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Chemical components of Fraxinus americana and Fraxinus pennsylvanica of northern Alabama

Twaskia S. Johnson

CHEMICAL COMPONENTS OF *FRAXINUS AMERICANA* AND *FRAXINUS PENNSYLVANICA* OF NORTHERN ALABAMA

by

TWASKIA S. JOHNSON

A THESIS

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We, the undersigned members of the Graduate Faculty of the University of Alabama in Huntsville, certify that we advised and/or supervised the candidate on the work described in this thesis. We further certify that we have reviewed the thesis manuscript and approved it in partial fulfillment of the requirements of the degree of Master of Science in Chemistry.

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ABSTRACT
The School of Graduate Studies
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Name of Candidate Twaskia S. Johnson

Title Chemical Components of *Fraxinus Americana and Fraxinus Pennslyvanica* Species of Northern Alabama

The scientific names for these two species of trees are *Fraxinus americana* and *Fraxinus pennsylvanica* also known commonly as white ash and green ash, respectively. The main components of the *Fraxinus* species W5 (female) and M3 (male) were isolated using preparative-high performance liquid chromatography (HPLC) and the structures identified by a Nuclear Magnetic Resonance (500MHz) instrument. The compound isolated in both species of *Fraxinus* was found to be syringin, a phenylpropanoid glycoside. Assays were conducted to measure the polyphenolic content, free radical scavenging capabilities, and the inhibition of cruzain in each sample of the two *Fraxinus* species. The crude extracts samples taken from each species did not exhibit 50% inhibition consistently over a span of 5 months. There was no correlation between the polyphenolic concentration, DPPH free radical scavenging activity, and the inhibition of cruzain. The HPLC profiling between male and female *Fraxinus* species was qualitatively similar but showed no apparent differences between the sexes in the five month period.

Abstract approval: Committee Chair

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Graduate Dean

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Chapter I

INTRODUCTION

1.1 *Fraxinus* Species

The *Fraxinus* species of North America are found in most habitats, which include river bottoms, mountainous regions, and drained upland sites. The scientific names for the two species of trees in this thesis are *Fraxinus americana* and *Fraxinus pennsylvanica* also known commonly as white ash and green ash, respectively. These two species of trees are in the olive family (Oleaceae). The trees grow rapidly reaching a height of 40 to 130 feet. The trees are not found to be very desirable around street corners or parks because of diseases and insects associated with this particular tree. The *Fraxinus* species of northern Alabama is decidous. Decidous trees are seasonal meaning once the leaves and the fruit of the trees reach full maturity they begin to fall off. *Fraxinus americana* and *Fraxinus pennslyvanica* have short life spans. The average life span for both species of *Fraxinus* is between 35-70 years.[1] It is rare to find an ash tree over 100 years old due to disease caused by the emerald ash borer beetles (*Agrilus planipenis or Argilus marcopoli*) from Asia. White ash is a good tree for large open areas, but too large for home landscapes unless providing shade is the primary objective. This species flowers in April-May, the male first, before appearance of the leaves; fruits and flowers during August-October, the seeds disperse during September-November. The pollen is already

airborne during the 7-10 days when the female is receptive. [2] Ash does not tolerate soil compaction or construction injury due to extensive roots. This species flourishes well in good soil and is probably not a good choice for poor, urban soils. It is not tolerant of heavy clay. The wood of white ash is valued for its strength, hardness, heavyweight, and elasticity. The white ash trees thrive well in wet lands and lowlands. It is the most common ash tree found in North America. The range of this particular species ranges from Alberta, Canada to Texas, Florida, Alabama, Georgia, and Tennessee. [2] Samaras flourish in the spring time providing a food source for the surrounding wildlife. White ash and green ash trees thrive in lowlands and wetlands; the ash has a pale gray diamond shape bark and green conical leaf buds as shown in Figure 1.1. The *Fraxinus pennslyvanica* trees thrive well in the wetlands and stream banks of northern Alabama. The bark of the branches of both species is very smooth with notches. The branches are illustrated in Figures 1.2 and 1.3. The samaras from both species of *Fraxinus* are green and the length of the samaras are two to seven centimeters long (see Figures 1.4 and 1.5). The fruit will reach mautrity when the color changes from green to brown. The key components of white and green ash trees of the leaves and bark are coumarone, flavonoids, sugars, and tannins.[3] It is readily available in many areas within its hardiness range. The diagram in Figure 1.6 shows the potential planting range for the two species of *Fraxinus* trees in North America.

Figure 1.1. Bark of *Fraxinus americana* (left) and *F. pennsylvanica* (right).

Figure 1.2. Branch and Leaflets from *Fraxinus americana.*

Figure 1.3. The Branch and Leaflets from *Fraxinus pennslyvanica.*

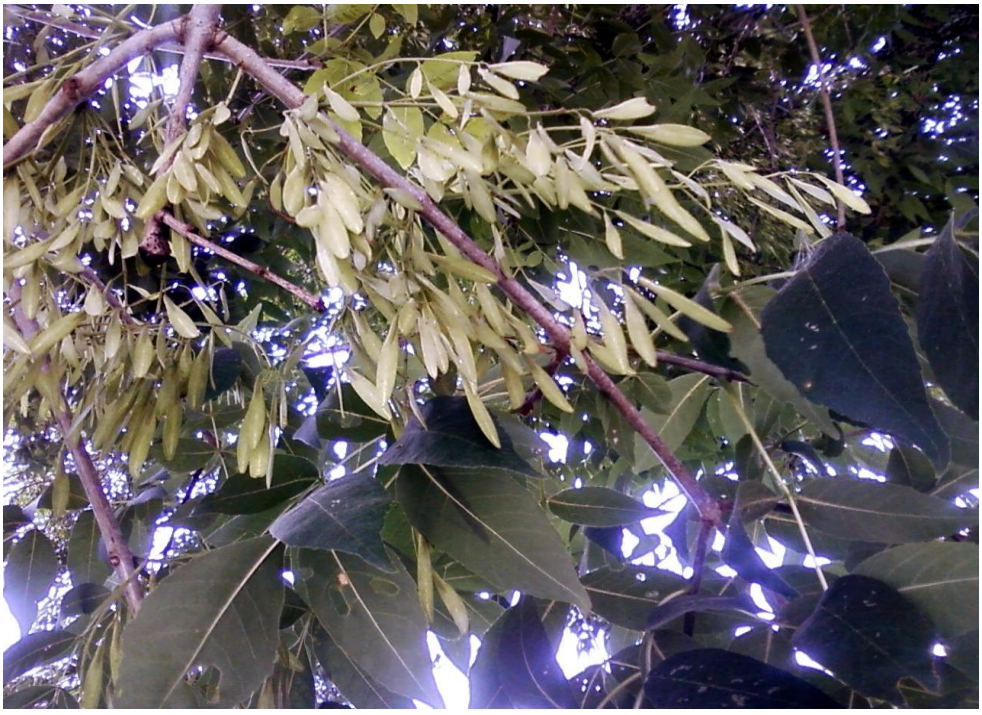


Figure 1.4. Samaras from *Fraxinus pennslyvanica*.

Figure 1.5. Samaras from *Fraxinus americana.*

1.2 Medicinal Usage

Native Americans appreciated its usefulness for tools and implements; it is used extensively today for tool handles. White ash was used by Native Americans for a variety of medicinal purposes such as arthritis, gout, bladder complaints, a laxative, a diuretic, fever, and for worm infections.[3] The leaves of the ash trees are used for lower leg ulcers and wounds. The bark is used as a tonic occasionally taken to reduce fever.

Fraxinus ornus and *F. excelsior* are small trees ranging from 15 to 16 feet which are indigenous to southern Europe, which extends to the southern borders of the Alps and as far as European Turkey also called Manna Ash. The Manna is a black sugary sap that is used for pharmaceutical purposes. They are cultivated in Italy for their extremely high concentration of sap. Manna is the sap generated from the slit bark of the trunk and

branches, and then dried. The manna is used as a laxative for children and pregnant women. It is also used for ailments where an easier elimination and softer stool is desirable, for example, anal fissures, hemorrhoids, and post-rectal or anal surgery. [3]

1.3 Experimental Procedure

Bark samples were taken from *Fraxinus americana* and *Fraxinus pennslyvanica* from different locations in the surrounding Huntsville area. Extraction procedures for each sample were performed each month to determine whether or not a chemical change had occurred in properties during a five month period from June to October. The analysis will determine whether there is a chemical difference in polyphenolics between male and female trees, the chemical difference between Monte Sano Mountain and surrounding Huntsville areas. We want to identify any chemical changes during the season. The over all goals are to analyze total polyphenolics and radical inhibition. HPLC instrument will be used to isolate and collect the components of both species. HPLC profiles will be taken each month for each species of *Fraxinus*.

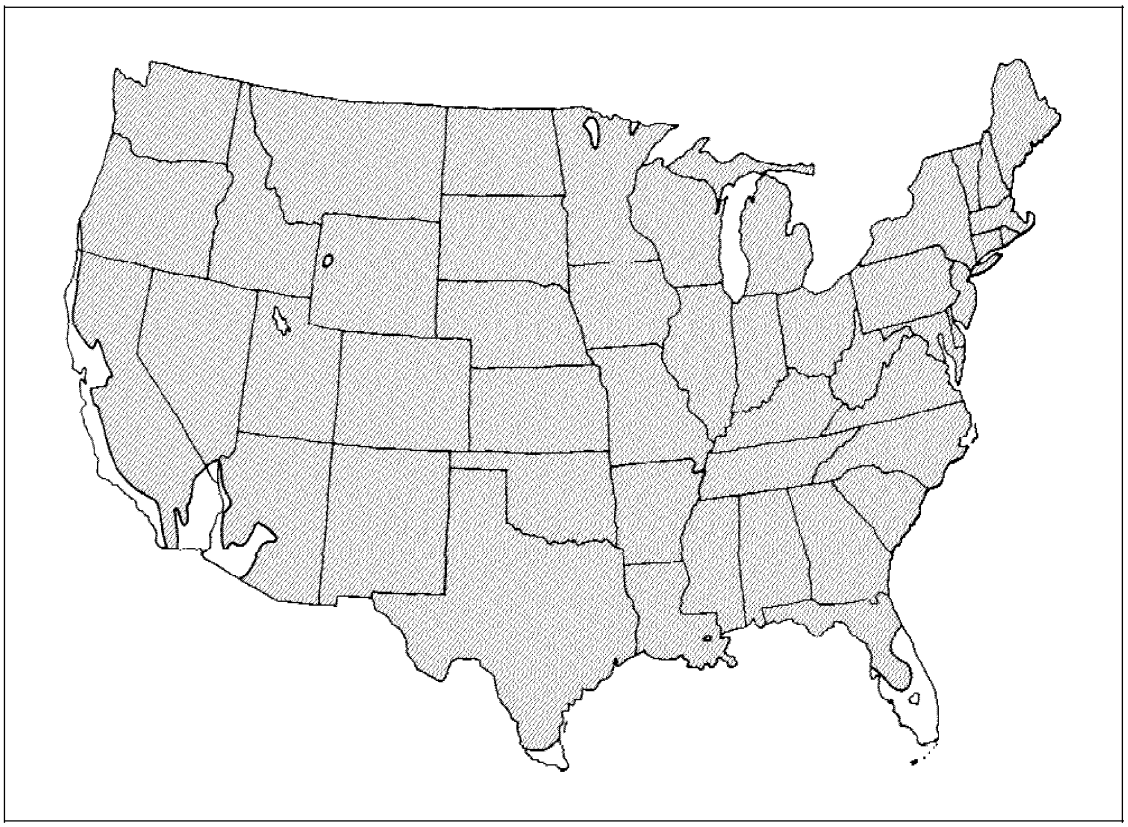


Figure 1.6. Shaded Area Represents the Potential Planting Range of White and Green Ash. [4]

1.4 Spectra Max M2

Spectra Max M2 is a multi detection micro plate reader. It is able to read up to 384-well micro plates. The Spectra Max M2 reader provides for an easier conversion and optimization of very low throughput and medium to high throughput assays. It is faster, gives precise results, and saves on reagents compared to spectrophotometric assays using a cuvette. Absorbance and florescence intensity assays can be run by issuing a single read command. Fluorescence spectroscopy is a zero background form of analysis that

utilizes the emission properties of specific molecules rather than the intensity to absorb certain wavelenghts of light. There is an energy gap between the ground and the excited state. This is achieved through the absorption of a photon. The excited species goes through a number of relaxation pathways through excess energy being loss by emitting a photon.[5] Absorbance is the amount of light that is absorbed by the chemical sample, which is calculated by the transmittance.[5] Transmittance is calculated by taking the ratio of the intensity (amount) of light leaving the chemical sample (I) to the intensity (amount) of light entering the chemical sample (I_o). There is no need for using filters with this instrument; instead it employs two scanning monochromators to determine optimal excitation and emission setting.

