glucose and glycogen levels are naturally elevated, glycogen does not accumulate in cancerous tissues due to the high levels of naturally available energy (Takahashi et al., 1999). Therefore, the FTIR
vibrational region with glycogen bond contributions (1185-900 cm\(^{-1}\)) may be a region of interest for distinguishing between normal and cancerous cells.

Finally, the lipid vibrational region (3010-2800 cm\(^{-1}\)) has also been reported to be a potential biomarker for cancer (Walsh et al., 2007). In a study using breast cancer tissue, Fabian et al., (2006) showed an increase in lipid-like material using FTIR that corresponded to cancer progression. A summary of the aforementioned cancer biomarker evidence using FTIR is illustrated below in Table 2.2.

**Figure 2.2. Tentative FTIR bands relating to cancer.**

<table>
<thead>
<tr>
<th>Absorption band/stretch (cm(^{-1}))</th>
<th>Assignment</th>
<th>Region of influence</th>
<th>Cancer speculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>~2830-2930</td>
<td>Fatty acids (1)</td>
<td>Lipids</td>
<td>Fabian et al., (2006) suggested that cancerous breast tissues have more lipid-like materials than benign tissues.</td>
</tr>
<tr>
<td>~1610-1680</td>
<td>Amide I</td>
<td>Protein secondary structure; α-helix (1645-1666 cm(^{-1})); β-sheets (1613-1637); Random coils (1637-1645); β-turns (1666-1682)</td>
<td>Shifts in secondary structure are thought to be spectroscopic biomarkers indicating structural disorder or stability (Kong &amp; Yu, 2007; Giambattista et al., 2010).</td>
</tr>
<tr>
<td>~1500-1700</td>
<td>Amide I and II (2)</td>
<td>Protein content</td>
<td>Protein compositional shifts within the amide regions are proposed to be indicative of protein content as it relates to cancer aggressiveness (Baker et al., 2008).</td>
</tr>
<tr>
<td>~1200-1500</td>
<td>Phospholipids and nucleotides (3)</td>
<td>Phosphodiester deoxyribose backbone</td>
<td>Conformational changes in the nucleotide region would be expected to alter gene expression and may correspond to cancer (Malins et al., 2003). Phosphodiester hydrolysis is also an important factor in DNA repair (Giambattista et al., 2010).</td>
</tr>
<tr>
<td>~1234, 1080, 970</td>
<td>Phosphates</td>
<td>PO(_4^\text{2-}) bonds</td>
<td>It has been reported that PO(_4^\text{2-}) bond intensities and shifts can be linked to divisional activity. Cell division is increased in a cancerous state (Walsh et al., 2007; Lasch et al., 2004; Tobin et al., 2004; Argov et al., 2002).</td>
</tr>
<tr>
<td>~900-1185</td>
<td>Polysaccharides (4)</td>
<td>Glycogen bonds</td>
<td>Cellular impacts on glycogen levels are reported to occur with cancer. This event has been outlined using FTIR in the glycogen bond spectral region (Takahashi, et al., 1999).</td>
</tr>
</tbody>
</table>
A significant gap in knowledge exists as to how health-promoting components of natural systems modulate metabolism to maintain health. Thus, the objective of this study was to monitor the metabolic alterations attributed to amenity grass phenolic extracts in human hepatocellular liver carcinoma cells, HepG2, using Fourier transform infrared (FTIR) spectroscopy. Recent research by the authors has demonstrated in vitro cancer antiproliferation and apoptotic induction capacities linked to phenolic extracts of understudied amenity grass species supina bluegrass \([\text{Poa supina Schrad.} \text{ 'Supranova']}\] and bermudagrass \([\text{Cynodon dactylon (L.) Pers. var. dactylon 'Sovereign']}\]. The results of this study illustrate amenity grass nutraceutical targets within the HepG2 metabolome compared to the chemopreventive influences of resveratrol. Resveratrol has documented chemopreventive impacts within all of the general stages of cancer progression (Lee, Zhang, & Sanderson, 2008). To our knowledge, this is the first investigation into the metabolic influences of resveratrol or amenity grass phenolics, as it relates to cancer, using FTIR spectroscopy.
MATERIALS & METHODS

Seed Sources

Supina bluegrass [Poa supina Schrad. ‘Supranova’] seed was generously provided by United Seed (Omaha, NE) and bermudagrass [Cynodon dactylon (L.) Pers. var. dactylon ‘Sovereign’] seed was purchased from Seeds West (Yuma, AZ).

Grass Samples

The amenity grass species used for this study included supina bluegrass [Poa supina Schrad. ‘Supranova’] and bermudagrass [Cynodon dactylon (L.) Pers. var. dactylon ‘Sovereign’]. Grasses were planted in May of 2010 and grown from seed in greenhouses at the University of Nebraska-Lincoln’s East Campus. Supina bluegrass samples were maintained under 15 hr ~ 30 °C max day / 9 hr ~ 17 °C min night growth conditions. Bermudagrass samples were maintained under 17 hr ~ 34 °C max day / 8 hr ~ 20 °C min night growth conditions in a separate warm season greenhouse. Relative humidity in the cool and warm season greenhouses ranged daily from 30-80% and 45-90% respectively. Supplemental lighting was supplied with halide lamps (200 mol photons m⁻² s⁻¹) and soil mixtures consisted of 34% peat, 31% perlite, 31% vermiculite and 4% screened topsoil, amended with 2 kg dolomite m⁻². Additionally, grasses were watered, fertilized (20-20-20 nutrient solution containing 200 ppm N) and trimmed as needed.

