The significant impact that this project is desired to have is to provide fundamental knowledge regarding the use of kudzu products for medicinal purposes, as it relates to antioxidant and anti-inflammatory effects. This is anticipated to be achieved by studying how the plant may alter the activity of several cytochrome P450 enzymes with known effects on human health. Different human cytochrome P450 isoforms were selected for this study based on their ability to convert relatively inert foreign chemical species into toxic metabolites. These include P450 2E1, 2A6, and 1A2. To analyze the selected enzymes in crude liver tissue each isoform had a unique substrate that was metabolized selectively.

Several different kudzu extracts were prepared for analysis. Each extract and the pure compound puerarin were tested for inhibition of the enzymes. The most inhibitory effects were observed with the interaction between cytochrome P450 1A2 and the ethanolic kudzu root extract and cytochrome P450 2C9 and the ethanolic kudzu root extract.
THE EFFECTS OF KUDZU (*PUERARIA LOBATA*) ON VARIOUS CYTOCHROME P450s

by

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A Thesis Submitted to the Faculty of The Graduate School at The University of North Carolina at Greensboro in Partial Fulfillment of the Requirements for the Degree Master of Science

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CHAPTER I

BACKGROUND

Cytochrome P450 enzymes play a major role in the metabolism of xenobiotics such as carcinogens, drugs, steroids, and pesticides. They also metabolize fat-soluble vitamins, and endogenous compounds such as eicosanoids and mammalian alkaloids. Cytochrome P450s are a microsomal superfamily of isoenzymes,[1] and are the most important enzyme system in human phase I metabolism of foreign chemicals. [2] Their role in phase I metabolism is catalyzing the oxidation of foreign chemicals that enter the body. [2] Regardless of what the compound is, it is important that the lipophilicity is reduced, so as not to accumulate in lipid membranes and that the compound is modified in such a way as to make it easier to remove. The general reaction through which cytochrome P450s accomplish such metabolism is shown in the reaction scheme NADPH + H+ + O2 + R → NADP+ + H2O + ROH, where NADPH is the biological electron donor, R is the substrate, and ROH represents the oxidized product. The electrons are supplied from NADPH via the intermediary action of a flavoprotein, NADPH-CYP450 reductase. [2] In many cases this can produce less toxic or non-toxic metabolites; however, in several instances cytochrome P450 metabolism can be undesirable and result in toxic metabolites. Reactive oxygen species (ROS) can be generated from cytochrome P450 metabolism that can cause oxidative stress. [3] In these instances, the inhibition of cytochrome P450 enzymes could actually limit the formation of ROS and cause an anti-
oxidant effect. In some cases, during the course of oxidizing certain compounds, cytochrome P450s can undergo mechanism-based, “suicide”, inactivation, destroying itself in the process. [3] Alternatively, the inhibition or induction of cytochrome P450s can result in drug interactions and promote toxicity or reduce therapeutic effects, thus understanding interactions between P450s and natural dietary supplements is extremely important when considering the use of such products. [2] When looking at mammalian cytochrome P450s as a whole, it is also important to keep in mind that the catalytic sensitivity of these enzymes can vary between organisms, thus understanding the relationship between P450 isoforms across different species is an important consideration when selecting model systems to study. For example, the rat, rabbit, and human liver contain the P450 2E1 isoform, and the rodent models are often used to simulate the human enzyme, so recognizing differences in the interactions between natural products and the 2E1 found in rabbit, rat, and human cells is an important facet of drug metabolism studies.

The current study will focus on potential interactions between the natural product kudzu and specific cytochrome P450 isoforms found in rabbit and human liver cells. It will address systems, along with identification of optimal extraction methods to maximize inhibitory effects. The isoforms selected for the study include P450 2C9, 2E1, 2A6, and 1A2 as described in the following sections.
Known Drug/Herb Interactions

Cytochrome P450 2C9

Cytochrome P450 2C9 has been well characterized. There are crystal structures/x-ray structures available for human cytochrome P450 2C9. The enzyme shows selectivity for small, lipophilic anions, such as ibuprofen. The hepatic total cytochrome P450 content within a normal human liver is comprised of approximately 20% of the cytochrome P450 2C9 isoform, so it is not unexpected that approximately 15% of clinical drugs are metabolized by this enzyme. However, mutations within this enzyme do exist, and can greatly influence its functionality. [4]

Recent studies have shown that cytochrome P450 2C9 inhibition may be the cause of several drug-drug / drug-herb interactions with warfarin. [5] Multiple components in marijuana smoke, such as CBD and CBN, have been shown to be significant inhibitors of cytochrome P450 2C9, and warfarin has been shown to be metabolized significantly by cytochrome P450 2C9. This seems to indicate that components in marijuana smoke may be contributing to side effects from warfarin, such as bleeding complications in patients who consume marijuana. Warfarin has been known to have a higher likelihood of complications than many medications, and this is just one example. A better understanding of cytochrome P450 2C9 inhibition may resolve many warfarin complications. [5]
**Cytochrome P450 2E1**

There are many other situations where specific cytochrome P450 activity could have a negative effect. A well-known situation would be mixing specific medications with alcohol. Various medications are labeled with a warning such as “do not take with alcohol”. Alcohol can promote the activity of specific cytochrome P450 enzymes. This may produce toxic metabolites when mixed with specific medications, such as Tylenol, by causing chemicals, such as acetaminophen, to be processed by the cytochrome P450 enzymes that display a promoted degree of activity when alcohol is consumed, such as cytochrome P450 2E1. When acetaminophen is metabolized by P450 2E1, it is oxidized to N-acetyl-p-benzoquinine-imine as illustrated in figure 4. This toxic intermediate can be excreted after it is conjugated to glucuronic acid and sulfate as displayed in the illustration below; however, the supply of glucuronic acid and sulfate may be depleted if this intermediate is produced in high concentrations. This intermediate will then start binding to cellular proteins and cause cell injury and death. [8]