1.5 Natural Product Drug Discovery

Exploitations of microbes, plants, and marine organisms have been the primary focus for finding new antioxidants through natural drug discovery and therapeutics. In addition to finding new antioxidants, new strategies need to be implemented in order to extract biological active agents from natural resources that continue to show promise. [6] Nature has provided the sources, the templates, or the prototypes for the majority of the drugs in use. This is owed in large part to their structural complexity and clinical specificity. However, only a small percentage of natural sources have been tested for potential therapeutic utility. New drug discovery strategies are now in place. Phytochemical natural products have also been introduced as anti-diabetic drugs, hormone antagonists, anticancer drugs, and agricultural and pharmaceuticals agents. [3]

The bark extracts from white and green ash trees produce secondary metabolites that affect cell growth. [6]

There is a growing need for new useful compounds in order to provide relief from degenerative illnesses. My research has been based on the hypothesis that trees native to North America have evolved libraries of polyphenolic compounds such as flavonoids, acetogenins, and tannins that exhibit antioxidant and free-radical scavenging activities, and that these materials can provide treatment for inflammatory illnesses as well as prevent age-related diseases such as cancers and cardiovascular diseases. The next step is to study the properties of flavonoids. Some agents have different defense mechanisms, similar to the human immune system. However, without the self preservation of our environment we will lose some of our most valuable assets. The cure for breast cancer, AIDS, and Alzheimer's may be out there, but without preservation of these critical habitats, we will lose some of our most useful species before we realize their medicinal value. To combat some of these issues, methods of cultivation have been put in place by combining production with traditional agricultural practices that lead to achievement of an appropriate balance in agro-forestry, therefore providing economic income to those communities that are impoverished.

There are certain criteria for the type of trees that are being selected for study. Several steps have been rationalized such as (a) trees from unique environmental settings, especially those with an unusual biology, and possessing novel strategies for survival are seriously under studied and (b) tress that have ethnobotanical history used by peoples that are related to the specific uses or applications of interest are selected for study.[6]

These plants are selected by direct contact with local people or via local literature; plants growing in areas of great biodiversity follow the prospect of housing great chemo diversity.[6] Proanthocyanidins or condensed tannins are polymeric flavonoid compounds widely distributed in the plant kingdom.[6] These compounds have been reported to demonstrate antibacterial, antiviral, anti-carcinogenic, anti-inflammatory, anti-allergic, and vasodilator activities. They have been found to inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, and to affect enzyme systems. [7, 8]

1.6 *Fraxinus* Phytochemistry

Chemical components have been isolated from the *Fraxinus* species which includes flavonoids, coumarone, secoiridoids, and phenylethanoids. The secoiridoids occur mainly in the form of glycosides and esters of hydroxyphenylethyl alcohols. Ligans, flavonoids, and simple phenolic compounds are also common, but they appear to have more limited distributions. [9] Extracts and metabolites have been found to possess anti-inflammatory, immunomodulatory, antimicrobial, antioxidative, skin regenerating, liver protecting, diuretic, and antiallergic activities. [9]

1.7 Tannins

Higher plants produce a variety of secondary compounds, including alkaloids, terpenes, and phenolics, and it is generally thought that these compounds apparently do not function in primary metabolism. Tannin is a name for a group of polymeric phenolic substances capable of browning or tanning leather or the precipitation of gelatin from solution. There are two groups of tannins: condensed and hydrolyzable tannins. The hydrolyzable tannins are based on gallic acid and the condensed tannins are more

numerous found in most every plant part, which are derived from flavonoid monomers. Tannins may be formed by condensations of flavan derivatives, which may have been transported to tissues of woody plants. Quinones are aromatic rings with two ketone substitutions. These compounds are subject to being colored causing a reaction to occur when a fruit or vegetable has been cut and left exposed to the elements. It takes on a brown coloration. Quinones and tannins are also responsible for plant pigmentation. The consumption of beverages containing tannins can either cure or prevent certain ills. Polyphenols, especially tannins, are well known for their ability to chelate heavy metals such as Fe^{3+}, and recently they have received much attention as potential antioxidants. [10] Teas and wines enriched in tannins are recommended as natural antioxidants in the human diet. Interactions of polyphenols with heavy metals can involve chelating, anti-oxidative activity against active oxygen species caused by heavy metals. Only a few reports described the action of tannins in the growing plants on heavy metal chelating or protection against active oxygen species caused by the heavy metal stress.[11] The tannin-rich plants, which are tolerant to excess Mn, are protected by the direct chelating of Mn by tannins and the typical brown spots on leaves exposed to Mn are actually caused by tannin oxidation.[12]An important aspect of phenols in plants is their role as lignin precursors, via their polymerization under natural lignification processes.[13] Quinones are known for providing a source of stable free radicals and are known to complex irreversibly with nucleophilic amino acids in proteins often leading to denaturing and the precipitation of the protein.

1.8 Free Radicals

Free radicals are any chemical species capable of independent existence possessing one or more unpaired electrons. Free radicals are formed from molecules via the breakage of a chemical bond such that each fragment keeps one electron (Free radicals may also be formed by collision of the non radical species by a reaction between a radical and a molecule, which must then result in a radical since the total number of electrons is odd), or by cleavage of a radical to give another radical. [14, 15, 16] Reactions result from the instability of the first formed radicals. The radicals may completely decompose or rearrange before reaction with other molecules or radicals present. The radical attacks a univalent atom, usually a terminal halogen or hydrogen in an abstraction reaction to give rise to a new radical. The problem with free radicals is that they have the ability to cause mutations, accelerate the aging process, as well as promote lesions of the tissues, thus disturbing the balance of the organism. Free radicals can be overproduced or the natural antioxidant defense weakened, first resulting in oxidative stress, and then leading to disorder continues to be the major cause of premature death worldwide. Oxidation of human low-density lipoproteins is considered an early step in the progression and eventual development of atherosclerosis, one of the leading causes to cardiovascular diseases. During the last few decades, research data have prompted a passionate debate as to whether oxidation, or specifically, oxidative stress mediated by free radicals, reactive oxygen species, or reactive nitrogen species, is a primary or secondary cause of many chronic diseases. As a result, scientific resources have focused to a large extent of the role that antioxidants could play to delay or prevent oxidative stress and consequently the incidence of chronic disorders. [16] Everyday people come

into contact with free radicals that are present in the air and produced in the human body. However, the harmful action of the free radicals can be blocked by substances known as free radical scavengers, which detoxify the organism. [17] The antioxidants reduce the risk for chronic diseases including cancer and heart diseases. Antioxidants occur naturally in food sources such as whole grains, fruits, and vegetables. The main characteristic of antioxidants is the ability to trap free radicals.[11] Free radicals have been known to oxidize proteins, lipids or DNA causing degenerative diseases. Antioxidant compounds like polyphenols, phenolic acids, and flavonoids scavenge free radicals such as peroxide, hydroperoxide, or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.[11] DPPH or 2,2 diphenyl-1-picryl hydrazyl is a stable free radical, which is violet in color, and has proven to be very useful in the determination of antioxidant properties of amines, phenols, or natural products; which also includes medicinal drugs and plant extracts.

1.9 Defensive Mechanism Prevention

There is a balance in the stomach between the aggressive digestive capabilities of acid plus pepsin and the mucosal barrier.[18] A plant-herbivore interaction has focused on the inhibitory effects of condensed tannins on protein and fiber digestion in mammals. The defensive nature of tannins as digestion inhibitors or toxins is dependent on the molecular characteristics of the tannins as it interacts with the physiological capability.[19] Tannins have strong inhibitory digestive capabilities. They act within the stomach of the animal by binding with the substrate to be digested usually by the proteins, carbohydrates, lipids, and also by inhibiting digestive enzymes. Toxins on the other

hand, interact with fundamental biochemical processes in cells.[18] Structural differences in condensed tannins vary in their effectiveness to deter browsers. Herbivores that consume tannin-rich shrubs and trees are known to produce proline-rich proteins in their saliva.[19] It was thought that over generations those ruminant animals have evolved and no longer need a defense mechanism for the inhibitory factors caused by enriched tannins. The exposure to this type of inhibiting plants controlled the metabolic rate of the animal. Because of the astringent properties of tannins, the herbivores intake of food is a lot less.[19] There are many factors that may influence the extent of digestive enzymes inhibition by tannins: the amount of protein in the diet, related amounts of various enzymes in the diet and the order in which they are encountered, formation of tannin-protein complexes prior to and following ingestion, and how various enzymes are affected by pH, type of tannin, and species and age of the animal.[19] Because tannins have such a wide array of affects on herbivores, it difficult to predict with any certainty how a particular tannin-containing forage will affect an animal without first understanding the characteristics of tannins. Several behavioral adaptations and several physiological mechanisms are available to browsers and grazers for reducing the activity of plant secondary compounds.[18] They include the following: formation of a less reactive complex, addition of functional groups, conjugation to change solubility, and alteration of metabolic rate. In a ruminant animal, the first line of defense is in the gut and the wall of the animal. If the first line of defense fails, then it will be absorbed and then transported to the liver. The toxic effects of tannins and other plant secondary compounds may be inactivated by the formation of non covalent complexes with other compounds that may be in the gut. The resulting complex must be less reactive or less

readily absorbed across the gut wall than the uncomplexed secondary compounds for this mechanism to be effective. Because of the reduced absorption, the complex may then be excreted in the feces of the animal. Although complex formation may be effective in the prevention of tannin absorption, reducing toxicity, it is also an anti-nutritional when tannins bind with dietary protein, forming precipitates, which then make dietary protein unavailable to the herbivores. Tannins are known to form soluble and insoluble binding complexes. The salivary proline-rich proteins produced by many browsers are considered to be a defensive strategy against these secondary metabolites. The tannin protein complex is synthesized in the following animals: deer, moose, beavers, bears and humans.[18] There are some environmental factors that may contribute the inhibition of tannins like pH, temperatures, and solution polarities are known to affect chemical reactivity. If an herbivore can alter the reactivity of tannins by modifying conditions in the gut, then the toxic or anti nutritional properties of these secondary plant compounds may be inactivated.[20] The pH is important because it governs the formation of tannin-protein complexes. Binding is particularly high at the iso-electric pH of the protein, and is much less strong at a high pH, where the phenolic groups of the tannins are ionized.

1.10 Coumarins

Coumarins are naturally occurring as benzopyrene derivatives.[21] In previous research, numerous compounds of coumarin derivatives with biological activity have been investigated.[21] The coumarins have been recognized to possess anti-inflammatory, antioxidant, anti-allergic, and anti-carcinogenic activities.[21] The hydroxycoumarins are typical phenolic compounds and therefore act as potent metal chelators and free radical

scavengers.[22] Coumarins are insoluble in water; however, 4-hydroxy substitution confers weakly acidic properties to the molecule that make it water soluble under slightly alkaline conditions. Coumarins are competitive inhibitors of vitamin K in the biosynthesis of prothrombin. [23] *F.ornus* is rich in hydroxycoumarins. Esculin, esculetin, fraxin, and fraxetin are the main components of the bark extract shown in Figure 1.7. Esculetin (6,7-dihydroxycoumarin) and diacetate exhibited a marked inhibitory effect on Newcastle diseases replication in cell cultures at concentrations of 36μM and 62μM respectively. Esculetin, fraxin, and fraxetin are mainly responsible for the antimicrobial properties of *F. ornus* bark extracts.