Phenolic Extraction

Phenolic extractions were carried out according to a modified procedure from Sarath et al. (2006). Soluble free cell wall phenolics were extracted twice (1:25 w/v) with 50% aqueous methanol (acidified to pH 5) followed by 50% aqueous acetone. Each
extraction was facilitated with 1 hr of mixing/vortexing and supernatants were pooled after centrifuging at 3000 g for 10 min. Sample pellets (residues) were vacuum-dried for 24 hr at 50 °C to remove residual solvents. Pooled supernatants were stored at -20 °C until drying or further testing.

**Cell Culture**

Human hepatocellular liver carcinoma (HepG2) cell lines were chosen as a model cancer line. HepG2 cells were cultured in Modified Eagle Medium Eagle (MEME) (Sigma, St. Louis, MO, USA) supplemented with 2.2 g L\(^{-1}\) sodium bicarbonate, 11 mL L\(^{-1}\) penicillin/streptomycin (50 units mL\(^{-1}\) penicillin with 50 µg mL\(^{-1}\) streptomycin), and 10% fetal bovine serum (FBS). Cell populations were grown in 75 cm\(^2\) culture flasks and maintained at 5% CO\(_2\), 95% air at 37 °C using a CO\(_2\) water-jacketed incubator (Thermo Fisher Scientific Inc., Forma Series II 3110, Waltham, MA). HepG2 cell populations from culture flasks were subcultured every 2-3 days after washing with 10x phosphate buffer saline (PBS) and trypsinization.

**Sample Preparation**

When HepG2 cell populations reached ~80% confluence, 0.7 mL cultured cell aliquots in MEME (3.5 x 10\(^6\) cells mL\(^{-1}\)) were briefly distributed to 24 well plates and incubated at 37 °C and 5% CO\(_2\) to form a partial monolayer. After 24 hr of incubation, the medium was removed and refreshed with growth medium [0.1% dimethylsulphoxide (DMSO) according to Zhang *et al.* (2008)] containing either amenity grass phenolic treatments (500, 125, or 31 µg mL\(^{-1}\) GAE) or resveratrol [(500, 125, or 31 µg mL\(^{-1}\)] Sigma, St. Louis, MO, USA]. After 18 hr of treatment incubation, the adherent cells were harvested after quenching metabolisms with ice-cold 10x PBS and trypsinization.
MEME (unsupplemented) was then added to harvested cells to deactivate the trypsin and the cell solutions were briefly centrifuged at 2000 rpm for 2 min (4 °C). Finally, cells were washed with 1x PBS, centrifuged (2000 rpm, 2 min, 4 °C), and resuspended in 0.15 mL of 1x PBS. For FTIR spectroscopy, 35 µL of each sample were pipetted onto a zinc selenide (ZnSe) plate and dried under vacuum (10 Hg) for 20 min at 35 °C.

**FTIR Spectroscopy**

Temporal changes in the mid infrared (IR) spectra of treated HepG2 cell populations were evaluated by comparison to untreated HepG2 populations. Specifically, BioWheel technology using a Bruker Equinox 55 FT-MIR equipped with a deuterated triglycine sulfate detector was used to acquire FTIR spectra. Samples were applied to a ZnSe plate, which contained 15 sample windows. FTIR spectra were recorded directly from the dried sample biofilms using the transmission mode. HepG2 treatment samples were concentrated to an optical density of ~ 0.9 absorbance units at 600 nm prior to FTIR analysis to ensure high and consistent signal to noise ratios. Each spectrum was recorded in the mid IR range (4000-500 cm⁻¹), at a resolution of 4 cm⁻¹, with 128 interferograms co-added, averaged, and apodized with the Blackman-Harris 3-Term function and then Fourier-transformed.

Recording of spectra, data storage, and all other spectral manipulations were performed using the Bruker Opus 4.0 Software. All spectra were independently and consistently baseline corrected, normalized to the amide I peak, and Fourier self-deconvoluted before averaging and analyzing using spectral manipulations. A total of 178 spectra were collected and averaged to determine biological treatment averages at each treatment concentration and the grand means of each treatment over all
concentrations. Culturing methods and sample plating techniques followed a randomized complete block design, which blocked for replicates. FTIR spectral areas were monitored and compared to the control through hierarchal cluster analysis (Ward’s algorithm) and presented in the form of dendrograms.
RESULTS & DISCUSSION

Previous research by the authors demonstrated HepG2 growth suppression and apoptosis induction capacities attributed to supina bluegrass and bermudagrass crude phenolic extracts in vitro. It was proposed that the diverse phenolic profiles and elevated ferulic acid (FA) content of amenity grasses could potentially lend these natural products as novel candidates for chemoprevention development. To further test this hypothesis, FTIR spectroscopy was implemented to evaluate the nutraceutical targets of amenity grass phenolic extracts (AGPE) within the HepG2 liver cancer cell line. These results were compared to the metabolic influences of resveratrol- an extensively studied polyphenolic compound with documented chemopreventive impacts on a variety of cell models, including HepG2 (Surh, 1999; Jang et al., 1997; Lee, Zhang, & Sanderson, 2008).

Characterization of phase I/II enzymes, as well as xenobiotic transporters have confirmed HepG2 cells as a suitable model for metabolic studies (Brandon et al., 2006). Therefore, the FTIR results for HepG2 cells directly treated with AGPE may conceivably be extrapolated to the human situation. Moreover, while intestinal absorption would invariably present the liver with metabolized AGPE products in vivo, some AGPE compound consistencies would be maintained in vitro considering the rapid absorption of free FA in the stomach and the intestinal absorption of free and glucuronidated FA (Olthof, Hollman, & Katan, 2001; Mateos, Goya, & Bravo, 2006).

