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Cytochrome P450 enzymes (CYP450) are the prominent member of a family of xenobiotic metabolizing enzymes that are collectively referred to as Phase 1 enzymes (1). Phase 1 enzymes are responsible for taking reactive oxygen molecules and introducing them to inactive nonpolar substrates. By doing this, the substrate becomes more polar which allows it to be easily excreted from the body (1). They catalyze a monooxygenase reaction that involves a substrate (RH) that is oxidized by the addition of one oxygen from molecular oxygen (O_2). These redox reactions can result in the production of reactive oxygen species which can cause oxidative stress in cells (1).

This study was designed to better understand the effect that essential oils had on the activity of Cytochrome P450 2C9. The goal was to determine the potency of essential oils that caused the activity of CYP2C9 to drop to 50% or lower. An initial screening process was done on all sixty essential oils to see which oils inhibited the activity of CYP2C9 by 50% or more. The screening process found that there were seventeen oils that had the potential to be potent inhibitors of CYP2C9. Those oils were: allspice, bergamot, cardamom, carrot seed, cinnamon leaf, citronella, clary sage, clove bud, elemi, eugenol, geranium bourbon, ginger, neroli, oregano vulgare, and vanilla absolute.

The seventeen oils that decreased the activity of CYP2C9 to 50% or less were then analyzed using Michaelis-Menten kinetics to determine the Ki of the oils. Of the seventeen oils tested, elemi had the lowest K_i values of 4.7 µg/mL and ginger had the highest K_i of 250 µg/mL. The Michaelis-Menten kinetics also allowed for the type of inhibition to be classified as well. Following the Michaelis-menten kinetics, reversibility studies and rate of inactivation studies were done on select essential oils to further classify the oils.

INHIBITION OF CYTOCHROME P450 2C9 BY ESSENTIAL OILS

by

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CHAPTER I

INTRODUCTION: CYTOCHROME P450'S

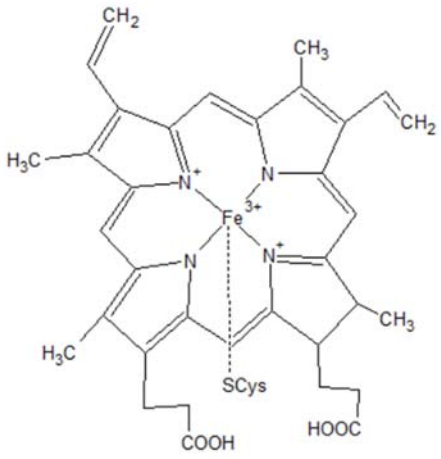
I.A. Cytochrome P450 Enzyme

Cytochrome P450's (CYP P450) are the prominent member of a family of xenobiotic metabolizing enzymes, collectively referred to as Phase 1 enzymes. Phase 1 enzymes are responsible for chemically transforming foreign species into more polar products that may be easily removed from the body (1). These reactions can occur by reduction, oxidation, cyclization, or decyclization. A problem that can be encountered by this metabolism is that cytochrome P450's convert inert xenobiotics and turn them into reactive compounds that in some cases are poisonous to the human body. Cytochrome P450's catalyze a monooxygenase reaction that involves a nonpolar substrate that is oxidized by the addition of one oxygen atom from molecular oxygen. In addition to reactive metabolites, these redox reactions can result in the production of reactive oxygen species which can lead to oxidative stress in cells (1). Reactive oxygen species include hydrogen peroxide, superoxide, hydroxyl radicals, and other related chemical species.

CYP 450's are part of a broader protein family called the heme proteins which are so-named because they use a heme prosthetic group to carry out oxidative chemistry (2). Figure 1 depicts what the heme center looks like. In the center of the hemeprotein there

is an iron atom that plays a key role in catalysis. The figure shows that the iron is in the center and bound to four surrounding nitrogens that are part of pyrrole rings. The iron center is also covalently bound to a cysteine ligand in the active site. In the resting state, there is also a water molecule bound to the iron center.