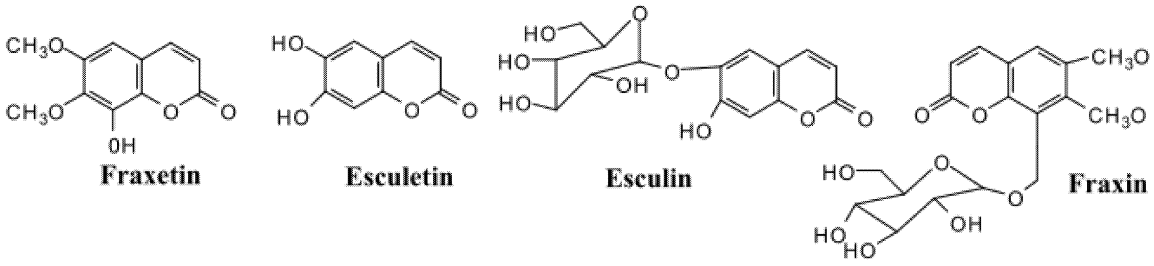


Figure 1.7. Main Components Found in *F.ornus.*

1.11 Secoiridoids

Secoirdoids are phytochemical components in the Oleaceae family. Components found in the *Fraxinus* species are 7-epiloganin and epiloganic acids are the intermediates in the biosynthesis of most of the oleoside-type secoiridoids present in *Fraxinus.*[24] Iriodoid glycosides have a very strong bitter taste. The glycoside is hydrolyzable. The glycoside changes color from being absolutely colorless to being blue upon hydrolysis with an enzyme or an acidic solution. Secoiridoids glycosides are formed by the rupture of the cyclopentane ring of the iridoids.[25] Secoiriodoids also show major activity on the

excretion of gastric juices and bile without any toxicity.[25] The secoiriodoids also play a major role as a crude drug for stomachaches.

1.12 Verbacoside

Verbacoside is a caffeoyl glycoside, also known as acetoside or kusaginin. [26] Verbacoside is found in the Oleaceae Family. Verbacoside is a hydroxycinnamic derivative found in olives, which exhibits cytotoxic and cytostatic activity against skin tumors by inhibiting protein kinase C activity. This compound has been used to repair brain oxidative damage caused by heroin consumption. [26]

1.13 Tyrosol

Tyrosol is a biophenol product of the olive fruit. This compound also exhibits cytostatic activity against McCoy cells and has the potential to be an active anti-tumoral compound.[27] Tyrosol exhibits anti-arrhythmic and cardio-protective actions by preventing stressor-induced change at the level of cyclic nucleotides in the myocardium.

1.14 Oleuropein

Oleuropein is another biophenol, which is one of the most abundant bio-phenol products found in olive leaves. It is found throughout the tree including all components such as the bark, peel, and the pulp. This secoiridiod prevents cardiac diseases by avoiding lipid oxidation acting on coronary dilation, also as antiarrhythmic action improves lipid metabolism and helps reduce obesity problems, protects enzymes, and hypertensive cell death in cancer patients. [27]

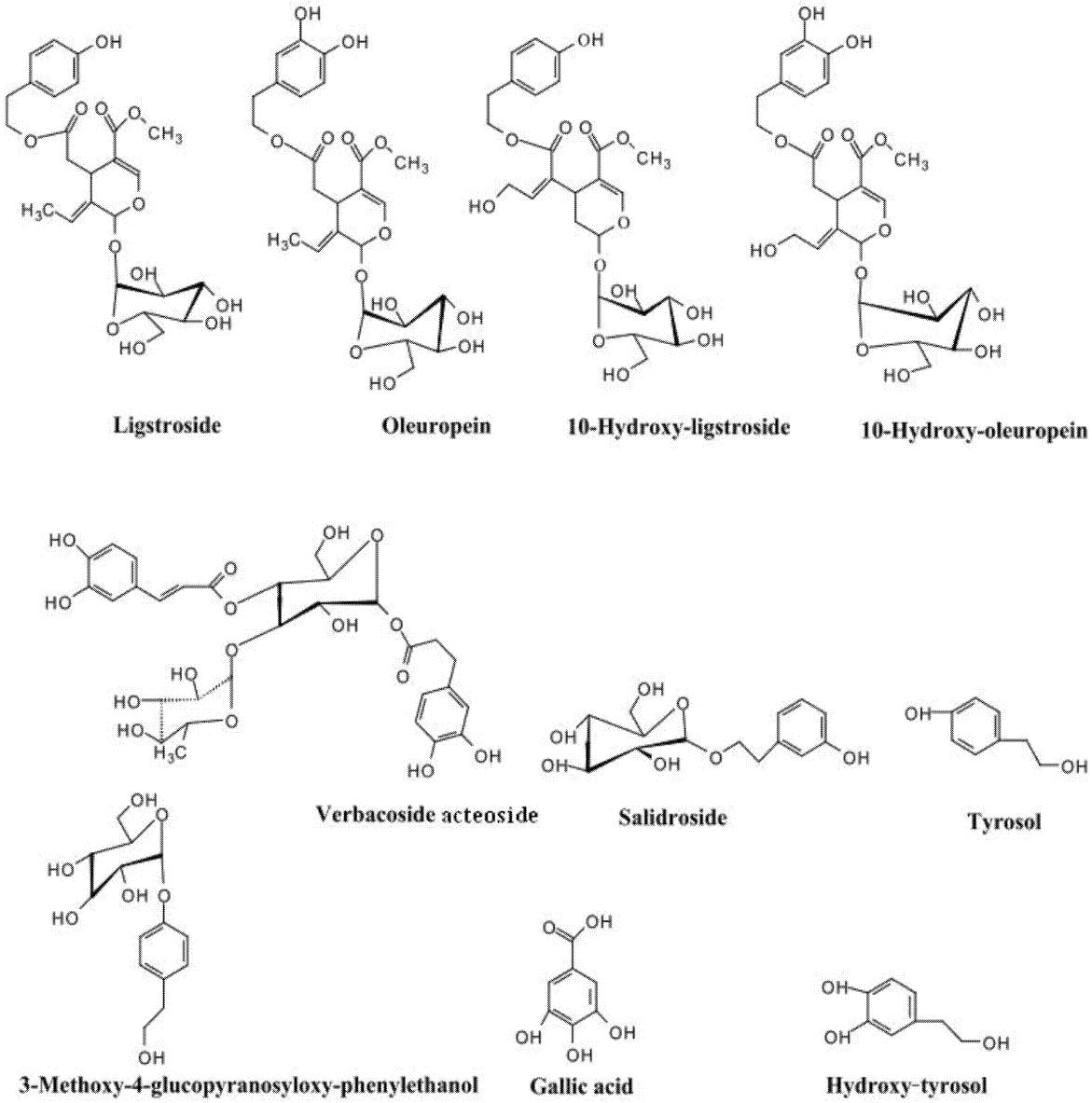


Figure 1.8. Chemical Components of White and Green Ash Species.

Chapter II

METHODS AND MATERIALS

2.1 Bark Extraction

Bark samples of *Fraxinus americana* were taken from two locations. The bark samples were collected in 2009 from Trail Creek Trail, Monte Sano Mountain, Huntsville, AL (32^{o} 19' 5'N -86^{o} 54' 8' W; elevation 494m) and from Oakwood Avenue, Huntsville, AL (34^{o} 44'57'N -86^{o} 36'7'W; elevation 192m). The bark was collected from the tree branches (M1-M7) and from the trunk of the trees (A-F and A1-A8). Fifty milliliters of methanol was used to extract each sample of the *Fraxinus* species. The bark was chopped and dried, then placed in a 250mL erylenmyer flask. Bark (9-12g) was extracted for each sample with methanol. The bark sample and solvent was stirred at room temperature for 24 hours. Each sample was extracted 5 to 6 times, and the extracts combined together to run HPLC profiles. Ten milligrams was used for bioassays for total phenolics, cruzain inhibition, and antioxidant activity for DPPH. Bark samples (W1-W7) from *Fraxinus pennslyvania* were also collected in 2009 from Wheeler Ridge Wildlife Refugee in Decatur,Alabama (34^{o}36'21'^{o}N-86^{o}59'0.03'W; elevation 171m). A series of bioassays was carried out using Spectra Max M2 for quanitative analysis to get information using the Prussian blue polyphenolic concentration cruzain inhibition, and DPPH antioxidant activity assays.

2.2 Prussian Blue Assay for Total Phenolics

The objective was to quantify total polyphenolic content in plant samples to localize the polyphenols, and to provide an initial characterization. Phenol content of the extract was determined by modification of the Prussian Blue Assay of Price and Butler (1977). Into each well of a 96-well microliter plate, 10µL of plant extracts were diluted with 100µL of water. Then 10µL of 0.10M $FeNH_4(SO_4)_2 \cdot 12H_2O$ in 0.10M HCl plus 10µL of 0.008M $K_3Fe(CN)_6$ were added to the samples, which were incubated for 20 minutes at ambient temperature. Absorbance at 720nm was determined with the (Spectra Max M2 spectrometer) against the reagent mixture with plant and bark extracts in 1% di-methylsulfoxide. Endpoint was the mode selected for this analysis. Endpoint is a single read made at one or more wavelengths. The Spectra Max M2 is able to select six separate wavelengths as an option with this instrument. Tannic acid was used as a standard. Tannic acid is white or yellowish astringent powder with a molecular formula of $C_{14}H_{10}O_9$. It is used as a denaturant and in tanning and textiles. It is derived from bark and the fruit of many plants and is commerically used to clarify wine and beer. The Folin-Dennis, Folin-Ciocaultou, and violet-complex tests were also used for total determination. The concentration of 0.10M HCl was prepared by bringing 8.3mL of the concentrated (12M) acid to one liter with distilled water. Ferric ammonium sulfates solution was prepared by diluting 48.2g of $FeNH_4(SO_4)_2 \cdot 12H_2O$ to one liter with 0.10M HCl to give a dark yellow solution. Filter solution from the precipitate if not dissolved

completely within a few days. Color will be a dark yellow. Stock potassium ferric cyanide solution was prepared by diluting 2.63g of 0.008M $K_3Fe(CN)_6$ to one liter with distilled water, giving a bright yellow solution. Tannic acid solution was prepared by dissolving 0.2g of tannic acid compound into 20mL of DMSO to make up 1% solution. The concentration of the sample was reported in mol/L for total phenolics. All calculations were reported on Excel Spreadsheet. Two different functions were used to calculate the absorbances: a linear function was used for absorbances <1 and an exponential function for absorbances >1.5. The final concentration was expressed in mmol/L of tannic acid equivalents as depicted in Figure 2.1.

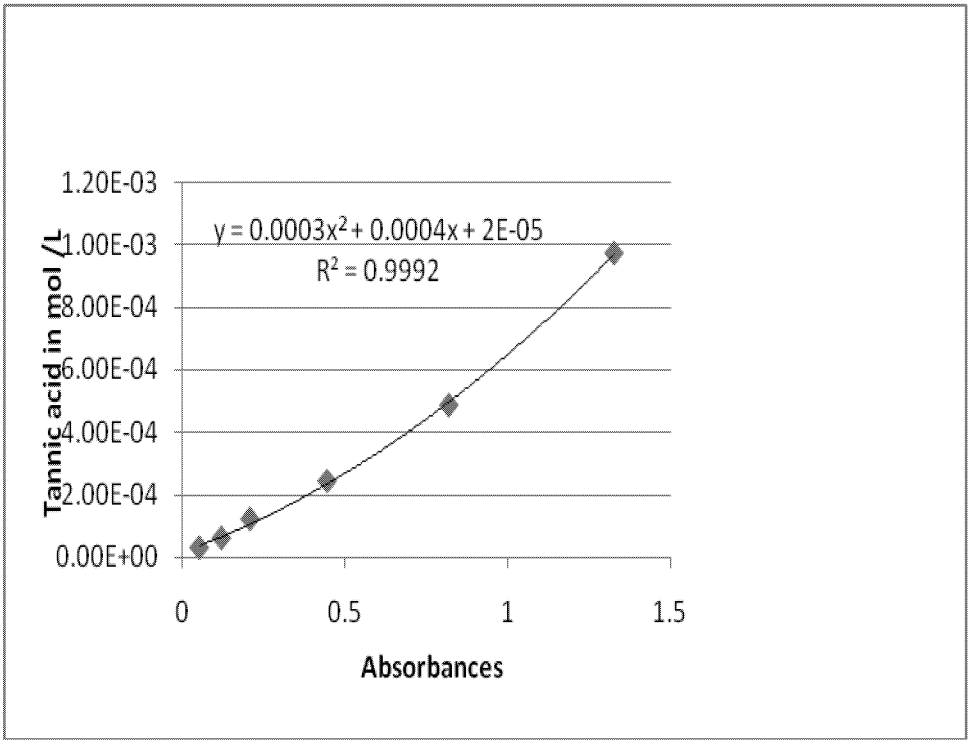


Figure 2.1. Exponential Calibration Curve for Polyphenolic Concentration.