Figure 2.2 depicts the preprocessed zero-order (A) and corresponding second derivative (B) FTIR spectra for all HepG2 technical replicates used in this study. These spectra represent three biological replicates of cells treated with 500, 125, or 31 µg mL⁻¹.
of the AGPE and resveratrol with each replicate analyzed three times via FTIR. Of the 108 total spectra generated, only three spectra were discarded before analysis due to inadequate absorbance. The discarded spectra originated from the same HepG2 biological replicate treated with 500 µg mL⁻¹ bermudagrass extract. The weak signals may have resulted from a lack of viable cells, which may have been due to excessive cytotoxicity within the cell population. For the remaining 105 spectra, multivariate analyses were carried out with the vector-normalized zero order data analyzed using the second derivative spectra.

To evaluate basic HepG2 biological differences attributed to phenolic treatment influences, an initial multivariate analysis was completed using a hierarchical clustering assessment (Ward’s algorithm). Figure 2.3A illustrates the frequency ranges utilized for the cluster analyses. These regions account for the mid-IR spectral windows of interest: CH stretching bonds from lipids (3000 to 2800 cm⁻¹); amide I and II functional groups associated with proteins (1700-1500 cm⁻¹); amide III/fatty acid/phosphate functional group combinations associated with DNA, nucleotides, and phospholipids (1500-1200 cm⁻¹); carbohydrates and the DNA/RNA phosphodiester backbone (1200-900 cm⁻¹). The heterogeneity/clustering of the technical reps are presented in Figure 2.3B. Although the large numbers of replicates make this figure difficult to review, the elevated phenolic treatment concentrations clustered away from the untreated controls respective of dose and treatment type. The occurrence of dose- and treatment-specific metabolic influences on HepG2 populations provided the level of confidence needed for analyses using the averages of biological replicates, as shown in Figure 2.4.
Figure 2.2. FTIR spectra obtained from 105 treated and untreated HepG2 replicates. A) Preprocessed zero-order spectra B) The corresponding preprocessed second derivative spectra.
Figure 2.3. Heterogeneity of HepG2 cells treated with either amenity grass phenolic extracts (500, 125, 31 µg mL\(^{-1}\) GAE) or resveratol (500, 125, 31 µg mL\(^{-1}\)) compared to untreated HepG2 controls. Cluster analyses were completed using Ward’s algorithm on vector-normalized zero order data that was analyzed using second derivative spectra. A) IR spectral regions used for cluster analyses B) Cluster analysis of all technical replicates corresponding to spectra in Figure 2.2A.
The multivariate analysis presented shows the clustering of treatment averages according to their co-expressed biochemical fingerprint. Therefore, similar fingerprints would be representative of similar biological functions, while increases in heterogeneity relate to shifts in the metabolic status of the cell. With this in mind, Figure 2.4 clearly displays treatment-induced metabolic alterations. As was expected, the severity of the treatment impacts appeared to be dose- and treatment-dependent. Most noteworthy, were the unique treatment-specific signatures on HepG2 biochemical constituents. For example, the metabolic influences of 500 mg mL$^{-1}$ bermudagrass treatments (BT) were similar to resveratrol’s influence at lower concentrations. In contrast, the 500 µg mL$^{-1}$ supina bluegrass treatments (SBT) and resveratrol treatments (RT) resulted in more dramatic HepG2 metabolic alterations, but these alterations were specific to each of the treatments. These results indicate that SBT, BT, and RT displayed appreciable HepG2 bioactivities, yet the mechanisms of action appeared to be treatment- and concentration-specific. Additionally, the reported differences in AGPE bioactivity supported the chemoprevention idiosyncrasies of particular amenity grass species for distinct biological influences.
Figure 2.4. Heterogeneity of HepG2 cells treated with either AGPE (500, 125, 31 µg mL\textsuperscript{-1} GAE) or resveratol (500, 125, 31 µg mL\textsuperscript{-1}) compared to untreated HepG2 controls. The multivariate analysis was performed using the averages of vector-normalized zero order data that was analyzed using the second derivative spectra for each biological replicate. A) Averaged IR spectra used for cluster analysis: untreated control (blue), bermudagrass treatments (orange), supina bluegrass treatments (red), resveratrol treatments (green). B) Hierarchical cluster analysis (Ward’s algorithm).

Before evaluating the spectral profiles as they relate to the down-regulation of cancer, a better understanding of the influences and physiological relevance associated with various treatment concentrations was necessary. Figure 2.5 illustrates the difference spectra of the preprocessed zero order data for supina bluegrass treated HepG2 cells at each of the concentration levels minus the untreated HepG2 control spectra.
Thus, respective peaks/valleys represent treatment-induced increases/decreases in vibrational bands for particular FTIR spectral constituents. Band alterations may intuitively reflect compositional increases/decreases in total constituent levels within a sample, or they may also reflect structural/conformational shifts that impact bond torsions, which lead to band differentials. For example, the onset of carcinogenesis induces an increase in free radical generation, which leads to the formation of lipid, protein, and DNA peroxidation products (Petibois & Deleris, 2006). These peroxidation products from carcinogenesis may be associated with structural alterations, which are detectable by FTIR spectroscopy.

In the lipid region (3000 to 2800 cm\(^{-1}\)), which is primarily attributed to membrane lipids (Kim et al., 2010), band intensity spikes resulted for the 500 \(\mu\)g mL\(^{-1}\) supina bluegrass treatments (SBT). Conversely, band decreases in the lipid region occurred for the lower concentrations of SBT. This suggests that SBT elicited dose-dependent lipid influences on HepG2 cells compared to the untreated control group. The occurrence of these contrasting results may be intimately linked to lipid peroxidation and apoptosis processes.

The lower concentrations of SBT displayed an overall decrease in HepG2 amide I/II protein band intensities (1700 to 1500 cm\(^{-1}\)). In contrast, the 500 \(\mu\)g mL\(^{-1}\) SBT displayed spectral qualities similar to the untreated control group except for the distinctive emergence of the \(~1640-1610\) cm\(^{-1}\) band. This band spike corresponds to the \(\beta\)-sheet region within protein secondary structures (Bourassa et al., 2010). Pathologies such as cancer have been shown to induce changes in the extent of hydrogen bonding among protein amide groups leading to conformational changes (Petibois & Deleris,
Therefore, the occurrence of treatment-induced alterations in the amide regions of carcinoma cells may be linked to the overall cancerous state of the cells/tissue.