Through inhibiting cytochrome P450 2E1, the formation of the toxic acetaminophen metabolite N-acetyl-p-benzoquinine-imine can be drastically reduced as shown in the study conducted by Hazai et al. [9]. In this study cytochrome P450 2E1 inhibitors 4-methyl pyrazol, Diethyl-dithiocarbamate, and disulfiram showed significant inhibition of the 2E1 isoform in a dose dependent manner. Disulfiram showed the most significant inhibition at about 20% at a 1 micromolar concentration and about 80% at a
50 micromolar concentration. This resulted in a reduction of the toxic metabolite produced from acetaminophen, NAPQI. [9]

Cytochrome P450 2E1 is also known to catalyze the activation of certain nitrosamines, such as N-nitrosodiethylamine and N-nitrosodimethylamine, into genotoxic compounds. Inhibition of these enzymes was shown to reduce the production of these compounds by approximately 60 percent. [10]

Cytochrome P450s can also be inhibited by certain foods, such as grapefruit. Grapefruit juice has displayed potent inhibition in assays for several cytochrome P450s including 3A4 and 2C9. Both Cytochrome P450 3A4 and 2C9 were inhibited by approximately 100% at 10% juice concentrations. This can be used to increase the bioavailability of certain medications, and in certain instances it can reduce the probability of ROS metabolites from cytochrome P450 metabolism. Kudzu extracts and products could potentially result in similar drug/natural product interactions. [11]

Cytochrome P450 2C9 is not the only isoform with the potential to experience drug/herb interactions. Cytochrome P450 2A6 metabolizes therapeutic agents such as halothane, valproic acid and losigamon, and cytochrome P450 2A6 can be significantly inhibited by chalepensin, a compound found in, *Rutagraveolens* L., Rue. Rue is commonly used in Asian cultures as an herbal remedy for a variety of ailments such as headache, wounds and rheumatics. Chalepensin was found to be a competitive inhibitor of CYP450 2A6, and the mechanism-based inhibitory properties displayed by the compound were significant enough to possibly result in chalepensin-induced toxicity.
through CYP2A6 inactivation, reaching up to over 60% inhibition at a 3 μM concentration of chalepensin. [12]

Similarly, cytochrome P450 1A2 can also be affected by drug/herb interactions. Nuciferine, a compound extracted from *Nelumbo nucifera* Gaertn leaves, has displayed modest, but significant inhibition of CYP450 1A2 both in vivo and in vitro. The IC50 value was determined to be 2.12 mmol/L in vitro; therefore, the inhibition of CYP450 1A2 was significant enough that it would increase the risk of toxicity from other CYP450 1A2 substrate drugs that may be co-administered. [13]

*Properties and uses of kudzu*

Kudzu is currently used in many parts of the world for a variety of reasons from antioxidant capabilities to anti-inflammatory properties. [6] We propose that certain health effects of kudzu could be the result of their ability to inhibit several different human cytochrome P450 enzymes such as cytochrome P450 2A6 and 2E1. In this regard, kudzu could be more efficiently utilized as a therapeutic agent if it was better known which individual cytochrome P450 enzymes are reacting with each of the chemical components of kudzu that contribute to the inhibitory effects.

The kudzu plant may be identified by looking for trifoliate, egg shaped, medium green leaves that are fuzzy to the touch. Kudzu leaves would be attached to long vines covered in small, bronze hairs. Trifoliate leaves are leaves that grow in sets of three from each node. The central leaf will have a leafstalk of about three quarters of an inch, and
the other leaves leafstalks will be much shorter. The seed pods look like hairy soy bean pods that are usually about two inches long. [7]

Kudzu has been well characterized with respect to its chemical composition as shown in figures 1, 2, and 3. [6] Puerarin is one of the most abundant compounds in kudzu root. Therefore, it is commonly used as a standard for quality control of medicinal materials produced from kudzu. This is part of the reason why puerarin was the first compound that was selected for testing in the current study. Puerarin has also been shown to possess antioxidant properties, hepatoprotective properties, anti-inflammatory properties, and etcetera. [6] A broad study was conducted to verify many of the beneficial effects attributed to puerarin. [6] The structure of puerarin is shown in table 1. The amount of different chemicals that are found in kudzu can vary to a substantial degree depending on where and when the plant is grown along with the preparation method used to produce the kudzu product; therefore, it is important to know the active constituents, so more accurate quality control standards can be established for a given application. [6]

The expected impact of this project is to increase the efficiency of the use of kudzu products for medicinal purposes, such as antioxidant and anti-inflammatory effects. This is anticipated to be achieved by contributing to the current knowledge about how the plant chemically aides in treatment of ailments by studying the impact kudzu products, and known chemical components of kudzu, have on several cytochrome P450 enzymes. This project may also develop a more efficient procedure for the preparation of medicinal kudzu products.
<table>
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<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>A5</th>
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<tbody>
<tr>
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<td>H</td>
<td>H</td>
<td>Glc</td>
<td>H</td>
<td>H</td>
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<tr>
<td>Daidzein</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Daidzin</td>
<td>H</td>
<td>Glc</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Genistein</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
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<tr>
<td>Genistin</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Formononetin</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Me</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Me</td>
</tr>
</tbody>
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**Figure 1. Structure of Known Flavonoids Found in Kudzu**

H: Hydrogen, OH: hydroxyl, Glc: glucose
Figure 2. Structures of Known Glycosides Found in Kudzu

<table>
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<th>Compound</th>
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<tr>
<td>Pueroside A</td>
<td>Glc 6-Rha</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Pueroside B</td>
<td>Glc</td>
<td>Me</td>
<td>Glc</td>
</tr>
<tr>
<td>(±)-puerol B 2-0-glucopyranoside</td>
<td>Glc</td>
<td>Me</td>
<td>H</td>
</tr>
<tr>
<td>kuzubutenolide A</td>
<td>Glc</td>
<td>H</td>
<td>H</td>
</tr>
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Glc: glucose, H: Hydrogen, Me: Methyl, Rha: rhamnose
Figure 3. The Structures of Several Additional Notable Constituents in Kudzu