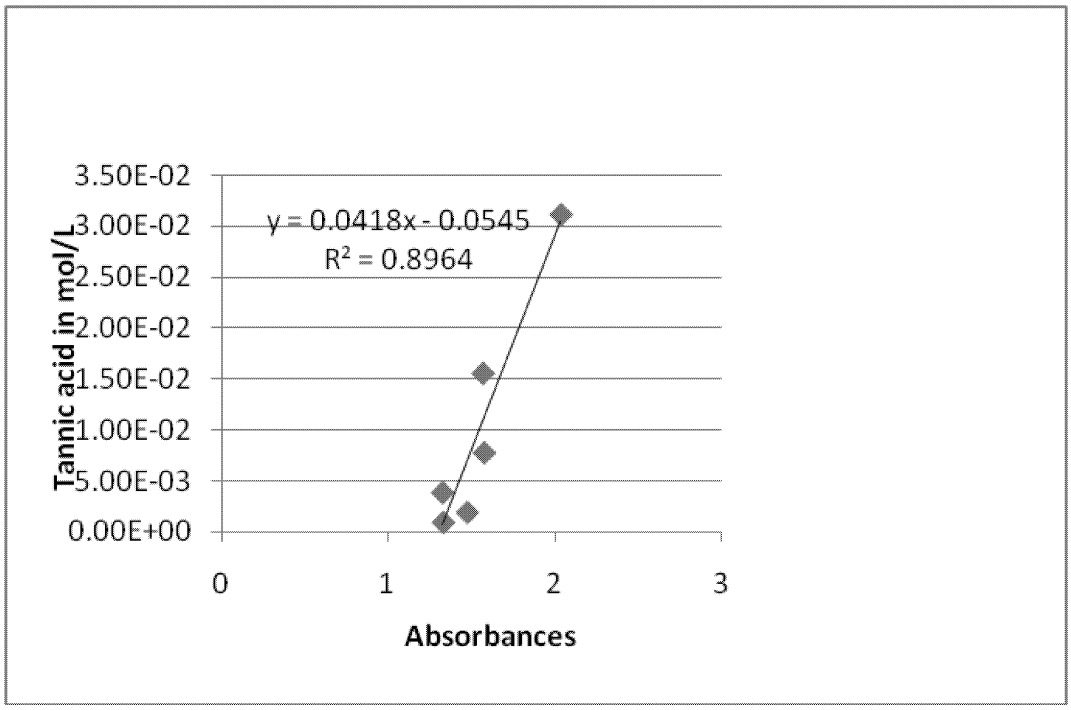


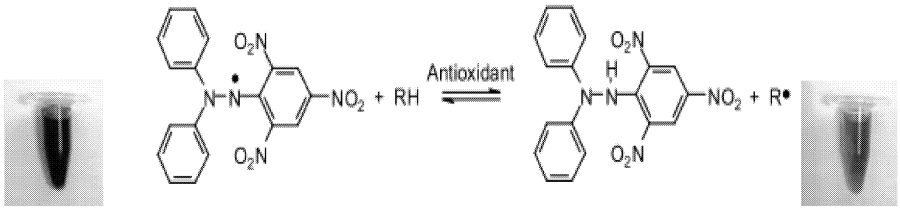
Figure 2.2. Linear Calibration Curve for Polyphenolic Concentration.

2.3 Antioxidant Activity of DPPH

DPPH has proven to be very useful in the determination of antioxidant properties of amines, phenols, or natural products, which also includes medicinal drugs and plant extracts. The *Fraxinus* extracts were measured for bleaching of the purple colored DMSO solution of 2,2-diphenyl-picryl-1-hydrazyl (DPPH) free radical. Into each well of the 96 well of the microplate, 100µL of various concentrations of the extracts in DMSO was added to 100µL of a 9.6 x 10^{-4}M concentration of DPPH. Before adding the DPPH free radical, a background reading of the plate was taken with just the extracts in DMSO

solution. After adding the free radical DPPH, extracts in various concentrations were allowed to react with the DPPH at room temperature for 30 minutes. The absorbance readings were taken at 515nm on the Spectra Max M2 instruments. The color changed from a dark purple to yellow when the reaction takes place. The standard concentrations were 0.1, 1, 2.5, 5, 7.5, and 10µg/mL of tannic acid was made up in DMSO. The inhibition of a free radical DPPH was calculated as $I\% = [A_{blank}-A_{sample})/ A_{blank} \times 100$. The A_{blank} is the absorbance of the control containing all reagents except the test compound. The A_{sample} is the absorbance of the test compound. Extraction concentrations providing 50% inhibition (IC_{50}) were calculated using the Reed-Muench method.[28] The (IC_{50}) is the test chemical concentration producing 50% inhibition of the endpoint measured. For example, DPPH and its reduction by an antioxidant are shown in Figure 2.3. The odd electron on the DPPH free radical gives a strong absorption of 515nm, which is purple in color. It turns yellow as the molar absorbtivity for the DPPH radical reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen for a free radical scavenging oxidant to form DPPH H. [11] To determine antioxidant activity, 0.1g of tannic acid was dissolved in 10mL of DMSO at several concentrations and placed into four different wells to obtain an average. The calibration curve was used as a screening process for antioxidant activity of plant extracts. Negative control factors were DMSO+DPPH. DPPH was determined colorimetrically at 515nm using the Spectra Max M2 plate reader by blanking against the control factor of DMSO+DPPH. The concentrations for the plant extracts were at 5000µg/mL, 500µg/mL, 50µg/mL, for each sample in order to determine the IC_{50} value. The free radical scavenging activities were expressed as IC_{50}, indicating the concentration

of a compound required for 50% reduction of DPPH indicated in the Excel spreadsheet. The blank or reference used was DMSO at 100µL for each well.



Dpph free radical **Reduction of free radical**

Figure 2.3. Reduction of DPPH by an Antioxidant at 515nm.

2.4 Cruzain Inhibition Assay

The activities of the bark extract were detemined by taking 10mg of crude extract and dissolving the extracts in DMSO to make up 1% solutions. The 70nM solution of cruzain was prepared by taking 10µL of the stock solution of cruzain in 10mL of the 0.1M sodium acetate buffer at the pH of 5.5. The 0.2mM fluorescence substrate stock solution was prepared by dissolving 0.065g of Z-Phe-Arg-AMC·HCL in 50mL of sodium acetate buffer. The 40µM substrate solution was prepared by using 5mM of DTT (dithiothreitol), 9.6mL of the 0.2mM concentrated substrate dissolved in 50mL of the sodium acetate buffer. The 0.1M of NaOAc buffer was prepared by dissolving 13.6g of $NaOAc \cdot 3H_2O$ in one liter of water. The sodium acetate buffer was brought to a pH of 5.5 using the Accumet AR 20 pH/conductivity meter. The DMSO was used as the negative control. The TLCK (N-tosyl-L-lysine- chloromethyl ketone), which will inhibit cruzain was used as the positive control. Cruzain solution (475µL) was mixed with 25µL

of the sample to make up 500µg/mL solution. In each well, 100µL of each component was placed in a 96 clear flat bottom well plate. Quadruplets of each sample and controls were placed in each well. In each well 100µL of the 40µM substrate was added. The plate was read using the Spectra Max M2. All samples, substrates and controls were maintained at 0^{o}C in an ice bath to avoid any decomposition. The 96 clear flat bottom well plates were read immediately. After mixing the plate for a few seconds, the well plate was measured in fluorescence. A total of nine measurements was taken with an excitation wavelength of 355nm and an emission wavelength of 460nm. The slope was measured over a five minute period. The information was then exported and saved to an Excel spreadsheet.

2.5 Rotary Evaporator

The Büchi rotary evaporator model 011 is a device used in chemical laboratories for the efficient removal of solvents. Extractions from the *Fraxinus* species were concentrated using a vapor duct, which acts as an axis of rotation for the sample. The sample is under a vacuum tight seal for the vapor being drawn off the sample. The evaporator bath is used to heat the samples as the solvent is being drawn. The cold finger or condenser is used as a chiller to collect the distilled solvent in a flask. The key advantages of the rotary evaporator are the centrifugal forces and the frictional forces between the wall of the rotating flask and the liquid samples result in the formation of a thin film of warm solvents being spread over a large surface. The rotational forces prevent violent boiling from happening. The only disadvantages are the single sample and nature and the possibility of the sample to boil violently because of the solvent low

boiling point. Samples containing compounds with high volatility are at risk at high temperatures.

2.6 HPLC

The overall goal of any chemical analysis is to separate a sample into its individual components in order to evaluate the mixture of the sample. Chromatography is a general technique that separates a mixture into its individual components or the analytes. The Varian Pro Star HPLC was used to analyze liquid extracts of bark extracts in order to separate pure compounds. The fundamental basis of HPLC consists of passing an analyte sample in a high pressure solvent through a mobile phase through a steel tube called a column. The analytes will pass through the column. They will interact between the stationary phase and the mobile phase. The rate at which each analyte elutes in the chromatogram is directly dependent on the polarities of each analyte. The method used for retaining these compounds is the reverse phase method. The reverse phase method uses hydrophobic interaction between solvents. Channel A is methanol and channel B consists of water. Polar molecules tend to elute early while nonpolar compounds elute later. Reverse phase columns have a nonpolar stationary phase and an aqueous mobile phase. The column used for analytical HPLC separation is the Sun fire C18, particle size is 5μm, ID 4.6mm, and the column length is 250mm. The part number is 186002560. The end fitting type is Waters, pH ranges from 2-8 and the particle shape is spherical. The process of reverse phase chromatography begins by first pumping the mobile phase through the column. The detector will show the retention of the molecules as they elute from the column. Separation is based upon two solvents hydrophobic character. The

C18 column is used to capture peptides or smaller molecules. The internal diameter influences the detection and selectivity in gradient elution. It also determines the amount of analyte that can be loaded onto a column. Low inside diameter gives better selectivity and less solvent use. Peaks from the bark extracts were isolated using methanol and water. Sample profiling was done using acidic conditions; 100µL of 90% formic acid was used in one liter of methanol and distilled water. The sample extracts were dissolved in methanol. Sample M3 was injected 30 times with an injection volume of 20µL each with a concentration of 1.43mg/mL. Sample W5 was also injected 30 times with an injection volume of 20µL each with a concentration of 1.14mg/mL. Both samples were isolated under neutral conditons using methanol and water as the mobile phase. The light source of the Pro Star 330 consists of a deuterium lamp with a wavelength set at 254nm for HPLC analysis. The deuterium lamp is capable of putting out light from 190 to 800nm. The light intensity is in the UV range of (<450nm). The flow cells control how much light is directed at each diode controlling the slit width. The slit widths can be operated at 8 or 16nm when the maxium amount of light is needed on each diode. In the UV, the light is highly dispersed. The diodes are actually centered at 219.65nm, 219.91nm, and 220.4nm. The diode assigned a value of 800nm really gets light 797nm to 811nm. This provides more light for each diode and allows the increase in signal away from the noise.[29] The photodiode array is a standard 512 element array. The light from the mirror is reflected onto the array and signals are created on each of the elements. The diode array measures a signal on each diode and identifies which one is responding to this wavelength for each slit setting.[29] Isolation of samples M3 (male) and W5

(female) were conducted on the Varian 330 HPLC and later identified using spectroscopic techniques.

2.7 Identification of Isolated Compound

The molecular formula for the compound isolated in both *Fraxinus* species is $C_{17}H_{24}O_9$ determined by ^{1}HNMR and ^{13}CNMR analysis, summarized on Table 3.9. The name of the compound isolated from both speceis of trees is called syringin, a phenylpropanoid glycosides which is abundant in many plants. Phenylpropanoids are a group of plant secondary metabolites derived from phenylalanine and having a wide variety of functions both as structural and signaling molecules.

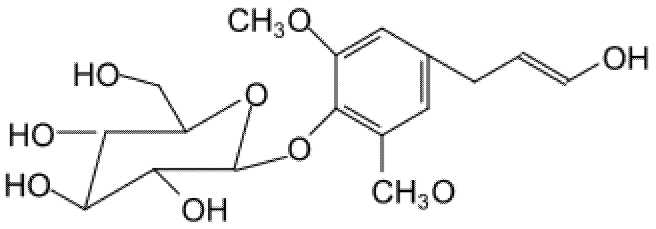


Figure 2.4. Structure of Syringin.