Figure 1. Cytochrome P450 Heme Iron III Center. This shows the covalent bond between the iron III center and the sulfur of the cysteine ligand. The four pyrole rings are shown, labeled A-D, as well as the four nitrogen ligands that surround the iron III canter (2).



Before the substrate can be hydroxylated the iron center needs to be prepared. Figure 2 shows the process of preparing the iron center. When cytochrome P450's are in the resting state, which has water present in the active site and all six ligands bound, the ferric iron center is in a low spin state. In this low spin state there is one unpaired electron. Once the substrate enters the active site, the water molecule is displaced which causes a change from a low spin state to a high spin state (4). When the iron center is shifted from a low spin state to a high spin state there is a splitting of d orbitals that occurs which results in three unpaired electrons as shown in Figure 3. After the substrate enters the active site and causes the ferric iron center to shift from a low spin state into a high spin state. This change in electronic state also causes a conformation change in the enzyme active site which causes the transfer of an electron from NADPH to a flavoprotein more favorable.

Figure 2. Oxidation and Reduction of the Iron Center of the Hemeprotein. This shows the progression of the preparation of the heme center in order for it to be able to interact with the substrate.

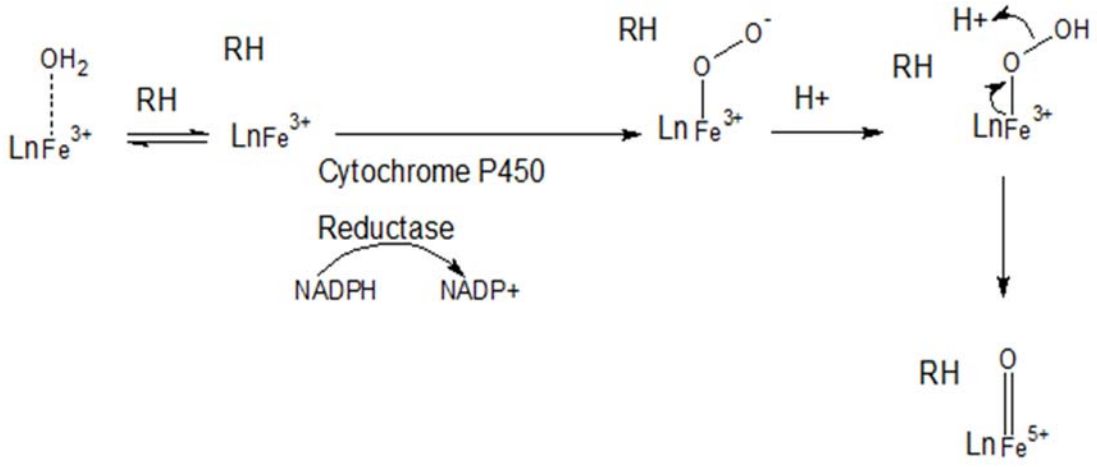
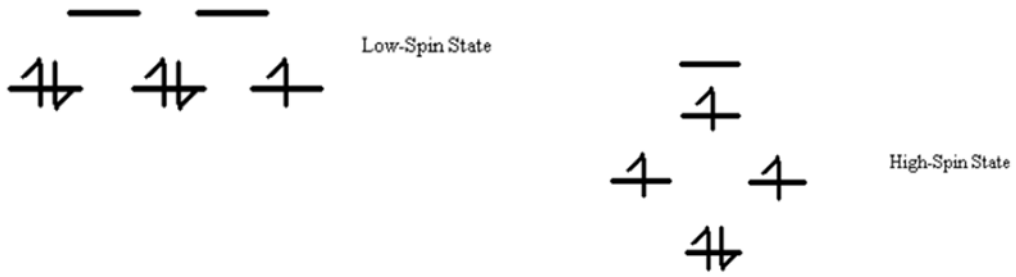