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<tr>
<th>Compound</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
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<tbody>
<tr>
<td>Kudzusapogenol A</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Kudzusapogenol C</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Kudzusapogenol A₁</td>
<td>Glc A²-ara²-rha</td>
<td>o-xyl</td>
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<td>OH</td>
</tr>
<tr>
<td>Kudzusapogenol A₂</td>
<td>Glc A²-gal</td>
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<td>OH</td>
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</tr>
<tr>
<td>Kudzusapogenol A₃</td>
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<td>Soyasapogenol B</td>
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Figure 4. Acetaminophen Metabolic Pathways Involving Cytochrome P450s
CHAPTER II
MATERIALS AND METHODS

**Preparation of Kudzu Extracts**

We have been utilizing a powdered kudzu root sample that was purchased from the Florida Herb House and grown in China. A simple procedure that was utilized to produce an ethanol extract from *Moringa oleifera* Lam. leafs for liver antioxidant studies was utilized for the current project as described below. [14] The procedure that was utilized began with obtaining about 10 grams of kudzu powder. The powder was inserted into a round bottom flask or jar with 100 mL of an 80 percent ethanol solution and a stir bar. The mixture was then allowed to stir for approximately 1 hour. The mixture was filtered with a Büchner funnel and filter flask, and the solution was then taken to the rotary evaporator to remove the ethanol. The rotary evaporator was set to 87 degrees and approximately 1 rotation every second. Once the ethanol was removed, the remaining extract was weighed and added to the desired amount of distilled water, normally 10 milliliters, and warmed on a hot plate until the extract went into solution. The extract was stored in the freezer.

**Preparation of Kudzu Extract Fractions**

A sample of the crude ethanolic kudzu root extract was partitioned utilizing a 35cc Sep-pak C18 filter cartridge provided by the waters division of the millipore.
corporation. The crude extract sample was introduced into the filter, and 5 mL aliquots of varying concentrations of ethanol were eluted through the filter and collected to obtain the extract fractions. An aqueous fraction, 10%, 20%, 40%, 70%, and 100% ethanol solution was utilized to make six different extract fractions. The extracts were then concentrated or diluted to obtain the desired concentration of each fraction.

**Cytochrome P450 Assays**

*Cytochrome P450 2A6 Assay using fluorometric detection*

The cytochrome P450 2A6 fluorometric assay reactions consisted of 150 μL of a 100 mM phosphate buffer solution at a pH of 7.4, 50 μM of coumarin and 20 μg of microsomal protein. The mixture was vortexed for 10 seconds before adding 1mM NADPH. The mixture was then vortexed for 5 seconds and inserted into the incubator at 37 degrees. After the solution was incubated for 20 minutes, the mixture was removed from the incubator and 25 μL of cold 2M hydrochloric acid was added to terminate the reaction. The mixture was then vortexed for 30 seconds to ensure that the reaction was thoroughly terminated. Then 450 μL of chloroform was added to the mixture. The mixture was then vortexed for approximately 30 seconds, and centrifuged at 3000 rpm for 5 minutes. Then 300 microliters of the bottom layer was added to separate vials containing 1 mL of sodium borate buffer. The mixture was vortexed for 30 seconds, and then centrifuged for 5 minutes at 3000 rpm. Then 800 μL of the top layer was taken for testing in the fluorometer. Quantification of the 7-hydroxycoumarin product was accomplished using an excitation wavelength of 370 nm, and by monitoring emission
from 400-550 nm. Due to inconsistencies related to the extraction required in this method, an alternative method was subsequently developed utilizing direct HPLC separation and detection of the product described in the next section.

**Cytochrome P450 2A6 Assay using HPLC detection**

The cytochrome P450 2A6 HPLC based assays are identical in procedure to the cytochrome P450 2E1 reactions except the substrate used was coumarin and the reactions were quenched with 10\(\mu\)L of 70% perchloric acid, placed on ice for 10 minutes, and then centrifuged for 5 minutes at 3000 rpm. Then the liquid samples were transferred to HPLC minivials for testing. The HPLC flow rate was 1.8 mL/minute. The LC run time was 9.05 minutes, the detector wavelength was 320 nm, and the mobile phase used was 20% acetonitrile.

**Cytochrome P450 2E1 Assay**

The cytochrome P450 2E1 reactions consisted of 130 \(\mu\)L of distilled water, 100 mM phosphate buffer at a pH of 7.4, 10 \(\mu\)g of microsomal protein, and 10 \(\mu\)M PNP. The mixture was then vortexed for 10 seconds, and 1 mM NADPH was added to reach a final volume of 200 \(\mu\)L. The mixture was then incubated at 37 degrees Celsius for 40 minutes. The vials were removed, and 400 \(\mu\)L of 3 percent, cold perchloric acid was added. The vials were placed in ice for 10 minutes, and then the vials were centrifuged for 5 minutes at 3000 rpm. A 500 \(\mu\)L aliquot of the sample was transferred to HPLC vials to be analyzed. The column used for all HPLC assays was a C\(_{18}\) column with a 5 \(\mu\) pore size and measured 15 cm by 4.6 mm. The flow rate for the 2E1 assay was 1.5 mL/minute,
with run times of 12.5 minutes and the detection wavelength was 340 nm, and the mobile phase was 20% acetonitrile.

Cytochrome P450 1A2 Assay

The cytochrome P450 1A2 HPLC based assay reactions consisted of 0.10 M phosphate buffer solution at a pH of 7.4, 97.5\(\mu\)M 7-ethoxycoumarin solution, and 20 \(\mu\)g of microsomal protein. The mixture was vortexed for 5 seconds before adding 1mM NADPH. The mixture was then incubated at 37 degrees Celsius. After the solution was incubated for 30 minutes, the mixture was removed from the incubator and 10\(\mu\)L of 70\% perchloric acid was added to terminate the reaction. The mixture was then vortexed for 5 seconds, set in ice for 10 minutes, and then centrifuged for 5 minutes at 3000 rpm. Then the liquid samples were transferred to HPLC minivials for testing. In this assay, the product was identical to that formed in the 2A6 assay, so HPLC conditions used were the same as those described in the previous section except the LC and detector run times were 16 minutes to allow enough time for the substrate to elute from the column.