Chapter III

RESULTS AND DISCUSSION

3.1 Polyphenolic Concentration

The Prussian Blue assay was conducted for the following samples W1-W7. W1, W2, W3, and W7 are the males and W4-W6 are female trees of *Fraxinus pennslyvanica.* Samples were collected from June to October (see Table 3.1). The months of July and August were the highest in polyphenolic concentration ranging from 11-36mmol/L. The male trees have a higher concentration than the female trees in the month of July and August. Samples recorded in the month of October showed significantly lower concentrations of polyphenols because the chemical make up has changed. Polyphenolic concentrations were analyzed from *Fraxinus americana.* Bark extracts were taken from the branches of the trees located on Monte Sano Mountain. Samples M1-M5 and M7 are all male species. M6 is a female white ash tree. These samples were collected from August to October as shown (see Table 3.2). Sample M7 remained consistent with a concentration between 5-6mmol/L. Samples showed a significant drop in concentration after the month of August. The male species of *Fraxinus americana* and *Fraxinus pennslyvanica* had higher concentrations than the female counterparts.

Samples A-F were taken from Toll Creek Trail on Monte Sano Mt. Samples B and E were female speices of *Fraxinus americana*; samples A, C, D, and F were male.

Samples A-F were taken from the trunk of the trees for the months of June and July measuring the total polyphenolic concentration. The Prussian Blue Assay results were all less than 1mmol/L for all samples taken from the trunk of the *Fraxinus americana* tree as shown in Table 3.3. There was a substantial difference in polyphenolic content from the bark of the branches and from the bark of the trunk of the trees. The eight male samples of bark extract were taken locally from Oakwood Park in Huntsville. The bark extracts were taken from the trunk of the tree showing a concentration of less than 1mmol/L of polyphenolic content for *Fraxinus americana.*

3.2 DPPH Antioxidant Activity

Another assay was conducted in order to determine the free radical scavenging capability of *Fraxinus pennslyvanica* and *Fraxinus americana* in order to inhibit 2,2 diphenyl-1-picryl-hydrazyl radical (DPPH). Bark extracts were taken from the branches of the two *Fraxinus* species. The free radical scavenging activities were expressed as IC_{50} values, indicating the concentration of a compound required for 50% reduction of 2, 2 diphenyl-1-picryl hydrazyl. W1,W2,W4, and W5 of the *Fraxinus pennslyvanica* ash trees showed an inhibition of almost 50% or greater for the month of June at 49, 70, 50, and 52 percent reduction of the free radical DPPH. The IC_{50} values were calculated on in ug/mL using the Reed Muench method in Table 3.4. W1 showed the most consistency of 50% inhibition throughout the five month period. The male species were significantly higher in inhibition than the female species from June to October.

Fraxinus pennslyvanica male and female species showed similar results during the seansonal period. The samples that were taken from the bark of the branches of the trees

much later in the year from August to October showed a decline in inhibitory activity of DPPH shown in Table 3.5. *Fraxinus* americana samples taken from June to July showed no inhibitory activity due to the lack of chemical activty present within the trunk of the tree. Samples M1-M5 and M7, which are all male species, have an inhibition of 52, 60, 49, 52, 53, and 67 percent. Sample M6 (female) results were negative for the inhibiting DPPH for the months of August and September.

3.3 Cruzain Inhibition

The bark extracts of both species of trees were analyzed for the inhibition of cruzain. The *Fraxinus pennslyvanica* extracts showed less than 50 % inhibition through the five month period. W1, a male tree is the most consistent sample with an inhibition of 88, 72, and 87 percent in the months of July, August, and October. Sample W4 has an inhibition of 64 percent in June and the sample was less than 50% percent for the other four months. *Fraxinus americana* extracts were showed less ability to inhibit cruzain. The female tree M6 had the highest inhibition of 62%. M1-M5 had an increase of inhibition of about 40% in the month of October.

3.4 HPLC Profiling

Crude extract samples taken from both species were analyzed over a five month period to identify any seasonal changes. Samples were prepared in methanol at various concentrations. The spectral data were analyzed using acidic conditions for better resolutions. Formic acid (100μL) was added to one liter of water and methanol as the mobile phase. The polyphenolic, cruzain inhibiton, and DPPH antioxidant activity showed notable differences in concentration and percent inhibition from the months of

June and October. The HPLC chromatograms for sample W2 and M3 for the months of June and October showed no seasonal changes. Chromatograms of both of the *Fraxinus* species in Figures 3.1-3.4 are similar qualitatively but quantatively different for the different months. Samples were analyzed in quadruplets under acidic conditions for reproducibility. W1 was used as a standard for both species of *Fraxinus*. There were no real changes from month to month. However, the compound syringin was isolated from samples W5 and M3 are only different in their retention times as shown in Figures 3.3-3.5.

3.5 Chemical Component of M3, W5

The structure was determined through using the Nuclear Magnetic Resonance (NMR 500 MHz) techniques. CD_3OD was the solvent used for ^{1}HNMR and ^{13}CNMR spectra. The compound was determined to be syringin, a phenylpropanoid glucoside. This glucoside is found in the bark of both species of male and female *Fraxinus.* Table 3.9 shows the compound chemical shifts of samples M3 and W5. The cruzain inhbition assay was conducted on both syringing samples [M3 (male) and W5 (female)]. Syringin was not appreciably inhibitory at a concentration of 1% in DMSO; sample M3 showed 32% (±%15) and sample M5 with 30% (±%16) inhibition at that concentration.

Table 3.1. Seasonal variation in polyphenolic concentrations (mM, standard deviations in parentheses) in *Fraxinus pennsylvanica* stem bark extracts.

Sample	June	July	August	September	October
W1 (♂)	5.78(3.93)	28.80(4.66)	36.40(13.14)	10.70(5.09)	9.15(1.63)
W2 (♂)	3.74(0.64)	19.06(4.88)	14.00(2.17)	3.76(1.96)	0.96(0.44)
W3 (♂)	15.42(3.78)	22.58(1.74)	11.73(2.58)	5.16(2.46)	1.44(1.33)
W4 (♀)	15.57(0.25)	20.62(3.54)	11.80(6.56)	7.24(2.86)	0.99(0.03)
W5 (♀)	16.48(0.58)	15.71(2.53)	15.54(2.55)	4.70(3.73)	3.04(1.79)
W6 (♀)	13.04(3.25)	16.06(3.47)	14.72(3.59)	6.37(2.57)	1.02(0.52)
W7 (♂)	14.15(2.56)	15.11(3.96)	15.09(1.62)	3.36(2.79)	0.97(0.06)

Table 3.2. Seasonal variation in polyphenolic concentrations (mM, standard deviations in parentheses) in *Fraxinus americana* stem bark extracts.

Sample	August	September	October
M1 (♂)	20.89(1.27)	5.75(1.88)	9.96(1.66)
M2 (♂)	6.52(3.55)	4.85(1.21)	6.02(1.86)
M3 (♂)	12.44(2.43)	5.58(0.21)	1.81(0.37)
M4 (♂)	14.40(2.98)	9.61(5.09)	5.76(0.73)
M5 (♂)	15.07(2.08)	8.57(1.67)	5.63(0.75)
M6 (♀)	16.64(2.33)	11.35(1.57)	5.52(1.37)
M7 (♂)	6.29(1.53)	6.46(2.34)	5.83(1.54)

Table 3.3. Polyphenolic concentrations (mM, standard deviations in parentheses) in *Fraxinus americana* trunk bark extracts.

Monte Sano Sample	June	July	Oakwood Sample	June	July
A (♂)	0.45(0.05)	0.37(0.06)	A1 (♂)	0.33(0.02)	0.50(0.11)
B (♀)	0.49(0.01)	0.64(0.15)	A2 (♂)	0.15(0.02)	0.46(0.09)
C (♂)	0.37(0.01)	0.36(0.04)	A3 (♂)	0.42(0.04)	0.29(0.11)
D (♂)	0.30(0.01)	0.64(0.06)	A4 (♂)	0.20(0.02)	0.34(0.05)
E (♀)	0.65(0.09)	0.89(0.28)	A5 (♂)	0.24(0.02)	0.50(0.20)
F (♂)	0.61(0.06)	0.64(0.12)	A6 (♂)	0.35(0.03)	0.31(0.06)
			A7 (♂)	0.25(0.03)	0.42(0.08)
			A8 (♂)	0.25(0.04)	0.73(0.21)

Table 3.4. Seasonal variation in DPPH radical scavenging activity (IC_{50}, µg/mL, standard deviations in parentheses) in *Fraxinus pennsylvanica* stem bark extracts.

Sample	June	July	August	September	October
W1 (♂)	311.5(4.2)	179.1(3.5)	132.3(3.0)	95.81(3.16)	107.9(1.7)
W2 (♂)	278.3(1.9)	271.6(7.5)	240.4(5.5)	260.3(7.4)	242.1(6.6)
W3 (♂)	378.1(8.3)	483.7(15.8)	284.8(9.2)	500.0(16.8)	410.2(17.7)
W4 (♀)	311.4(10.8)	451.2(13.2)	233.9(4.1)	194.3(5.3)	147.7(4.8)
W5 (♀)	322.6(14.9)	311.5(13.3)	212.8(7.3)	211.9(6.6)	358.2(17.8)
W6 (♀)	803.3(25.8)	391.9(13.0)	199.9(5.3)	263.5(10.9)	166.5(6.2)
W7 (♂)	312.0(4.5)	390.1(17.2)	316.5(18.0)	607.4(14.9)	481.8(17.5)

Table 3.5. Seasonal variation in DPPH radical scavenging activity (IC_{50}, µg/mL, standard deviations in parentheses) in *Fraxinus americana* stem bark extracts.

Sample	August	September	October
M1 (♂)	62.96(1.47)	119.9(15.1)	265.3(9.6)
M2 (♂)	55.18(0.78)	146.5(6.2)	205(11.6)
M3 (♂)	115.7(7.5)	240.5(10.7)	136.7(6.4)
M4 (♂)	61.41(0.43)	106.0(2.62)	286.6(21.8)
M5 (♂)	107.2(3.5)	359.0(22.9)	209.1(7.9)
M6 (♀)	266.5(12.9)	368.2(21.5)	266.3(13.5)
M7 (♂)	291.0(8.7)	1019(62)	274.2(36.8)

Table 3.6. Seasonal variation in cruzain inhibitory activity (% inhibition at 250 µg/mL, standard deviations in parentheses) in *Fraxinus pennsylvanica* stem bark extracts.

Sample	June	July	August	September	October
W1 (♂)	39.45(18.04)	88.09(3.21)	73.28(1.24)	44.89(2.46)	78.38(8.81)
W2 (♂)	20.07(26.63)	61.80(2.14)	21.92(3.01)	45.61(0.47)	-0-
W3 (♂)	24.28(11.74)	8.43(2.90)	37.69(1.05)	18.08(3.00)	-0-
W4 (♀)	64.74(14.42)	3.01(1.94)	22.01(2.71)	14.61(2.47)	8.76(6.73)
W5 (♀)	33.74(16.58)	17.95(3.93)	12.91(4.22)	29.56(4.80)	36.90(7.94)
W6 (♀)	-0-	5.66(5.57)	12.08(6.53)	4.44(4.65)	14.72(5.08)
W7 (♂)	3.51(27.02)	13.68(4.81)	7.63(2.25)	12.78(4.32)	41.34(8.98)

Table 3.7. Seasonal variation in cruzain inhibitory activity (% inhibition at 250 µg/mL, standard deviations in parentheses) in *Fraxinus americana* stem bark extracts.

Sample	August	September	October
M1 (♂)	7.14(2.43)	44.36(0.75)	45.45(6.80)
M2 (♂)	27.65(1.68)	28.84(3.18)	-0-
M3 (♂)	26.72(0.42)	30.32(1.13)	23.79(7.57)
M4 (♂)	22.97(1.37)	11.41(2.08)	-0-
M5 (♂)	27.52(2.78)	30.68(0.22)	-0-
M6 (♀)	62.86(0.88)	53.58(1.15)	-0-
M7 (♂)	50.64(1.21)	19.52(1.48)	-0-

Table 3.8. Cruzain inhibitory activity (% inhibition at 250 µg/mL, standard deviations in parentheses) in *Fraxinus americana* trunk bark extracts.