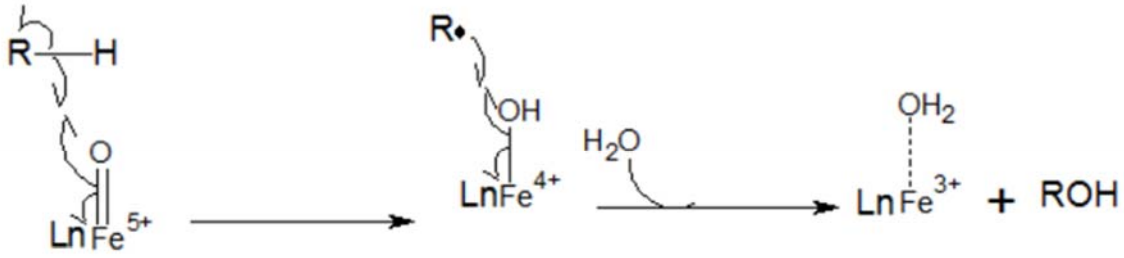
Figure 3. Difference between Low Spin and High Spin States. This shows what the spin state looks like when water is covalently bound to the heme center (low spin state) and when the water is displaced upon the substrate binding in the active site (high spin state).



Once in this high spin state, the ferric center can be reduced to a ferrous center by a single electron transfer reaction from a flavoprotein, which got the electron donated from NADPH. Once the ferric center is reduced to the ferrous center, molecular oxygen can bind to the ferrous center through a radical reaction, shown in Figure 2**.** Due to the proximity of the Cys ligand, molecular oxygen is more reactive than normal. Once molecular oxygen is bound, the ferrous iron is oxidized back to a ferric iron center (5). The next step in the preparation of the iron center is dealing with the radical charge on oxygen. A single electron transfer occurs between another flavoprotein, which got its electron from the same molecule of NADPH, and the radical oxygen. Then the negatively charged oxygen is reduced into a peroxo group. Next the peroxo group is either protonated twice by a water molecule present in the active site or one of the amino acid side chains surrounding it and oxidizes the iron center into a ferryl heme center. In

this ferryl state, the heme center can now interact with the nonpolar substrate. Once the ferryl heme center is present it immediately reacts with the nonpolar substrate via a radical mechanism, as shown in Figure 4 (6).

Figure 4. Interaction of the Ferryl Iron Center with the Nonpolar Substrate via a Radical Mechanism in which there is a Hydrogen Abstraction and Oxygen Rebound Reaction to form the Hydroxylated Product.