Cytochrome P450 2C9 Assay

The cytochrome P450 2C9 HPLC based assay reactions consisted of 0.10 M phosphate buffer solution at a pH of 7.4, 60\(\mu\)M dichlofenac, and 20 \(\mu\)g of microsomal protein. The mixture was vortexed for 5 seconds before adding 1mM NADPH. The mixture was then inserted into the incubator at 37 degrees Celsius. After the solution was incubated for 40 minutes, the mixture was removed from the incubator and 100\(\mu\)L of a 94\% acetonitrile, 6\% glacial acetic acid solution was added to terminate the reaction. The
mixture was then vortexed for 5 seconds, set in ice for 10 minutes, and then centrifuged for 5 minutes at 4000 rpm. An aliquot of the liquid samples were transferred to HPLC minivials for analysis. The mobile phase utilized for this procedure was 50% acetonitrile with a 20 mM phosphoric acid concentration. The LC and detector run times were 15 minutes with a flow rate of 1 mL per minute and a detection wavelength of 220 nm.

**Inhibition studies**

To probe for inhibition, the assays described above were carried out in the presence of various kudzu extracts along with the compound puerarin to assess the ability of each to interfere with cytochrome P450 metabolism. Concentrations used in the individual experiments are described in the results section.
CHAPTER III
RESULTS AND DISCUSSION

**Effects of Kudzu Extracts and Puerarin on Rabbit Liver Cytochrome P450s**

*Inhibition of rabbit liver P450 2E1*

The rabbit enzyme model was chosen initially as a model for the human cytochrome P450 activity based on its availability and accepted similarity to the human enzyme.

*Inhibition of Rabbit Liver P450 2E1 by an Ethanolic Kudzu Root Extract*

The 2E1 isoform of P450 is important toxicologically because of its ability to activate toxins, thus it was the first isoform chosen for evaluation of the inhibitory effects of kudzu extracts and puerarin.
Figure 5. Dose Dependent Effects of an Ethanolic Kudzu Root Extract on the Metabolism of p-nitrophenol by Cytochrome P450 Enzymes Found in Rabbit Liver

The ethanol extract from kudzu root powder was examined first, and as shown in figure 5, this extract produced modest inhibition of cytochrome P450 2E1 activity at concentrations ranging from 20-201\,\mu g/mL. Approximately 13\%, of the activity was lost when a 20 \,\mu g/mL concentration was included in the reaction, and this inhibition increased to approximately 45\% at the highest dose utilized, 201\,\mu g/mL.
Inhibition of Rabbit Liver P450 2E1 by a Kudzu Root Extract Prepared Utilizing Chloroform

2E1 Inhibition by Kudzu Chloroform Extract

Figure 6. The Results from a Rabbit Cytochrome P450 2E1 Dose Dependent Assay Conducted in the Presence of a Kudzu Root Extract Prepared Utilizing Chloroform Rather than Ethanol

No inhibition was observed in the chloroform extract, as shown in figure 6. Chloroform is a very non-polar solvent that is used to extract non-polar constituents of the plant, and since P450 active sites tend to be non-polar, generally good inhibitors are isolated from this fraction. The data shows that there are no non-polar constituents in the kudzu extract with strong P450 inhibitory properties. Although the inhibition was not dramatic, it is significant that at the lowest dose utilized, there was measureable inhibition. The dose response curve was not typical of a competitive reversible inhibitor,
as one would expect a larger effect upon increasing the concentration by 10-fold (200 μg/mL).

*Inhibition of Rabbit Liver P450 2E1 by Puerarin*

**2E1 Inhibition by Puerarin**

![Graph showing inhibition of P450 2E1 by Puerarin](image)

*Figure 7. The Effects of Puerarin on Cytochrome P450 2E1 Metabolism was Studied through a Dose Dependent Assay Utilizing Rabbit Liver Microsomes*

Minor inhibition was exhibited, though only 25%, in the presence of puerarin at a 1mM concentration. This concentration is extremely high, and unlikely to be of any physiological significance indicating a P4502E1-puererin interaction in vivo is highly unlikely. The results shown in figure 7 involved the rabbit isoform; however, a
comparison with the human P4502E1 is necessary before a conclusive statement regarding drug interactions in the human body could be made.

**Inhibition of rabbit liver P450 2A6**

**Inhibition of Rabbit Liver P450 2A6 by an Ethanolic Kudzu Root Extract**

The 2A6 isoform of P450’s has similar importance toxicologically as the 2E1 isoform because of its analogous ability to activate toxins, thus it was the second isoform chosen for evaluation of the inhibitory effects of kudzu extracts and puerarin.

**2A6 Inhibition by an Ethanolic Kudzu Extract**

![Graph showing 2A6 Inhibition by an Ethanolic Kudzu Extract](image)

**Figure 8. The Results from a Dose Dependent Rabbit Cytochrome P450 2A6 Assay Performed in the Presences of an Ethanolic Kudzu Root Extract**
No inhibition was displayed in the presence of the ethanolic kudzu root extract up to 35 mg/mL; however, at the highest concentration used 150 mg/mL a substantial reduction in activity was observed, suggesting that certain components of the kudzu are capable of inhibiting this isoform. The average background activity was removed from these results, so the apparent negative activity at the highest concentration represents close to complete inhibition. Based on the results shown in figure 8, the rabbit P450 2A6 is not significantly inhibited by the ethanolic kudzu root extract, as even 35 mg/mL is extremely high for physiological significance.
Inhibition of Rabbit Liver P450 2A6 by Puerarin

Figure 9. Results Obtained from a Dose Dependent Assay Testing the Effects of Puerarin on Rabbit Cytochrome P450 2A6

Experiments were carried out in duplicate, and the results of each individual trial are shown in figure 9. The results displayed minor inhibition. Approximately 30% inhibition in the presence of puerarin at a 0.231 mM concentration with the peak area decreasing from about 68K to 48K was observed and ~66% inhibition in the presence of puerarin at a 1mM concentration with the peak area decreasing from 68K to 25K.
Inhibition of rabbit liver P450 1A2

Inhibition of Rabbit Liver P450 1A2 by an Ethanolic Kudzu Root Extract

The 1A2 isoform of P450’s has similar importance toxicologically as the 2E1 and 2A6 isoform because of its analogous ability to activate toxins, thus it was the second isoform chosen for evaluation of the inhibitory effects of kudzu extracts and puerarin.