Monte Sano Sample	June	July	Oakwood Sample	June	July
A (♂)	6.24(4.64)	26.94(2.66)	A1 (♂)	-0-	-0-
B (♀)	6.41(2.88)	23.86(1.64)	A2 (♂)	-0-	1.61(2.91)
C (♂)	6.16(3.36)	5.67(4.96)	A3 (♂)	-0-	-0-
D (♂)	1.71(3.69)	20.62(2.77)	A4 (♂)	-0-	24.85(0.73)
E (♀)	4.53(2.40)	9.42(3.93)	A5 (♂)	-0-	10.92(5.44)
F (♂)	20.36(1.99)	26.95(1.22)	A6 (♂)	-0-	-0-
			A7 (♂)	-0-	-0-
			A8 (♂)	-0-	17.95(1.62)

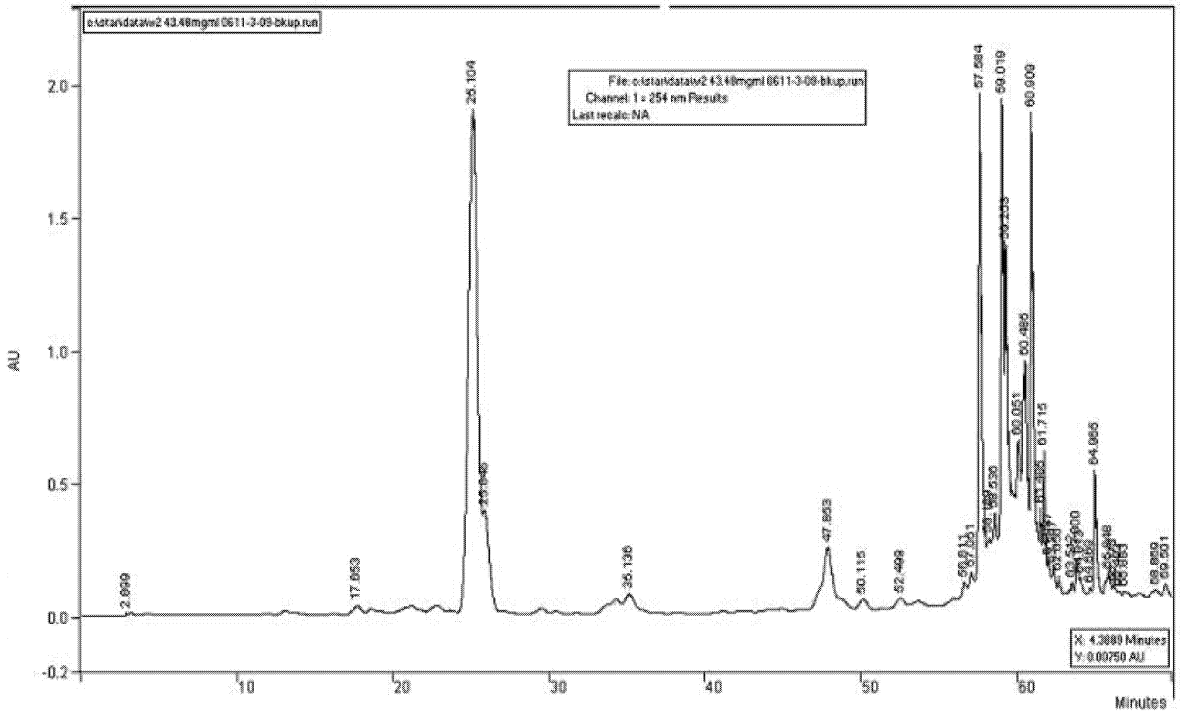


Figure 3.1. HPLC Spectrum of W2 ♂ in June.

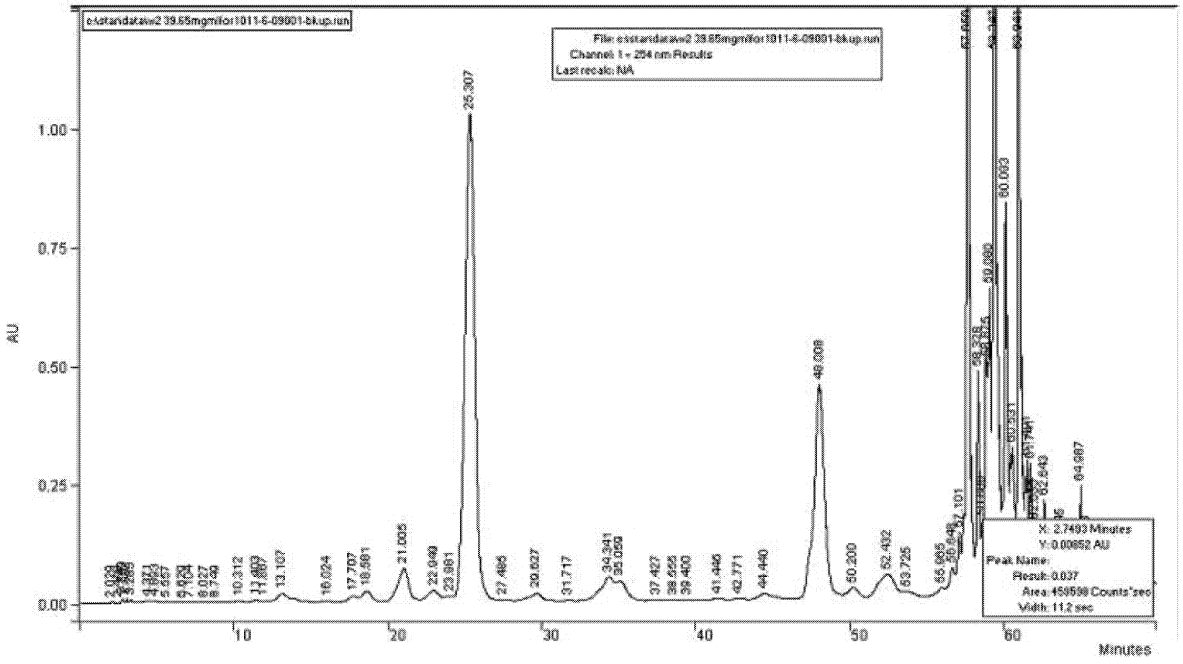


Figure 3.2. HPLC Spectrum of W2 ♂ in October.

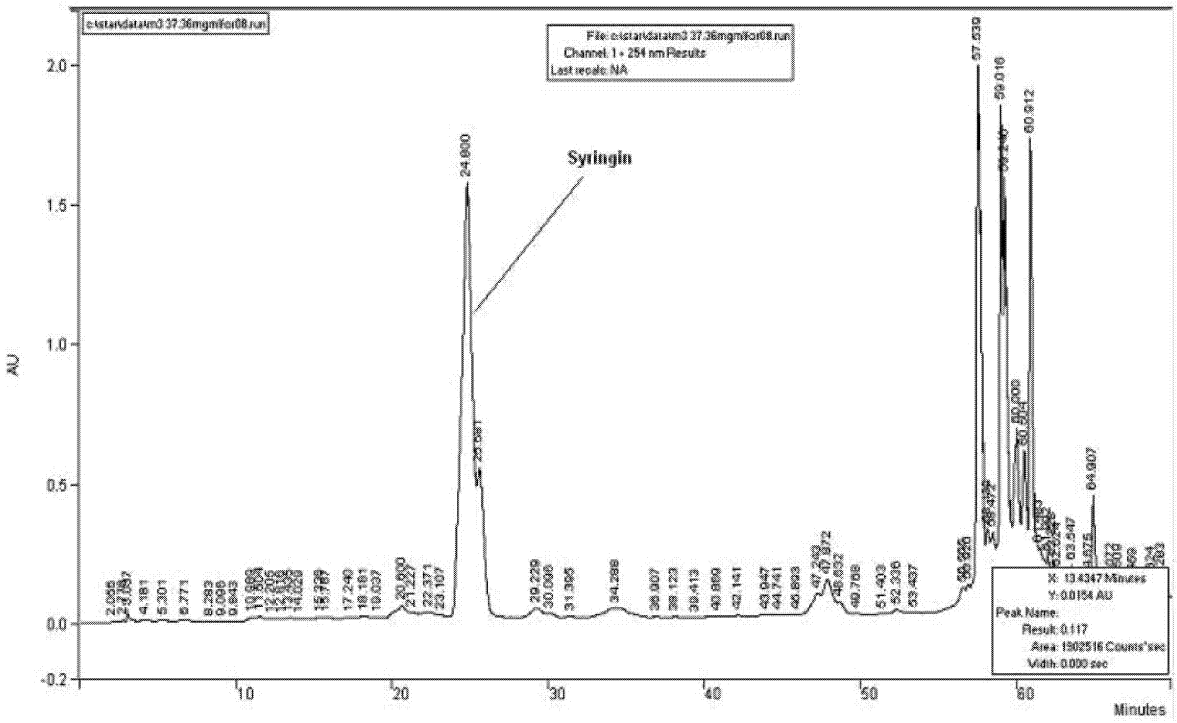


Figure 3.3. HPLC Spectrum of M3 ♂ in August.

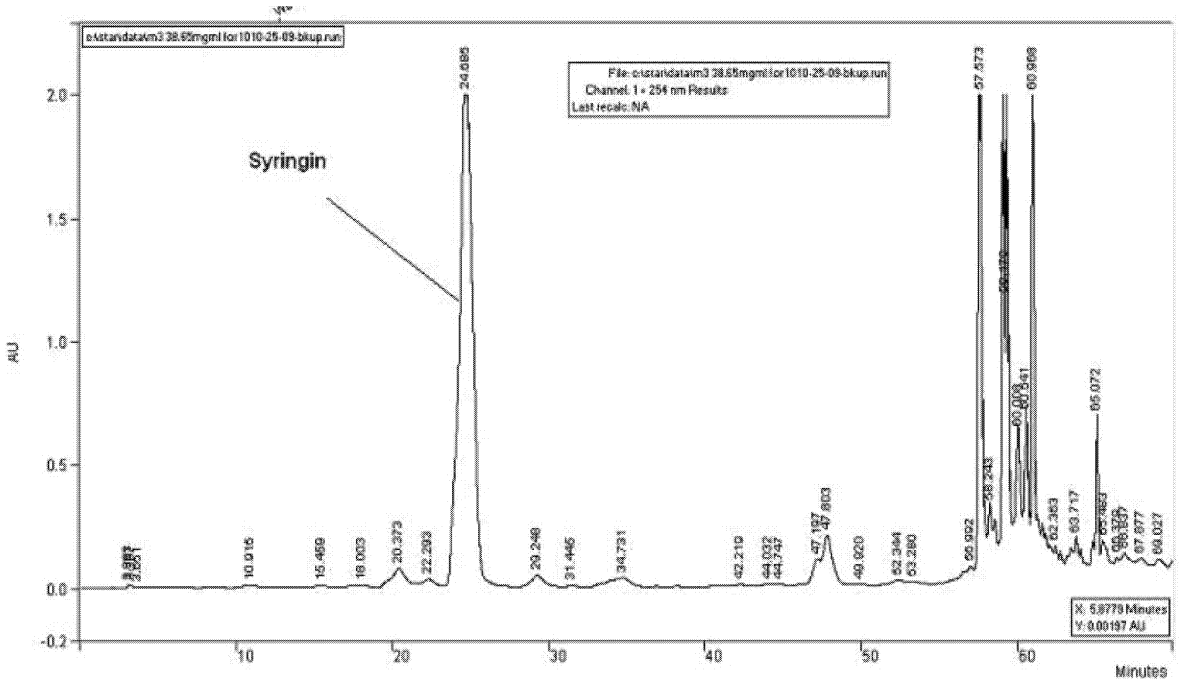


Figure 3.4. HPLC Spectrum of M3 ♂ in October.