Within the phosphate and nucleotide region (1500-1200 cm\(^{-1}\)), the lower SBT concentrations elicited HepG2 band spikes, while a decline in band intensities occurred for 500 µg mL\(^{-1}\) SBT. Liu et al., (2001) reported decreases in band intensities within the nucleic acid spectral region following the onset of apoptosis in leukemic cells. The band differentials for SBT within this spectral region may be intimately linked to HepG2 nucleotide modifications at lower SBT concentrations and the onset of HepG2 apoptosis at the higher SBT concentration.

Significant increases in HepG2 vibrational bands also occurred for the lower SBT concentrations within the polysaccharide/carbohydrate region (1190-900 cm\(^{-1}\)). Yet, the 500 µg mL\(^{-1}\) SBT elicited negligible influences on HepG2 cells in this spectral region. Strong glucose and glycogen bands occur in this carbohydrate region, so band shifts may reflect alterations in cellular energy metabolism. It has been reported that in organs such as the liver and cervix, glycogen levels are lower in cancer cells compared to the levels in corresponding normal cells (Sahu & Mordechai, 2005). Further investigations are needed to determine whether this glycogen-related event is relevant to our SBT difference spectra results, or even to \textit{in vitro} models in general.
Figure 2.5. Average FTIR differences of preprocessed zero order spectra for supina bluegrass treated HepG2 cells at A) 31 µg mL⁻¹ B) 125 µg mL⁻¹ C) 500 µg mL⁻¹ GAE. The contributions of the untreated control spectra have been subtracted from the supina bluegrass treatment (SBT) spectra at each concentration level.

Figure 2.6 illustrates the spectra for bermudagrass treatments (BT) at each concentration level minus the control. The BT difference spectra differed from SBT in that the two higher BT concentrations displayed the most qualitative similarities. Within the lipid region, slight increases in HepG2 lipid vibrational bands occurred for the higher BT concentrations, while declines in band intensities were apparent for the 31 µg mL⁻¹ BT. Difference spectra results for each BT concentration were relatively similar within the amide I/II region, except for one well-defined spectral inverse at ~1650 cm⁻¹. This inverse displayed a major band spike for the higher BT concentrations at ~1650 cm⁻¹.

The IR spectral band at ~1650 cm⁻¹ has been attributed to α-helix protein secondary structures (Bourassa et al., 2010; Kong & Yu, 2007). Giambattista et al., (2010) proposed that shifts within this region could serve as a ‘spectroscopic biomarker’ for structural disorder relating to apoptosis induction. The details surrounding this amide shift in bermudagrass treated HepG2 cells needs to be further investigated.
Within the phosphate and nucleotide region, the higher BT concentrations were qualitatively similar to the HepG2 untreated control group. Conversely, the difference spectra for the 31 µg mL⁻¹ BT displayed a drastic spike in band intensities in the ~1300-1200 cm⁻¹ range. This spectral region is comprised of protein (amide III) constituents and symmetrical stretching of phosphodiester groups associated with the DNA backbone (Kim et al., 2010). Within the polysaccharide/carbohydrate region, an interesting dose-dependent occurrence was also detected: 31 µg mL⁻¹ BT demonstrated a decrease in HepG2 band intensities; 125 µg mL⁻¹ BT displayed increases in band intensities; and 500 µg mL⁻¹ BT and the untreated control group demonstrated similar HepG2 band intensities. Considering the previous report by the authors that demonstrated HepG2 apoptosis induction at 500 µg mL⁻¹ BT, these results offer an interesting perspective into treatment- and dose-dependent apoptotic effects within liver cancer cells.

**Figure 2.6.** Average FTIR differences of preprocessed zero order spectra for bermudagrass treated HepG2 cells at A) 31 µg mL⁻¹ B) 125 µg mL⁻¹ C) 500 µg mL⁻¹ GAE. The contributions of the untreated control spectra have been subtracted from the bermudagrass treatment (BT) spectra at each concentration level.
Resveratrol’s bioavailability and accumulation in the liver has been well documented (Vitrac et al., 2003; Goldberg, Yan, & Soleas, 2003) in addition to resveratrol’s chemopreventive influence in vitro using liver cancer models (Sun et al., 2002; Delmas et al., 2000). While most of the resveratrol results presented in Figure 2.7 represent physiologically irrelevant doses, this study was more concerned with elucidating the reported biochemical mechanisms of resveratrol using FTIR spectroscopy. In contrast to the AGPE difference spectra, the concentration-dependent differences in response to HepG2 resveratrol treatments (RT) were predominately due to degrees of band intensity, rather than contrasting metabolic events.

Within the lipid region, the difference spectra for each of the RT concentrations displayed increases in HepG2 lipid bands— an event consistent with both of the 500 µg mL⁻¹ AGPE treatments. Resveratrol’s influence on the HepG2 amide I/II protein region was very similar to that of the bermudagrass treatments. Given the qualitative nature of FTIR difference spectra, it is not clear whether the spectral similarities between resveratrol and bermudagrass in the amide region suggest similar influences (or a lack thereof) as compared to the untreated HepG2 control group. What can be deducted from the amide results are the unique impacts on protein secondary structure displayed by supina bluegrass treated HepG2 cells.