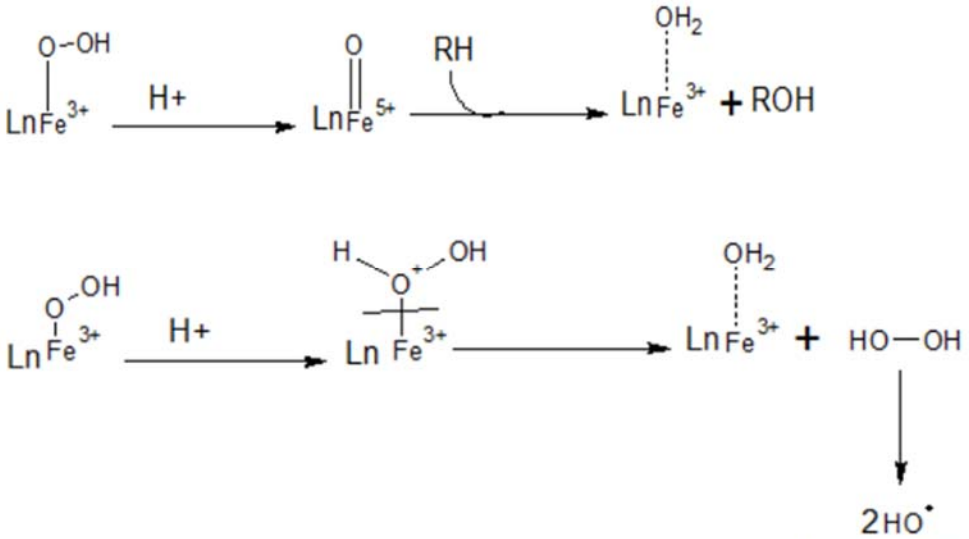


In step 1 the nonpolar substrate interacts with the ferryl heme center. In this reaction the single bond between the hydrogen and the rest of the nonpolar substrate splits, with one electron going to the adjacent carbon and the other forming a bond with the oxygen attached to the ferryl center. In order for the OH bond to be formed an electron has to be donated back to the heme center causing a reduction in the heme center. In step 2, the radical substrate is now going to interact with the OH group attached to the heme center by another radical mechanism. When the bond between the heme center and the OH group is broken, a new bond is formed between the substrate and the OH group and the heme center is again reduced to a ferric state (6). After the

hydroxylation of the substrate, a free water in the active site will covalently bond to the heme center and return the cytochrome P450 active site to its resting state.

All of the events described above refer to the productive pathway of cytochrome P450's, shown in Figure 4. In some instances there is a chance for an uncoupling reaction to happen, which results in the production of hydrogen peroxide. This path way is known as the unproductive pathway, shown in Figure 5 (7).

Figure 5. The Two Different Metabolic Pathways that can Occur in the CYP450's Catalytic Cycle. Top: the productive pathway which shows progression from the peroxo heme center to the hydroxylated product. Bottom: the nonproductive pathway which shows the progression from the peroxo heme center to the release of hydrogen peroxide and the CYP450 back in its resting state.



Hydroxide Radicals

This uncoupling pathway occurs when the oxygen attached to the ferric iron center is protonated instead of water being released from the iron center. When the oxygen directly connected to the iron center is protonated it causes the release of hydrogen peroxide. In order to return to the resting state a water molecule will come and bind to the heme center. Once hydrogen peroxide is released it can be degraded into to hydroxide radicals which causes oxidative stress. In some instances, this uncoupling reaction can occur when the substrate is interacting with the ferryl heme center in which the electron transfer is not coordinated correctly in the catalytic cycle (8).

Cytochrome P450's are responsible for metabolism of many xenobiotics that enter the body. However, this family of enzymes are also involved in the activation of inactive compounds that can potentially be toxic to the liver. An example of this is when acetaminophen is bioactivated into a reactive, N-acetyl-p-benzoquinonimine (NADPQI). NADPQI is a reactive metabolite that can bind to DNA, RNA, and proteins which leads to cellular necrosis (8).

I.B. Inhibition of CYP450 Enzymes

As stated previously, cytochrome P450 enzymes are responsible for detoxifying the body of xenobiotics, which includes the metabolism of pharmaceutical compounds (9). Like other enzymes present in the human body, the activity of CYP450 enzymes toward a specific substrate can be altered by the presence of a competing compound or related substrate. The focus of this study is on cytochrome P450 inhibition by nonpolar organic compounds found in various essential oils (9).