**Figure 10. Effects of Varying Concentrations of an Ethanolic Kudzu Root Extract on 7-ethoxycoumarin Metabolism by Cytochrome P450 1A2 Enzymes Found in Rabbit Liver**

As displayed in figure 10, modest inhibition of cytochrome P450 1A2 was displayed in the presence of an ethanolic kudzu root extract. Roughly 25% inhibition was displayed at the lowest concentration, 20 µg/mL, with the peak area reducing from about 25,000 to 18,000 and increased to near 50% inhibition at the highest concentration, 201
µg/mL with the peak area reducing from about 25,000 to 13,000. Similar to the results in figure 5, the dose response curve is not typical of a competitive reversible inhibitor. Based on the observed results, it is apparent that the 1A2 isoform is more susceptible to inhibition by the ethanolic kudzu extract than the 2E1 isoform.

**Inhibition of Rabbit Liver P450 1A2 by a Kudzu Root Extract Prepared Utilizing Chloroform**

![Graph](image)

**Figure 11. Effects of Varying Concentrations of a Kudzu Extract Prepared Utilizing Chloroform on 7-ethoxycoumarin Metabolism by Cytochrome P450 1A2 Enzymes Found in Rabbit Liver**

Figure 11 shows minor inhibition of approximately 29%, at 201 µg/mL; whereas, the ethanolic kudzu root extract achieved this at approximately a 10 fold less concentration. The ethanolic kudzu root extract also started to show notable inhibition at a much lower concentration. The chloroform kudzu root extract only showed 4%
inhibition at 20 µg/mL, and the ethanolic kudzu root extract showed about 25% inhibition at the same concentration.

**Inhibition of Rabbit Liver P450 1A2 by Puerarin**

![1A2 Inhibition by Puerarin](image)

**Figure 12. The Effects of Varying Concentrations of Puerarin on 7-ethoxycoumarin Metabolism by Cytochrome P450 1A2 Enzymes Found in Rabbit Liver**

As shown in figure 12, the rabbit liver cytochrome P450 1A2 isoform displayed 3% inhibition in the presence of a 50 µM concentration of puerarin. Considering this is a pure compound, puerarin seems as though it would have a minor impact on the total inhibitory potential against rabbit liver cytochrome P450 1A2 metabolism displayed by any extract.
**Inhibition of rabbit liver P450 2C9**

The 2C9 isoform of P450’s has a different importance toxicologically compared to the other isoforms tested previously. This isoform metabolizes many pharmacological and commonly consumed compounds. Some of these compounds have been known to be commonly involved in drug-drug and drug-herb interactions such as warfarin. For this reason, it was a good candidate for evaluation of the inhibitory effects of kudzu extracts and puerarin.

**Inhibition of Rabbit Liver P450 2C9 by an Ethanolic Kudzu Root Extract**

![Graph showing 2C9 Inhibition by an Ethanolic Kudzu Extract](Figure 13. The Effects of an Ethanolic Kudzu Root Extract on Dichlofenac Metabolism by Cytochrome P450 2C9 Utilizing Rabbit Liver Microsomes)
A significant amount of inhibition was displayed as illustrated in figure 13, 74% inhibition, in the presence of the ethanolic kudzu root extract at a concentration of 20 μg/mL. The interaction has an IC50 value of approximately 15 μg/mL.

Inhibition of Rabbit Liver P450 2C9 by a Kudzu Root Extract Prepared Utilizing Chloroform

2C9 Inhibition by Kudzu Chloroform Extract

![Graph depicting inhibition by Kudzu Chloroform Extract](image)

Figure 14. The Effects of Varying Concentrations of a Kudzu Extract Prepared Utilizing Chloroform on Dichlofenac Metabolism by Cytochrome P450 2C9 Enzymes Found in Rabbit Liver

Figure 14 displays minor inhibition. Roughly 12% inhibition was displayed at a 49 μg/mL concentration of the extract. This is approximately 6 fold less of an effect compared to the ethanolic kudzu root extract at a 20 μg/mL concentration. Once again the
ethanolic kudzu root extract displayed much more inhibitory potential than the kudzu chloroform extract.

Inhibition of Rabbit Liver P450 2C9 by Puerarin

Figure 15. The Effects of Puerarin on Dichlofenac Metabolism by Cytochrome P450 2C9 in Rabbit Liver Microsomes

Figure 15 illustrates minor inhibition. Approximately 18% inhibition was displayed, in the presence of puerarin at a concentration of 25 μM. Similar to the rabbit cytochrome P450 1A2 isoform, puerarin seems to be displaying a minimal contribution to the inhibitory potential displayed by any extract obtained from the kudzu root.
Effects of Kudzu Extracts and Puerarin on Human Liver Cytochrome P450 in Live S9 Fraction

After utilizing the rabbit liver model, the results were utilized to conduct a more targeted, activity guided study with the human liver model due to the more limited supply of enzymes. The human liver model was utilized to more accurately represent in-vivo testing with human subjects.

Inhibition of human liver P450 2E1

Inhibition of Human Liver P450 2E1 by an Ethanolic Kudzu Root Extract

Human 2E1 Inhibition by an Ethanolic Kudzu Extract

![Graph showing the effects of an Ethanolic Kudzu Root Extract on PNP Metabolism by Cytochrome P450 2E1]

Figure 16. The Effects of an Ethanolic Kudzu Root Extract on PNP Metabolism by Cytochrome P450 2E1
To more accurately investigate possible interactions the ethanolic kudzu root extract would exhibit in vivo with cytochrome P450 2E1, human liver microsome testing was carried out. As illustrated in figure 16, approximately 20% inhibition was displayed in the presence of 49 μg/mL of the crude ethanolic kudzu root extract. This is comparable to the results of 23% obtained utilizing rabbit liver enzymes.