While subtle band inconsistencies were exhibited between RT concentrations in the phosphate and nucleotide region, for the most part, the spectra were qualitatively similar to the untreated HepG2 controls. The same cannot be said for RT in the polysaccharide/carbohydrate spectral region. Glycogen vibrational bands have been reported to occur from ~1045-1015 cm⁻¹ in cancer cells (Takahashi et al., 1999; Kim et
al., 2010; Petibois & Deleris, 2006). Of the three RT concentrations, the 125 µg mL⁻¹ RT was the only concentration with significant influence (intensity decrease) on the aforementioned glycogen band. In contrast, the 500 µg mL⁻¹ RT displayed remarkable influence on nearly every spectral region (except glycogen) within the polysaccharide/carbohydrate window. These results indicate significant interaction with nucleotide backbones and/or carbohydrate polymers by resveratrol at excessive concentrations.

**Figure 2.7.** Average FTIR differences of preprocessed zero order spectra for resveratrol treated HepG2 cells at A) 31 µg mL⁻¹ B) 125 µg mL⁻¹ C) 500 µg mL⁻¹. The contributions of the untreated control spectra have been subtracted from the resveratrol treatment (RT) spectra at each concentration level.

The recent and rapid evolution in understanding the underlying molecular alterations associated with carcinogenesis offers the possibility of targeting specific malfunctioning molecules and pathways to attain more effective and *rational* cancer therapy (Neergheen et al., 2010). *Rational* cancer therapy approaches should involve natural products/chemicals that are highly accessible, affordable, and selective toward
specific carcinogenic conditions. A myriad of research over the past two decades has elucidated numerous underlying phenolic chemopreventive mechanisms, which range from inhibition/reversal of genotoxic alterations, suppression of proteases and cell proliferation, and modulation of apoptotic and signal transduction pathways (Chen & Kong, 2005; De Flora & Ferguson, 2005; Hwang et al., 2007). Specifically, resveratrol has been shown to induce apoptosis (Lee, Zhang, & Sanderson, 2008), delay cell entry to mitosis (Delmas et al., 2000), and modulate growth factor induced signaling cascades (De Ledinghen et al., 2001) in liver cancer studies.

Using the breadth of reports in the literature of resveratrol’s mechanistic influence on liver cancer, the cluster analyses and difference spectra results listed above present some intriguing insights into the therapeutic/preventive potential of AGPE. These preliminary FTIR results have shown mechanistically assorted chemopreventive potential for AGPE. While the 500 µg mL⁻¹ bermudagrass treatments demonstrated HepG2 metabolic influences similar to the known chemoprevention agent resveratrol, supina bluegrass treatments displayed distinct metabolic influences. Thus, supina bluegrass extracts could provide a mechanistic alternative and/or complement for liver cancer chemoprevention. Not only do these results reveal the therapeutic idiosyncrasies associated with AGPE, but they also affirm the subjective importance of understanding the associated idiosyncratic nature of natural products, profiles, and compounds for disease treatment options.

The ferulic acid-rich AGPE profiles may also confer potential selectivity toward liver cancer *in vivo*. Free ferulic acid (FA) has been shown to be readily absorbed *in vivo* and its bioavailability is not limited by intestinal and hepatic metabolism (Adam et al.,
Furthermore, the fractional availability of FA for tissues has been reported to be ~50% of the absorbed dose (Adam, et al., 2002), which is quite substantial considering that quercetin was only reported to have an available dose absorbance of ~9% (Crespy et al., 1999). In addition to the potential selectivity of nutraceutical amenity grasses, AGPE may also offer select advantages over phenolic compounds in isolation such as resveratrol. Cancer therapy and prevention approaches have recently adopted the use of total plant extracts due to the synergistic effects displayed by natural phytochemical cocktails, which may intervene at multiple carcinogenic stages (Neergheen et al., 2010). It is well documented in the literature that natural plant extracts contain numerous phenols in balanced proportions, which may demonstrate superior metabolic influences (Ray et al., 2004). A number of studies have reported the synergistic effects of phenolic compounds in concert (Olsson et al., 2004; Seeram et al., 2005; Ray et al., 2004).

After assimilating the chemoprevention potential and unique novel qualities associated with AGPE concentrations, an evaluation of the relationship between phenolic metabolic influences and specific carcinogenic events was undertaken. Thus, a visual assessment of second derivative spectra was employed to evaluate AGPE nutraceutical targets within the HepG2 metabolome compared to the chemopreventive influences of resveratrol. This second derivative analysis only assessed the 500 µg mL\(^{-1}\) GAE amenity grass treatments and the 125 µg mL\(^{-1}\) resveratrol treatments. While the 31 µg mL\(^{-1}\) resveratrol treatments represent a concentration (~135 µM) near physiological relevance (Vitrac et al., 2003), the cluster analysis and difference spectra results showed the influence of the 31 µg mL\(^{-1}\) treatments to be consistent to that of the 125 µg mL\(^{-1}\) resveratrol treatments. Therefore, a visual analysis of the 125 µg mL\(^{-1}\) resveratrol spectra
offered more pronounced metabolic shifts, while still maintaining a concentration level that could extrapolate to in vivo situations. As for the 500 µg mL\(^{-1}\) GAE supina bluegrass and bermudagrass treatments, these concentrations represented the greatest degree of HepG2 metabolic influence. Moreover, AGPE concentrations are most likely overstated due to the crude nature of the extracts (lack of purity) and the colorimetric standard curve-based concentration estimations. However, the limited characterization and the lack of purity associated with AGPE profiles make physiological relevance speculations difficult for this preliminary study. Additionally, increasing the purity of crude AGPE may also diminish their synergistic contributions.