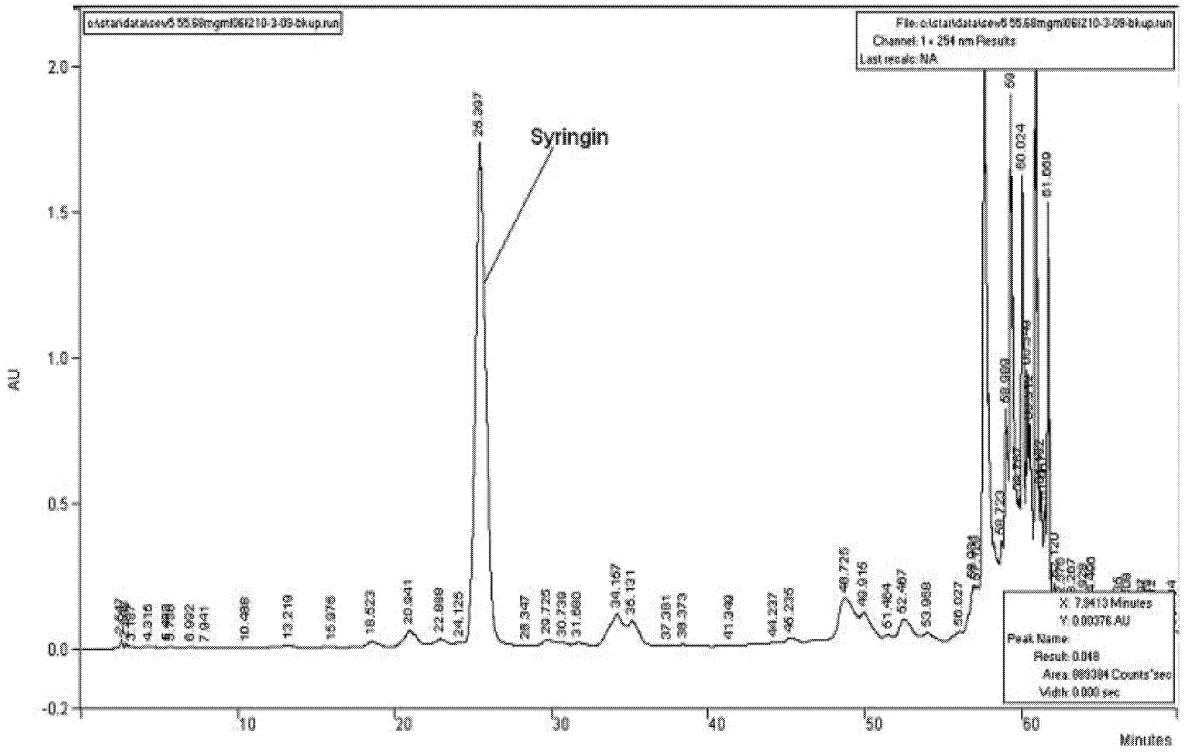
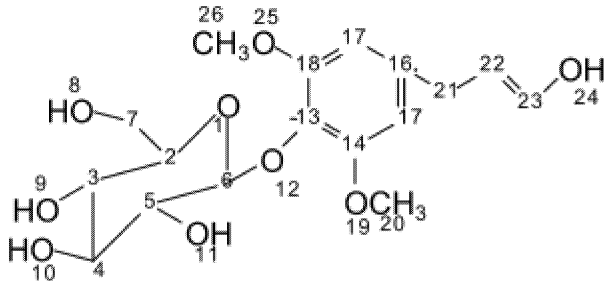


Figure 3.5. HPLC Spectrum of W5 ♀ in June.

Table 3.9. NMR Chemical shifts of syringin in methanol-d_4.



Atom No.	^{13}C NMR	^{1}H NMR
2	79.59	3.69(m,1H)
3	72.56	4.54(m,1H)
4	79.06	4.29 (m,1H)
5	76.94	4.02(m, 1H)
6	106.58	6.78 (s,1H)
7	63.80	3.79(dd,1H)
13	136.51	6.56(d, 1H)
14	155.58	
15	106.68	6.78 (s,1H)
16	113.20	
17	131.28	6.36 (dt,1H)
18	155.57	
20	58.27	3.85(s,3H)
21	64.80	3.35(d,2H)
22	106.67	6.56(t,1H)
23	136.51	4.20(dd,1H)
26	57.04	3.85(s,3H)
8,9,10,11,24		4.13(s,5H)

Chapter IV

CONCLUSION

In August, both species of *Fraxinus* started to decline in phenolic concentation from 15-20mM to 4mM in October. Male samples that were taken from the the trunk of white ash trees exhibited no significant biological or chemical activity present in the trunk of these trees. Samples taken from Oakwood Avenue, Huntsville, AL (A1-A8), and from Toll Creek Trail (A-F), Monte Sano Mountain, Huntsville, AL, showed <1mM polyphenolic concentration and <30% inhibition of cruzain. There were no free radical scavenging capabilites present in the trunk bark of the white ash trees. The DPPH free radical scavenging capaability of the *F. pennslyvanica* ash trees and the *F.americana* ash trees did not show consistent IC_{50} value inhibition during the five month period for each sample. The seasonal variation of inhibition of cruzain showed that W1 had the most inhibition of the seven samples taken. The two young juvenile male trees W2 and W3 showed no inhibiton in October, possibly due to the lack of maturity as well as the change in season. The seasonal variations of *Fraxinus pennslyvanica* and *Fraxinus americana* did not show a consistentcy of 50% throughout the five month period. There were 14 samples that were analyzed in quadruplets on HPLC. All chromatograms analyzed under neutral and acidic conditions showed the same pattern of peaks. Samples (M3 and W5) showed slight inhibitiory activity to cruzain 32±15% and

30±16%, respectively. Because of the close proximity of both species, the results in inhbition of cruzain, free radical scavenging capability, and the polyphenolic content were similar between *Fraxinus pennslyvanica* and *Fraxinus americana*. Interestingly, there was no apparent correlation between polyphenolic concentration, cruzain inhibitory activity, or DPPH radical scavenging activity. Cruzain inhibition and free radical inhibition are apparently due to other compounds besides polyphenolics in these trees. The chemical profiles of male and female Fraxinus extracts were qualitatively similar; there were no apparent chemical differences between the sexes of these two species.

APPENDIX

NMR SPECTRA

Appendix figures present all the ^{1}HNMR, gCOSY, gHSQC,and gHMBC NMR spectra, which were recorded in $CDOD_3$ on a (500MHz) Varian spectrometer operating system. The chemical shifts are referenced to solvent signals, which are shown in the value of ppm.

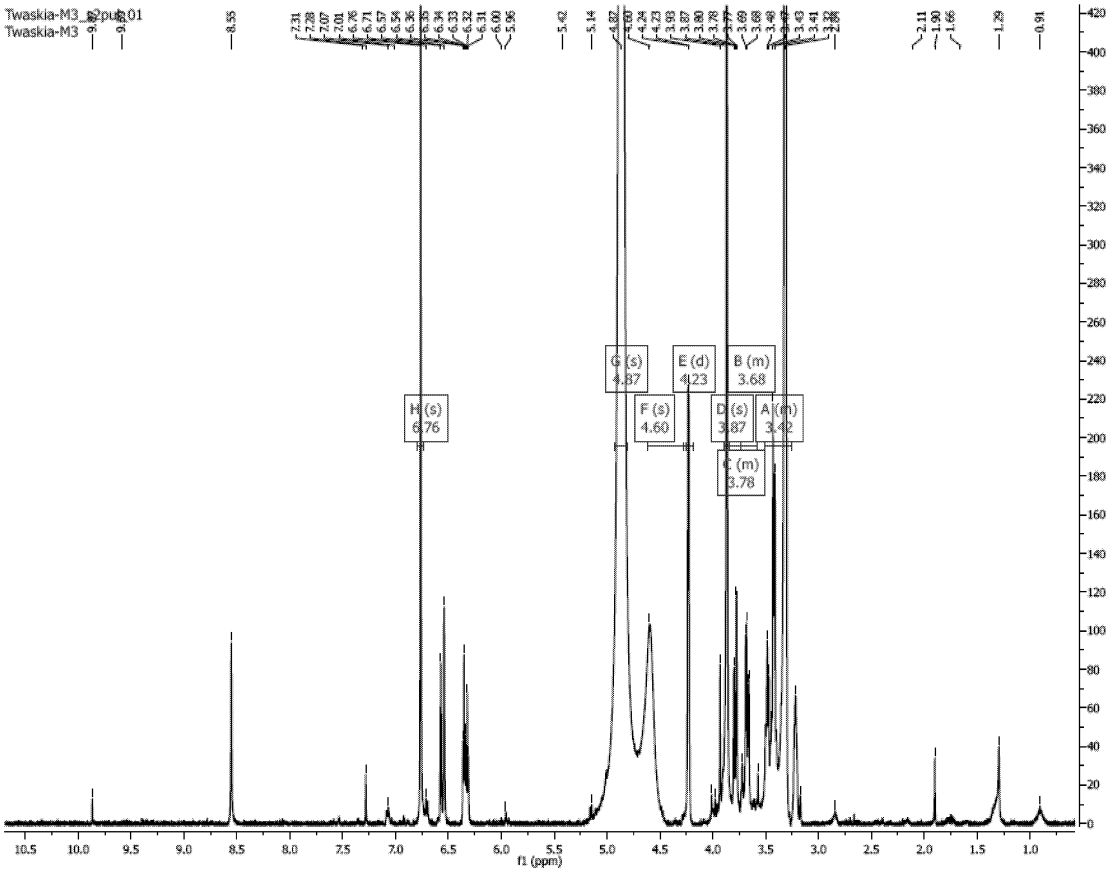


Figure A.l. ^{1}HNMR Spectrum of Syringin.

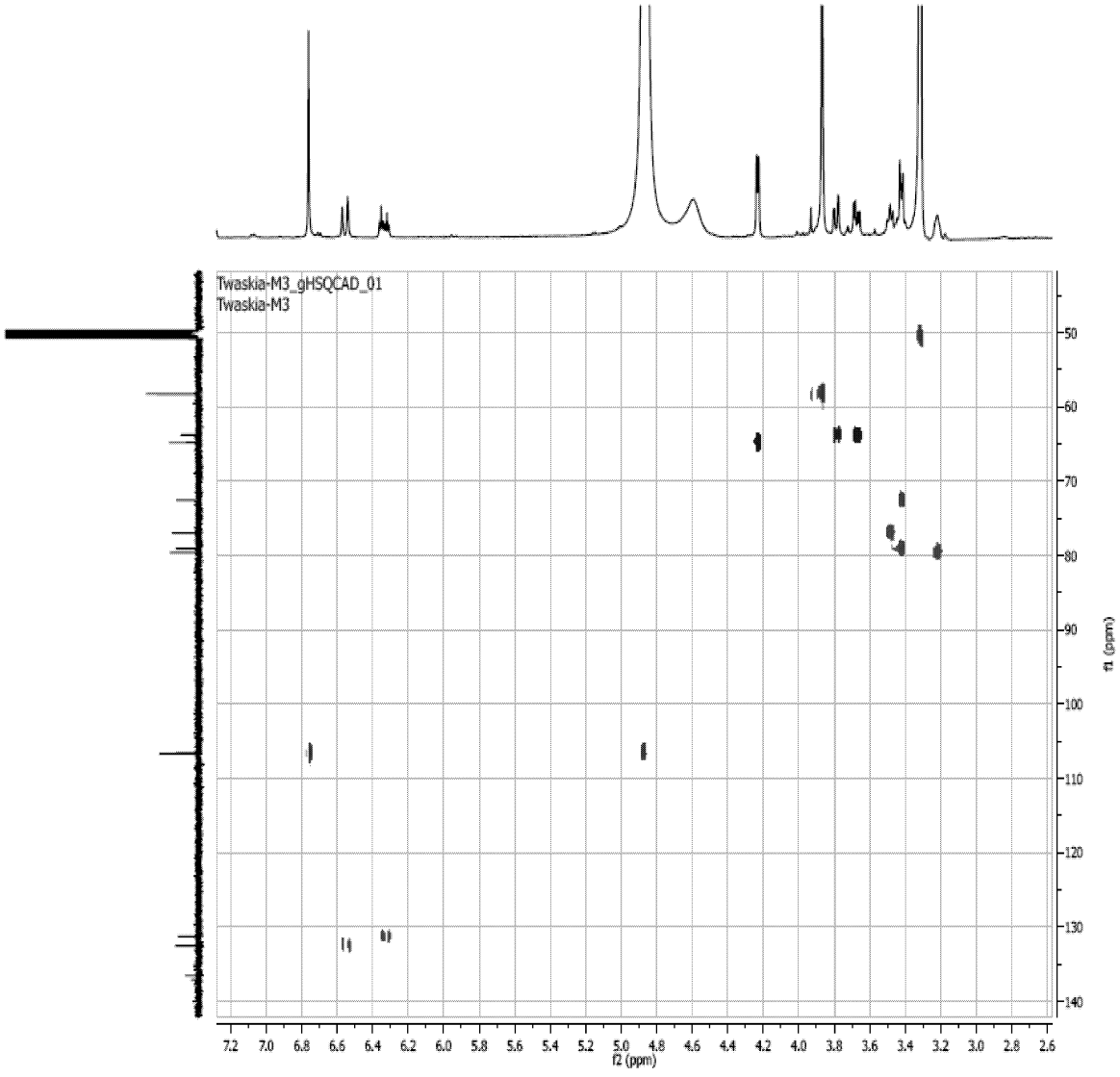


Figure A.2. gHSQC NMR Spectrum of Syringin.