**Inhibition of Human Liver P450 2E1 by Puerarin and a Kudzu Root Extract Prepared Utilizing Chloroform**

![Human 2E1 Screening](image)

**Figure 17. The Effects of Varying Concentrations of Puerarin and a Kudzu Extract Prepared Utilizing Chloroform on Dichlofenac Metabolism by Cytochrome P450 2C9 Enzymes Found in Human Liver**

To more accurately investigate possible interactions the kudzu root extract that was prepared utilizing chloroform would exhibit *in vivo* with cytochrome P450 2E1, human liver microsome testing was carried out. The results are shown in figure 17.
Approximately 12% inhibition was displayed in the presence of 49 µg/mL of the crude chloroform kudzu root extract, and approximately 18% inhibition was displayed in the presence of puerarin at a 25µM concentration. This is comparable to the results of 7% inhibition obtained utilizing rabbit liver enzymes with a 49 µg/mL concentration of the crude chloroform kudzu root extract. Puerarin was not used in the same concentration in the rabbit liver testing; however, there was also no significant inhibition displayed.

*Inhibition of human liver P450 2A6*

**Inhibition of Human Liver P450 2A6 by an Ethanolic Kudzu Root Extract**

Human 2A6 Inhibition by an Ethanolic Kudzu Extract

![Bar chart showing inhibition of coumarin metabolism by Cytochrome P450 2A6 utilizing human liver microsomes.](image)

**Figure 18. Effects of an Ethanolic Kudzu Root Extract on Coumarin Metabolism by Cytochrome P450 2A6 Utilizing Human Liver Microsomes**
A minor amount of inhibition was displayed, 15% inhibition, in the presence of the ethanolic kudzu root extract at a concentration of 49 μg/mL, as shown in figure 18. These results suggest that the human cytochrome P450 2A6 isoform is more susceptible to inhibition by compounds in this extract than the rabbit cytochrome P450 2A6 isoform. The rabbit liver cytochrome P450 isoform did not display inhibition with up to a 35 mg/mL concentration of the ethanolic kudzu root extract.

_Inhibition of Human Liver P450 2A6 by Puerarin and a Kudzu Root Extract Prepared Utilizing Chloroform_

**Figure 19. Effects of a Kudzu Root Extract Prepared Utilizing Chloroform on Coumarin Metabolism by Human Cytochrome P450 2A6**
The results illustrated in figure 19 display a minor amount of inhibition, 15% inhibition, in the presence of puerarin at a 101 \( \mu \text{M} \) concentration. Minor inhibition was also displayed by the kudzu root extract prepared utilizing chloroform, 16% inhibition at a 201 \( \mu \text{g/mL} \) concentration.

**Inhibition of human P450 1A2**

**Inhibition of Human P450 1A2 Supersomes by an Ethanolic Kudzu Root Extract**

**Human 1A2 Inhibition by an Ethanolic Kudzu Extract**

![Graph showing peak area vs. micrograms per milliliter](image)

*Figure 20. Effects of an Ethanolic Kudzu Root Extract on 7-ethoxycoumarin Metabolism by Cytochrome P450 1A2 Utilizing Human Supersomes*
The results from figure 10 prompted a comparison between results obtained from rabbit liver microsomes and supersomes; therefore, a dose dependent assay was conducted utilizing cytochrome P450 1A2 supersomes in the presence of the crude ethanolic kudzu root extract. Significant inhibition, approximately 61%, was displayed in the presence of the crude ethanolic kudzu root extract at 49 µg/mL. The IC50 value is approximately 17 µg/mL.

**Inhibition of Human Liver P450 1A2 by a Kudzu Root Extract Prepared Utilizing Chloroform**

**Human 1A2 Inhibition by Kudzu Chloroform Extract**

![Graph showing peak area vs. micrograms per milliliter for two trials](image)

**Figure 21. Effects of a Kudzu Root Extract Prepared Utilizing Chloroform on 7-ethoxycoumarin Metabolism by Human Cytochrome P450 1A2 Enzymes**
To more accurately predict any in vivo interactions, human liver microsomes were used to observe any interaction between a kudzu root extract prepared utilizing chloroform and human cytochrome P450 1A2. The results are illustrated in figure 21. Similar to the rabbit model, no significant inhibition was displayed. The human and rabbit liver models displayed 7% and 6% inhibition respectively in the presence of a 49 µg/mL concentration of the chloroform kudzu root extract; however, there was a notable difference between the rabbit and human liver microsomes at a 201 µg/mL concentration. The rabbit and human liver cytochrome P450 1A2 microsomes showed 30% and 19% inhibition, respectively, at a 201 µg/mL concentration of the chloroform kudzu root extract.
Inhibition of Human Liver P450 1A2 by Puerarin

Figure 22. Effects of Puerarin on Human Cytochrome P450 1A2 Metabolism of 7-ethoxycoumarin

To more accurately investigate possible interactions puerarin would exhibit in vivo with cytochrome P450 1A2, human liver microsome testing was carried out. As illustrated in figure 22, approximately 15% inhibition of human cytochrome P450 1A2 was displayed in the presence of puerarin at a 50 μM concentration. Puerarin has not shown significant inhibition across all studies. These results suggest that the human cytochrome P450 1A2 isoform is more susceptible to inhibition by the components within the ethanolic kudzu root extract and puerarin than the rabbit cytochrome P450
1A2 isoform. The human and rabbit liver microsomes showed about 15 and 3 percent inhibition, respectively, in the presence of puerarin at a 50 μM concentration.

**Inhibition of human P450 2C9**

**Inhibition of Human Liver P450 2C9 by an Ethanolic Kudzu Root Extract**

Human 2C9 Inhibition by an Ethanolic Kudzu Extract

![Figure 23. Effects of an Ethanolic Kudzu Extract on Human Cytochrome P450 2C9 Metabolism of Dichlofenac](image)

An assay with limited extract concentrations was performed to determine promising human cytochrome P450 interactions with the ethanolic kudzu root extract. As displayed in figure 23, modest inhibition was exhibited by human cytochrome P450 2C9 enzymes in the presence of an ethanolic kudzu root extract. Modest inhibition was observed at 49 μg/mL approximately 49% inhibition was displayed.
Inhibition of Human Liver P450 2C9 by an Ethanolic Kudzu Root Extract

Human 2C9 Inhibition by an Ethanolic Kudzu Extract

Figure 24. Effects of an Ethanolic Kudzu Extract on Human Cytochrome P450 2C9 Metabolism of Dichlofenac

After obtaining the results from figure 23, a dose dependent assay was performed to further investigate the potential inhibitory effects the ethanolic kudzu root extract may display against cytochrome P450 2C9 metabolism. The results are shown in figure 24. Modest inhibition was observed, approximately 35% inhibition was displayed at 49 \( \mu g/mL \), and about 41% inhibition was displayed at 201 \( \mu g/mL \). The difference in results at the 49 \( \mu g/mL \) concentration may be due to the fact that the batch of human liver...
microsomes was depleted with the test documented in figure 23 and a new batch was started.