The onset of carcinogenesis is generally associated with increases in free radical generation, which ultimately lead to the formation of lipid, protein, and DNA peroxidation products. Within the lipid region, a number of FTIR studies have established tissue-specific lipid biomarkers for cancer diagnostic purposes. Most of these studies investigated quantitative changes in oxidized lipid bands induced by carcinogenesis (Petibois & Deleris, 2006). The lipid region (~3100-2800 cm\(^{-1}\)) is composed of membrane lipids consisting of symmetric CH\(_2\) (~2924, 2850 cm\(^{-1}\)), asymmetric CH\(_3\) (~2958, 2873 cm\(^{-1}\)), and symmetric CH (~3010 cm\(^{-1}\)) vibrational bands (Kim et al., 2010; Petibois & Deleries, 2004). Gasparri & Muzio, (2003) reported an increase in the CH\(_2\)/CH\(_3\) ratio that correlated to the onset of apoptosis, but believed this event was related to cell growth and not specific to apoptosis. In contrast, Tsuzuki et al., (2004) showed that treatment induced apoptosis in tumor cells can occur via lipid peroxidation. This is concerning for this study considering the possibility of phenolic pro-oxidation. Because these conflicts of interest could be encountered within the
contexts of our experimental design, a quantitative lipid analysis was not pursued. Furthermore, as detailed in Figure 2.8, the qualitative similarities between the HepG2 treatment spectra in the lipid window suggest a lack of unique molecular targets in this spectral region.

**Figure 2.8.** Second derivative FTIR spectra in the lipid region obtained from treated and untreated HepG2 treatment averages; 500 µg mL⁻¹ GAE bermudagrass treatments (orange), 500 µg mL⁻¹ GAE supina bluegrass treatments (red), 125 µg mL⁻¹ resveratrol treatments (green), and untreated controls (blue).

The most sensitive mid IR region to protein secondary structural components was the amide I region (~1700-1600). A second derivative analyses of the amide I region allows for the identification of secondary structures present in proteins (Kong & Yu, 2007; Susi & Byler, 1983). Knowing that pathologies such as cancer have been shown to induce conformational changes in protein amide groups (Petibois & Deleris, 2006), numerous studies have used shifts in the amide I region to successfully evaluate the biopotentials of cancerous and healthy cell/tissues (Baker et al., 2008; Fabian et al., 2006; Lasch et al., 2004). **Figure 2.9** illustrates the amide I protein region for phenolic treated
and untreated HepG2 cells. A second derivative analysis of this region allows for the identification of α-helix (1645-1666 cm⁻¹), β-sheet (1613-1633 cm⁻¹), random coil (1633-1645 cm⁻¹), and β-turn (1670-1690 cm⁻¹) protein secondary structures.

In Figure 2.9, the most drastic shift in HepG2 protein secondary structure was detected for 500 µg mL⁻¹ supina bluegrass treatments, while the resveratrol and bermudagrass treatments displayed remarkable consistency throughout the amide region. This occurrence was also confirmed by the difference spectra results (Figure 2.5). In comparison to the untreated HepG2 controls, the 500 µg mL⁻¹ GAE supina bluegrass treated cells displayed a dramatic conformational shift toward β-sheet structures. On the contrary, the influences of bermudagrass (500 µg mL⁻¹ GAE) and resveratrol (125 µg mL⁻¹) were more related to increases in alpha helical structures (Figure 2.6 & 2.7), rather than to the direct induction of conformational alterations. Recent investigations in the literature have suggested that these conformational changes in the amide I region may be intimately related to apoptotic and necrotic processes.

Using heat stressed Jurkat cells, Giambattista et al., (2011) noted a large spike in the β-sheet band (corresponding decline in the α-helix band) and concluded that this band appearance was a necrotic biomarker. The Giambattista et al., (2011) paper stated that the β-sheet band spike in heat stressed cells was due to nucleotide carbonyl stretching and ring breathing modes. The appearance of the β-sheet band in the Giambattista paper corresponded to annexin–V flow cytometry results that demonstrated high levels of necrosis. In another study using HL60 cells, Gasparri & Muzio, (2003) identified necrotic cells by the appearance of a band shoulder at 1621 cm⁻¹. Though, Gasparri’s group reported that levels of α-helix and β-sheet structures were unaffected in both
apoptotic and necrotic cells. As seen in Figure 2.9, the distinct emergence of the β-sheet band (encompassing the ~1621 cm⁻¹ absorbance band) was evident in supina bluegrass (500 µg mL⁻¹ GAE) treated HepG2 cells. Upon further investigation into the appearance of this band in cellular systems, some inconsistencies were uncovered concerning the direct correlation between necrosis and β-sheet conformational shifts.

The activation of caspase proteases has been reported to initiate the apoptotic cascade (Seifried et al., 2007; Murugan et al., 2010; Logue & Martin, 2008). Within this apoptotic cascade, caspase-activated DNase (CAD) is a key apoptotic protein that degrades DNA through the action of caspases (Uegaki et al., 2005). Uegaki et al., (2005) was the first to demonstrate the spontaneous aggregation of CAD into amyloid fibrils in the presence of low pH and salt. Analyses of the CAD domain structure have revealed a protein conformation consisting of one α-helix and a five stranded β-sheet (Orengo, Jones, & Thornton, 1994). Moreover, structural analyses of CAD fibrils revealed not
only cross-β structures, but also an α-helix structure; a unique secondary structure for amyloid fibrils (Uegaki et al., 2005). These conformational properties are significant due to their possible relationship to the β-sheet shift in the 500 µg mL⁻¹ supina bluegrass treated HepG2 cells. In a previous study by the authors, HepG2 apoptosis, and not necrosis, was observed for 500 µg mL⁻¹ supina bluegrass treatments using the annexin-V flow cytometry assay. Thus, the appearance of the β-sheet band and the absence of α-helix band reductions may be explained by CAD activation in HepG2 cells treated with the elevated supina bluegrass concentration. It is postulated that the fibrillogenesis of CAD proteins may have been facilitated through the use of phosphate buffered saline (PBS) during the cell harvesting procedure and the subsequent dehydration of acidified (apoptotic characteristic) HepG2 samples before analysis.