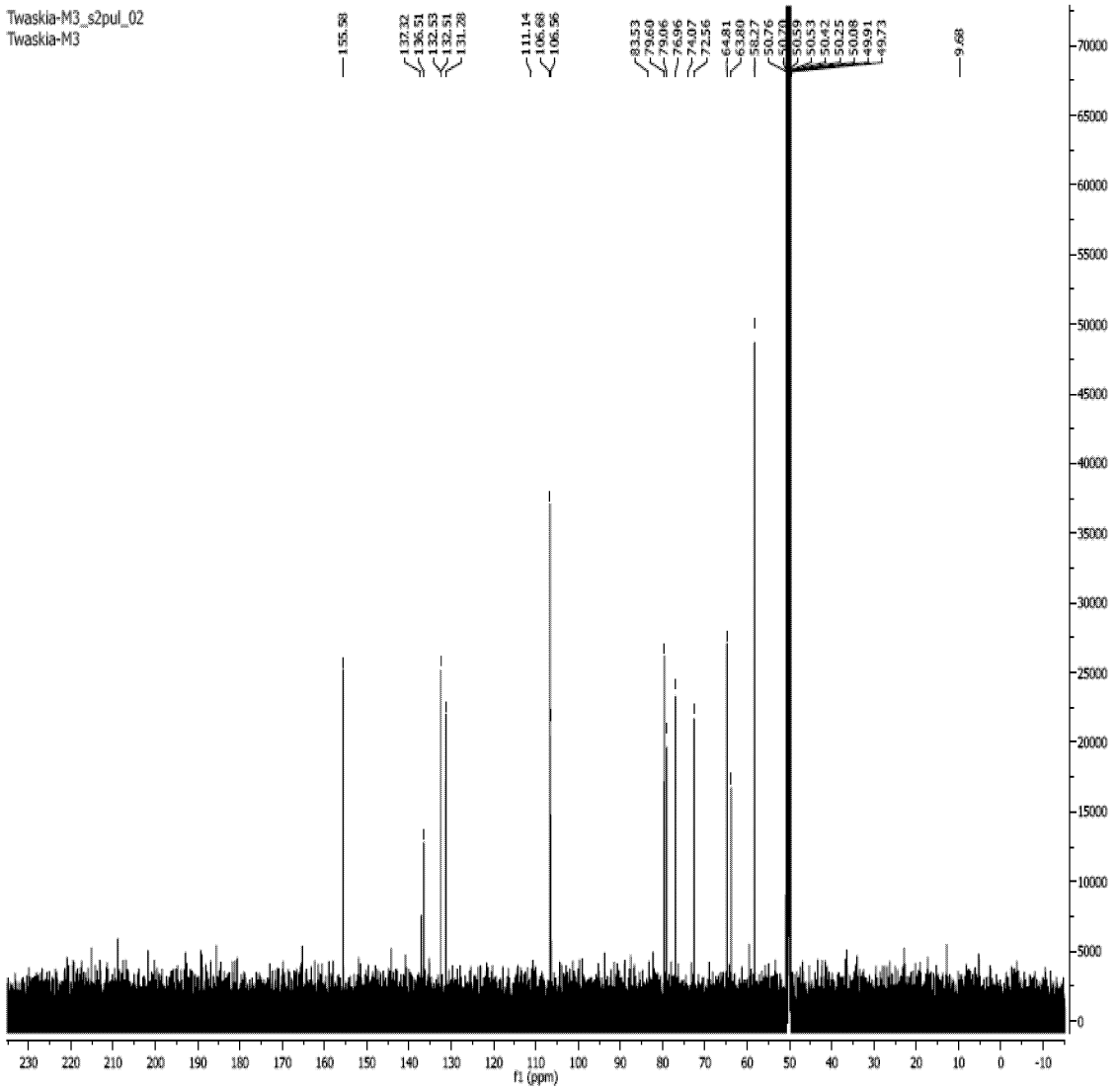


Figure A.3.^{13}CNMR Spectrum of Syringin.

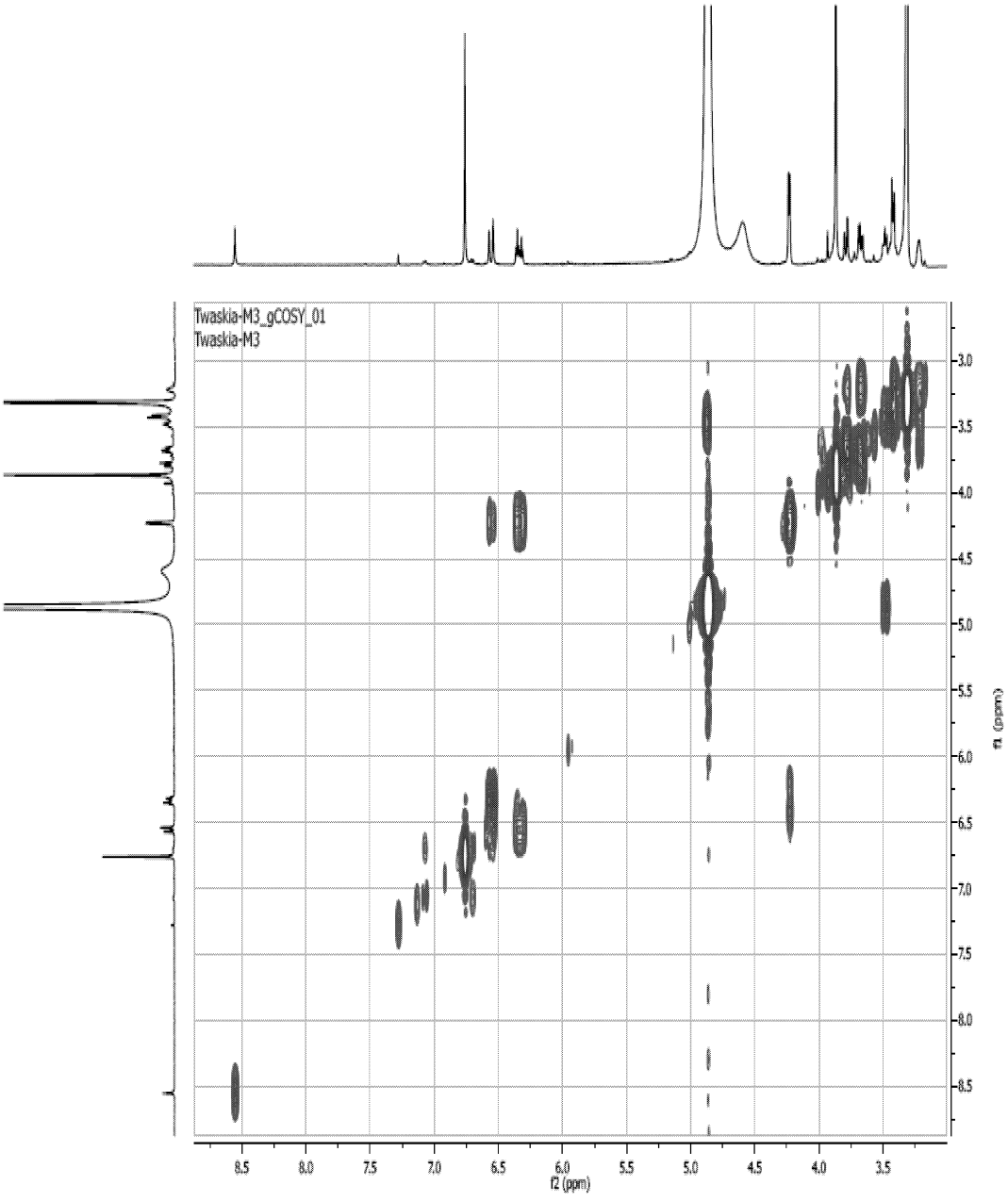


Figure A.4. gCOSY Spectrum of Syringin.

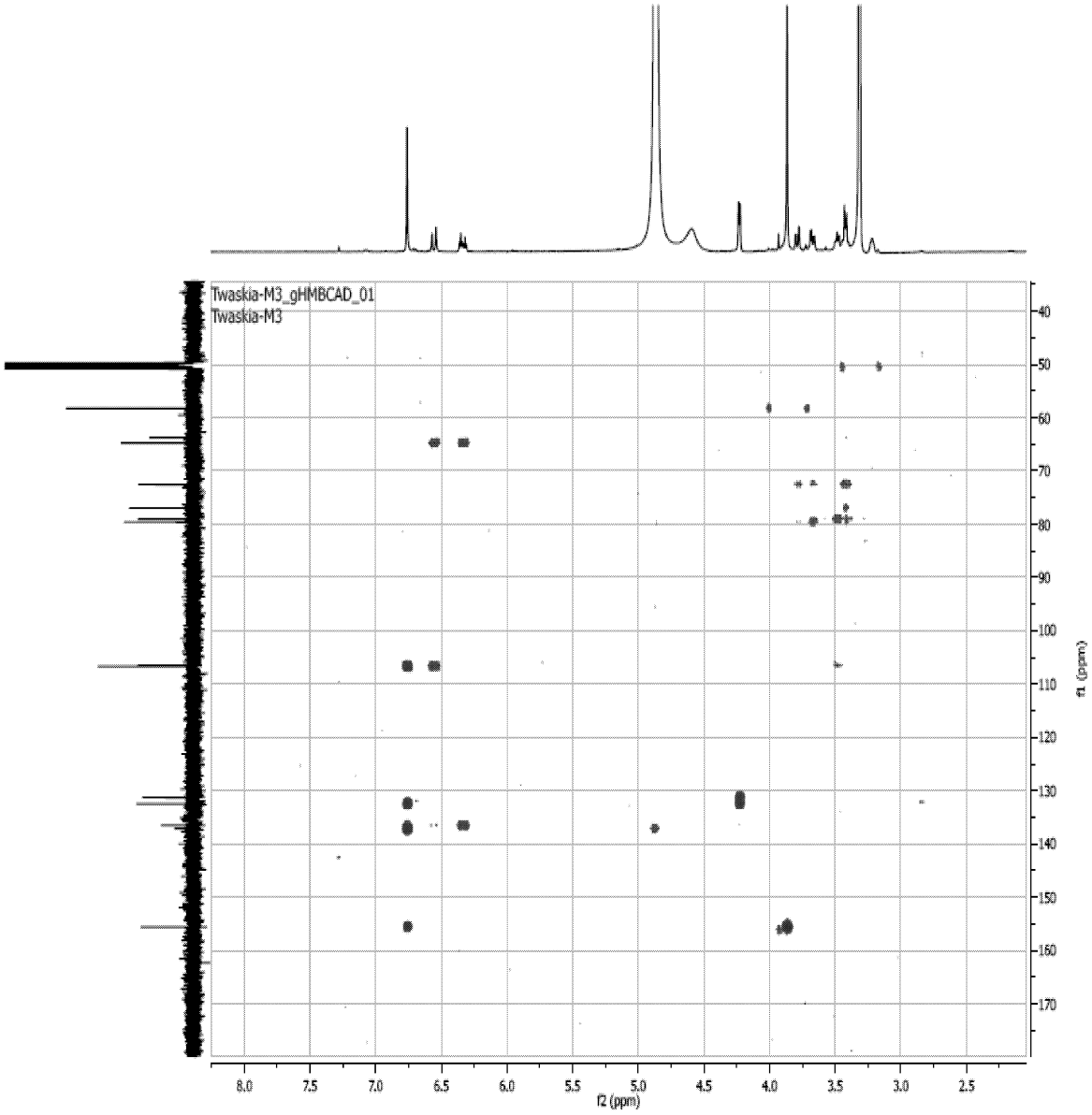


Figure A.5. gHMBC Spectrum of Syringin.

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