_Inhibition of Human Liver P450 2C9 by Various Possible Inhibitors_

![Graph showing 2C9 Inhibition by the 70% Ethanolic Fraction](image)

**Figure 25. The Most Substantial Effects Observed From Any Ethanolic Kudzu Extract Fraction on Cytochrome P450 2C9 Metabolism of Dichlofenac**

The 2C9 isoform displayed modest inhibition under certain circumstances, so a more complete screening of the isoform was performed. The results from the most significant interaction are displayed in figure 25. Puerarin displayed no inhibition against the 2C9 supersome metabolism, and the chloroform extract showed little signs of inhibition. Significant inhibition, approximately 56%, was displayed at a 50 μg/mL concentration of the 70% ethanol fraction prepared from the crude ethanolic kudzu root extract. Minor inhibition was observed by the kudzu root extract prepared utilizing
chloroform, 16% at 201 μg/mL. No significant inhibition was displayed in the presence of puerarin. The kudzu extract prepared utilizing chloroform showed several notable interactions during cytochrome P450 testing. The kudzu root extract prepared utilizing chloroform displayed approximately 29% inhibition against rabbit liver cytochrome P450 1A2 metabolism of dichlofenac at a 201 μg/mL concentration and 12% inhibition of rabbit liver cytochrome P450 2C9 at a 29 μg/mL concentration.

Effects of Active Ethanol Fractions on Individually Expressed 1A2 and 2C9

Supersomes

The supersome model was utilized to further investigate the most significant interactions found in the human and rabbit liver models due to its high activity and the selective nature of the enzymes. Supersome samples only contained the human isoform of interest.
Inhibition of human liver P450 1A2 supersomes

Inhibition of Human Liver P450 1A2 Supersomes by an Ethanolic Kudzu Root Extract

1A2 Inhibition by Ethanolic Kudzu Extract Fractions

Figure 26. Effects of Several Ethanolic Kudzu Extract Fractions on Cytochrome P450 1A2 Metabolism of 7-ethoxycoumarin

Cytochrome P450 1A2 showed the second most potential for inhibition in the presence of the crude ethanolic kudzu root extract; therefore, a broad screening was conducted utilizing the ethanolic kudzu root extract fractions and isolated human liver supersomes. As shown in figure 26, the 20% ethanol fraction prepared from the crude ethanolic kudzu root extract showed the most potential for inhibition. Approximately 40% inhibition was displayed in the presence of 50 μg/mL of the 20% ethanol fraction. Modest inhibition was also displayed in the presence of the 70% ethanol fraction.
prepared from the crude ethanolic kudzu root extract. No significant inhibition was displayed by any other extract fraction.

*Inhibition of P450 1A2 Supersomes by the 20% Ethanol Fraction of an Ethanolic Kudzu Root Extract*

![Graph showing 1A2 Inhibition by the 20% Ethanol Fraction](chart.png)

**Figure 27. Effects of the Ethanolic Kudzu Extract Fraction with the Most Substantial Interaction on Human 1A2 Supersome Metabolism of 7-ethoxycoumarin**

The results presented in figure 27 prompted a dose dependent assay to investigate the affects that the 20% ethanol fraction prepared from the crude ethanolic kudzu root extract would have on cytochrome P450 1A2 metabolism. Supersome samples were utilized for this assay. Modest inhibition of the cytochrome P450 1A2 supersomes was exhibited in the presence of this fraction obtained from the crude ethanolic kudzu root extract; approximately 43% inhibition was displayed at 50 µg/mL. Approximately 65%
inhibition was observed at the highest concentration, 200 μg/mL. The IC50 value is approximately 40-90 μg/mL. The crude ethanolic extract inhibits 50% at 20 μg/mL, while the most active fraction (20%) only shows 43% inhibition at 50 μg/mL.

**Inhibition of P450 2C9 supersomes**

**Inhibition of P450 2C9 Supersomes by Ethanolic Kudzu Root Extract Fractions**

2C9 Ethanolic Kudzu Extract Fraction Screening

![Figure 28. Effects of Several Ethanolic Kudzu Extract Fractions on Cytochrome P450 2C9 Metabolism of Dichlofenac](image)

To further investigate the results from figure 28, an activity guided partial fractionation was conducted utilizing the ethanolic kudzu root extract. The extract fractions from the crude ethanolic kudzu root extract were obtained to enrich and further
isolate compounds responsible for the inhibition observed by the crude ethanolic kudzu root extract. A screening of all extract fractions was performed. Minor inhibition was displayed in the presence of the aqueous fraction and the 10% ethanol fraction prepared from the crude ethanolic kudzu root extract, approximately 14% inhibition at an 11 \( \mu \text{g/mL} \) concentration.

*Inhibition of P450 2C9 Supersomes by Ethanolic Kudzu Root Extract Fractions*

**2C9 Ethanolic Kudzu Extract Fraction Screening**

![Graph showing peak area vs. micrograms per milliliter/fraction](image)

*Figure 29. Effects of Several Ethanolic Kudzu Extract Fractions on Cytochrome P450 2C9 Metabolism of Dichlofenac*

To further investigate the results from figure 24, an activity guided partial fractionation was conducted utilizing the ethanolic kudzu root extract. As shown in figure
29, the extract fractions from the crude ethanolic kudzu root extract were obtained to enrich and further isolate compounds responsible for the inhibition observed by the crude ethanolic kudzu root extract.