The opposed hypothesis generated for this present study compared to the investigations by Uegaki et al., (2005) and Gasparri and Muzio, (2003), could be a result of differences in cell models. Likewise, the appearance of similar amide I conformational shifts could also reflect a progressed stage of cell death that may or may not be specific to apoptosis or necrosis. Although necrosis and apoptosis can result from the same oxidative stress, their death mechanisms are different (Halliwell, 1994). Dying cells are engaged in a cascade of molecular events that are reversible until a first irreversible process takes place (Galuzzi & Kroemer, 2009). Knowing this, it is reasonable that apoptosis and necrosis may share certain spectroscopic biomarkers depending on the various stages of cell death. Other recent studies have demonstrated the occurrence of protein unfolding events induced by polyphenolic ligand binding. In a FTIR study analyzing bovine serum albumin (BSA) unfolding, Bourassa et al, (2010)
showed that polyphenol binding induced a major decrease in \( \alpha \)-helix structure, which correlated to an increase in \( \beta \)-sheet structure. For our present study, the \( \beta \)-sheet spikes were not suspected to be a result of protein unfolding because decreases in \( \alpha \)-helix structure were not apparent.

Nucleic acids and phosphates also exhibit significant mid IR vibrational bands within biological samples (Benedetti et al., 1997). FTIR analyses enable the identification of carcinogenesis-associated conformational changes in nucleotide base and backbone structures (Malins et al., 2003). Moreover, the onset of apoptosis in biological samples has been shown to affect the IR band intensities of nucleotide structures. Apoptosis is marked by cell shrinkage, nuclear condensation, and DNA fragmentation (Murugan et al., 2010; Seifried et al., 2007; Mandelker, 2008). It has been proposed that chromatin condensation occurring during apoptosis may result in elevated DNA mid IR vibrational bands, thus resulting in only a partial visibility of the nucleic acid bands (Tobin et al., 2004; Gasparri & Muzio, 2003). Many studies have reported band intensity decreases within the nucleic acid region following apoptosis (Mantsch & Chapman, 1996; Giambattista et al., 2011). Furthermore, other studies have reported increases in nucleic acid band intensities associated with necrotic cells (Chiriboga et al., 1998).

The first nucleotide shift of interest occurred near the amide region at \( \sim 1715 \text{ cm}^{-1} \) and can be observed within the second derivative spectra presented in Figure 2.9. Malins et al., (2003) attributed the \( \sim 1715 \text{ band} \) to thymine residues (\( \text{C}_4=\text{O} \) stretching) associated with DNA bases. According to Figure 2.9, the 500 \( \mu \text{g mL}^{-1} \) supina bluegrass treatments elicited a distinct shift in the thymine band. In the nucleotide region presented in Figure 2.10A, a similar shift for all treatments occurred at \( \sim 1400 \text{ cm}^{-1} \) band. Giambattista et al,
(2011) reported that ~1400 cm\(^{-1}\) band shifts may be linked to alterations in the methylation state of amino acid side chains and fatty acids that corresponds to the progression of carcinogenesis. The spectral shift at ~1237 cm\(^{-1}\) for supina bluegrass treated HepG2, which is presented in Figure 2.10B, is reportedly associated with conformational changes in the DNA sugar phosphate (PO\(_2^-\)) backbone (Malins et al., 2004). Malins’ group suggested that band shifts toward higher wavelengths within this phosphate absorbance peak were indicative of increasing tumor aggression in prostate tissues. Therefore, the ~1237 cm\(^{-1}\) shift in the FTIR spectrum of supina bluegrass treated HepG2 cells may be linked to a decrease in carcinogenic aggression.

In general, all of the treatments exerted various influences within the nucleic acid and phosphate spectral region. Yet, the most drastic and distinct metabolic influences occurred for supina bluegrass treatments. The difference spectra results presented in Figure 2.11 comparatively detail the overall decreases in band intensities for all treatments in the ~1500-1200 cm\(^{-1}\) spectral window. As mentioned earlier, it has been reported that nucleic acid vibrational bands decline in intensity following apoptosis. As compared to the untreated HepG2 controls, the most severe decrease in nucleic acid/phosphate vibrational bands resulted in response to the 500 µg mL\(^{-1}\) GAE supina bluegrass treatments- another factor supporting our hypothesis that the β-sheet shift of the SBT treated cells in the amide region was not necessarily correlated to the onset of necrosis.
Figure 2.10. Second derivative FTIR spectra in the nucleic acid and phosphate region obtained from treated and untreated HepG2 treatment averages; 500 µg mL⁻¹ GAE bermudagrass treatments (orange), 500 µg mL⁻¹ GAE supina bluegrass treatments (red), 125 µg mL⁻¹ resveratrol treatments (green), and untreated controls (blue).
Figure 2.11. Average FTIR differences of preprocessed zero order spectra for treated HepG2 cells at 500 µg mL\(^{-1}\) GAE bermudagrass treatments (orange), 500 µg mL\(^{-1}\) GAE supina bluegrass treatments (red), 125 µg mL\(^{-1}\) resveratrol treatments (green). The contributions of the untreated control spectra have been subtracted from each treatment spectra and the results are presented in the nucleic acid and phosphate spectral window (~1500-1200 cm\(^{-1}\)).