A screening of all extract fractions was performed. Out of all of the extract fractions prepared from the crude ethanolic kudzu root extract, the 70% ethanol fraction displayed the most inhibition, 32% inhibition at 50 μg/mL.

**Inhibition of P450 2E1 supersomes**

**Validation of Puerarin Data Using Supersomes**

![2E1 Supersome screening Puerarin](image)

**Figure 30. Effects of Puerarin on Human Cytochrome P450 2E1 Supersome Metabolism of PNP**

For comparison, a screening of the 2E1 isoform was conducted with supersomes as well. As shown in figure 30, approximately 37% inhibition was displayed in the
presence of puerarin at a 49 μg/mL concentration. This indicates that the supersomes for the 2E1 isoform are also more susceptible to inhibition by this compound than the 2E1 human liver microsomes. No inhibition was displayed when investigating this interaction with human liver microsomes. Puerarin showed little notable inhibition throughout all studies. The most notable interactions involving puerarin were the human liver cytochrome P450 1A2 interaction which displayed about 15% inhibition at a 50 μM concentration, the rabbit liver cytochrome P450 2E1 isoform which displayed about 25% inhibition at a 1mM concentration, and the rabbit liver cytochrome P450 2A6 interaction which displayed 30% inhibition at a 0.231 mM concentration.
Table 1. Rabbit Liver Microsome Results

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Puerarin</th>
<th>Chloroform extract</th>
<th>Ethanol Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Puerarin did not show signs of inhibition.</td>
<td>~29% Inhibition at 201 micrograms/mL</td>
<td>~25% inhibition at 20 micrograms/mL</td>
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<tr>
<td>2C9</td>
<td>The dose dependent assay showed no signs of inhibition.</td>
<td>~12% inhibition at 49 micrograms/mL</td>
<td>Complete inhibition was observed at 49 micrograms/mL</td>
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<tr>
<td>2E1</td>
<td>~25% Inhibition at 1 mM</td>
<td>No Inhibition</td>
<td>~25% inhibition at 20 micrograms/mL</td>
</tr>
<tr>
<td>2A6</td>
<td>30% at .231 mM</td>
<td>The preliminary screening indicated that this potential interaction was not worth further investigation.</td>
<td>No Inhibition up to 35 mg/mL</td>
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</table>
Table 2. Human Liver/Supersome Results

<table>
<thead>
<tr>
<th>Human/Supersomes</th>
<th>Puerarin</th>
<th>Chloroform extract</th>
<th>Ethanol Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>~15% inhibition at 50 micromolar concentration</td>
<td>The preliminary screening indicated that this potential interaction was not worth further investigation.</td>
<td>~ 61% inhibition at 49 g/mL, IC50 ~17 g/mL (supersomes)</td>
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<tr>
<td>2C9</td>
<td>The preliminary screening indicated that this potential interaction was not worth further investigation.</td>
<td>The preliminary screening indicated that this potential interaction was not worth further investigation.</td>
<td>~35% inhibition at 49 micrograms/mL</td>
</tr>
<tr>
<td>2E1</td>
<td>The preliminary screening indicated that this potential interaction was not worth further investigation.</td>
<td>The preliminary screening indicated that this potential interaction was not worth further investigation.</td>
<td>~12% inhibition at 201 micrograms/mL(supersomes)</td>
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<tr>
<td>2A6</td>
<td>The preliminary screening indicated that this potential interaction was not worth further investigation.</td>
<td>The preliminary screening indicated that this potential interaction was not worth further investigation.</td>
<td>The dose dependent assay showed no signs of inhibition.</td>
</tr>
</tbody>
</table>
Table 3. Human Liver/Supersome Results Continued

<table>
<thead>
<tr>
<th>Human/Supersomes</th>
<th>Ethanol Extract Fractions</th>
<th>Ethanol Extract Fractions of Interest</th>
<th>Ethanol Extract Additional Fractions of Interest</th>
<th>Initial Screening of Extracts</th>
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</thead>
<tbody>
<tr>
<td>1A2</td>
<td>20% fraction ~40% inhibition at 50 micrograms/mL</td>
<td>20% fraction ~43% inhibition at 50 micrograms/mL IC50 ~ 40 micrograms/mL (supersomes)</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>2C9</td>
<td>The other Ethanol Extract fractions showed little to no inhibition.</td>
<td>~32% inhibition at 50 micrograms/mL</td>
<td>~14% inhibition at 11 micrograms/mL</td>
<td>~49% inhibition at 49 micrograms/mL</td>
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<tr>
<td>2 E1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>~20% inhibition at 49 micrograms/mL</td>
</tr>
<tr>
<td>2A6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
</tbody>
</table>
Activity Guided Study Summary

Figure 31. Summary of Activity Data for All Models, Extracts, and Pure Compounds Tested

The isoforms in red displayed modest or significant interactions and the isoforms in green displayed little to no interaction.
The data gathered from the assays represented in figure 33 shows that the ethanolic kudzu root extract is a slightly more potent inhibitor of Cytochrome P450 2E1 enzymes than Puerarin alone. Cytochrome P450 1A2 and 2E1 displayed modest dose dependent inhibition in the presence of the ethanolic Kudzu root extract. Throughout the study multiple inhibitory interactions were observed; however, the two most substantial interactions were the reactions the 1A2 and 2C9 isoforms had in the presence of the ethanolic kudzu root extract. A comparison of the inhibition observed by all four isoforms in the presence of the different microsomes indicates that the supersomes have the most susceptibility to the inhibitory properties displayed by these extracts, and the rabbit liver microsomes displayed the least susceptibility to any inhibitory properties displayed by these extracts.

The chloroform extract showed little inhibitory potential across the entire study. This is unexpected because P450 sites are generally non-polar, and the chloroform extract would have contained the more non-polar compounds from the kudzu root samples. Puerarin also displayed little inhibitory potential throughout the study. Puerarin was expected to display more inhibition because it is used as a standard for quality control in many kudzu products. The 1A2 supersomes also displayed substantially more susceptibility to the ethanolic kudzu root extract than the human liver microsomes and the rabbit liver microsomes. From the results obtained, kudzu appears to be safe for human consumption.
REFERENCES


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