A number of potential cancer biomarkers in the polysaccharide and carbohydrate regions (~1200-900 cm\(^{-1}\)) have been reported in the literature. In Figure 2.12A, band alterations at ~1120 cm\(^{-1}\) were visible for all HepG2 treatments. According to Giambattista et al, 2011, the ~1120 cm\(^{-1}\) vibrational peak is attributed to lactic acid. Increases in cellular lactic acid production (Warburg effect) are linked to overactive glycolysis, which can be associated with increased hypoxia in cancer cells (Weljie & Jirik, 2010). The difference spectra presented in Figure 2.13 allows for a comparative analysis of HepG2 lactic acid concentrations among treatments. At ~1120 cm\(^{-1}\), AGPE had negligible effects on lactic acid production, while the 125 µg mL\(^{-1}\) resveratrol treatments demonstrated a substantial decrease in the lactic acid band. Hypoxia in cancer cells has been proposed to promote increases in cancer aggression and the onset of hypoxia has been shown to modulate a cellular shift away from oxidative...
phosphorylation toward glycolysis (i.e. Warburg effect: increases in lactic acid production) (Weljie & Jirik, 2010).

Another band shift was detected at ~1088 cm\(^{-1}\) (**Figure 2.12A**). This vibrational peak is associated with the symmetric phosphate bonds (PO\(_2\)) of DNA backbone components (Malins et al., 2003). Each of the treatments induced a similar phosphate shift toward ~1090 cm\(^{-1}\). Additionally, the difference spectra results in **Figure 2.13** once again illustrated substantially less band intensities for resveratrol treated cells. The absorption bands < ~1050 cm\(^{-1}\) are primarily attributed to ribose phosphate main chain vibrations (Malins et al., 2004) and glycogen/glucose bands (Kim et al., 2010; Takahashi et al., 1999). As shown by **Figure 2.12B**, all treatments demonstrated considerable influence on HepG2 energy metabolism and nucleotide backbone components.

According to the difference spectra results in **Figure 2.13**, the spectral trends were once again consistent for resveratrol and bermudagrass treatments, while a spectral inverse was apparent from ~1040-980 cm\(^{-1}\) for supina bluegrass treatments. Malins *et al.*, reported that the ~1020 cm\(^{-1}\) band is attributed to ribose phosphate main chain vibrations.
Figure 2.10. Second derivative FTIR spectra of the polysaccharide and carbohydrate and region (\(~1200-900~\text{cm}^{-1}\)) obtained from treated and untreated HepG2 treatment averages; 500 \(\mu g~\text{mL}^{-1}\) GAE bermudagrass treatments (orange), 500 \(\mu g~\text{mL}^{-1}\) GAE supina bluegrass treatments (red), 125 \(\mu g~\text{mL}^{-1}\) resveratrol treatments (green), and untreated controls (blue).
Figure 2.11. Average FTIR differences of preprocessed zero order spectra for treated HepG2 cells at 500 µg mL\(^{-1}\) GAE bermudagrass treatments (orange), 500 µg mL\(^{-1}\) GAE supina bluegrass treatments (red), 125 µg mL\(^{-1}\) resveratrol treatments (green). The contributions of the untreated control spectra have been subtracted from each treatment spectra and the results are presented in the carbohydrate spectral window (~1200-900 cm\(^{-1}\)).

In conclusion, evidence has been presented to show the effects of crude amenity grass extracts on the biochemical fingerprint of HepG2 liver cancer cells \textit{in vitro}. These influences were evaluated for chemopreventive and chemotherapeutic biomarkers as compared to the known chemopreventive phenolic compound, resveratrol. In addition to the therapeutic appeal associated with amenity grass nutraceuticals in terms of (i) elevated absorption and tissue selectivity, (ii) low cost to patients/consumers, (iii) global accessibility, (iv) inconsequential impacts on the global food supply; amenity grass nutraceuticals offer additional cancer specific advantages. For example, the results presented above outline the novel qualities associated with the molecular targets of specific amenity grass species. This could prove to be a key attribute for cheap and effective personalized medicines. Additionally, the synergistic and interactive effects of natural phytochemical cocktails can provide combination therapies/prevention programs...
that simultaneously influence multiple molecular targets. Furthermore, natural extracts may be able to achieve treatment efficacy beyond the reach of single compound-based drug by reducing toxicity potentials as lower doses of individual compounds within a cocktail can be administered (Neergheen et al., 2010).

Our results showed that FTIR spectroscopy is a viable tool for discriminating between molecular targets within cancer cells. To our knowledge this is the first study to use FTIR spectroscopy to comparatively investigate the underlying mechanisms of action associated with natural chemopreventive agents. Our results clearly demonstrated the nutraceutical properties of supina bluegrass and bermudagrass. The molecular targets of bermudagrass (125 and 500 µg mL⁻¹ GAE) were most similar to the documented impacts of resveratrol (31 and 125 µg mL⁻¹). In contrast, distinct molecular targets for supina bluegrass treatments (500 µg mL⁻¹ GAE) were exhibited—especially on protein secondary structure. Both bermudagrass and supina bluegrass treatments also affected nucleotide base and backbone structures. According to Turner, (2001), the corresponding conformational changes would be expected to influence gene expression and the fidelity of transcription and replication. However, it was not believed that the unique influences reported in supina bluegrass treated HepG2 cells were linked to necrosis. Previous work by the authors demonstrated negligible HepG2 necrosis (annexin V assay) and cytotoxicity (MTT assay) associated with supina bluegrass treatments within the same concentration range. It is conceivable that some of the unique shifts within the supina bluegrass spectra represent a progressed cellular stage of programmed dismantling (i.e. apoptosis).
Future investigations into the chemopreventive potential of amenity grasses should begin to address the physiological relevance of doses for \textit{in vivo} testing and \textit{in vivo} absorption and distribution. Finally, additional studies are needed concerning the ability of amenity grasses to selectively target carcinogenesis without adversely impacting healthy tissues. According to Weljie & Jirik, (2010), “The ability to preferentially target cancer cells without incurring significant toxicity to normal cells represents one of the ‘Holy Grails’ of cancer therapeutics.” Natural phytochemical cocktails may be one of the most viable possibilities for realizing this highly sought-after therapeutic quality.
LITERATURE CITED