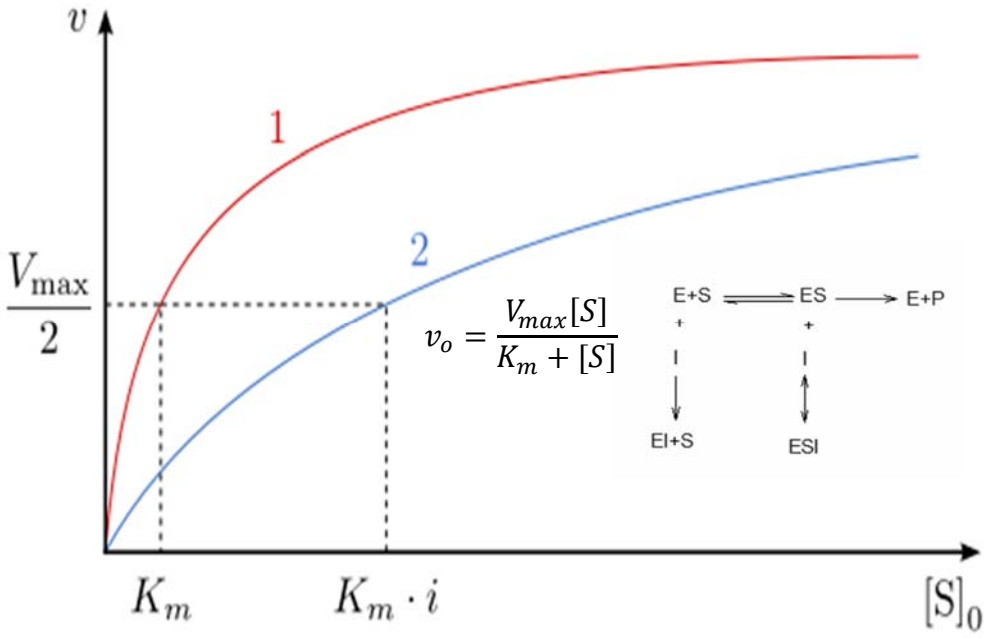
An inhibitor is any molecule that blocks the enzymatic metabolism of the desired substrate, which leads to a decrease in the overall CYP450 enzymatic activity. When the metabolism of a drug by CYP450 is inhibited it can lead to an increase in the drug's availability in the body and thus increasing the probability for adverse drug effects. How the inhibitor binds to the active site determines the type of inhibition that is occurring, which can fall in two categories: irreversible and reversible (9).

A reversible inhibitor binds to the CYP450 active site prior to the substrate being oxidized. The effects of a reversible inhibitor only last as long as the inhibitor is present in the active site (10). The way that a reversible inhibitor binds to the active site is usually through either lipophilic attractions to the amino acid side chains present in the active site or through hydrogen bonding. For example, imidazole derivatives typically have a tight binding affinity due to their higher nucleophilicity and thus have a higher attraction to the heme iron center when compared to the substrate. Alcohols and ketones tend to have weaker binding affinities for binding to the heme iron center and are classified as weak irreversible inhibitors (10).

Irreversible inhibitors often bind covalently to the enzyme. By binding covalently to the active site, the inhibitor chemically alters the enzyme structure which permeantly deactivates the enzyme. When the inhibitor is activated by the enzyme prior to inactivation, these inhibitors are referred to as "suicide inhibitors". Irreversible inhibitors of P450 enzymes usually involve chemical modification of the heme cofactor (11).

The Michaelis-Menten model is universally used to determine enzyme activities of CYP450 enzymes. As seen in Figure 6, the Michaelis-Menten model demonstrates the relationship between activity and the substrate concentration. The V_{max} represents the maximum rate of product formed after the saturation of all available enzymes by the substrate. The other variable that is able to be determined by this model is K_m, which represents the substrate concentration at V_{max}. The K_m shows where 50% of the substrate is bound to the enzyme. When an inhibitor is introduced to this model, it can be used to describe the nature of the interaction between CYP 450 enzymes and the molecules that inhibit the enzyme. There are several types of inhibition for CYP450 enzymes that can be shown by Michaelis-Menten kinetics: competitive inhibition, uncompetitive, and noncompetitive inhibition. Competitive inhibition is where the substrate and the inhibitor are competing for the same active site. When one binds it will block the other and vice versa (12). In noncompetitive inhibition, the inhibitor reduces the activity of the enzyme and will bind equally to the enzyme if it has bound substrate or not (12). Uncompetitive is the most common form and occurs when inhibitors bind only when the substrate is bound to the active site first.

Figure 6. This Shows the Michaelis-Menten Model of Enzyme Kinetics for Competitive Inhibition. This illustration shows the nonlinear relationship between reaction rate V and substrate concentration [S], along with the corresponding V_{max} and K_m constants.

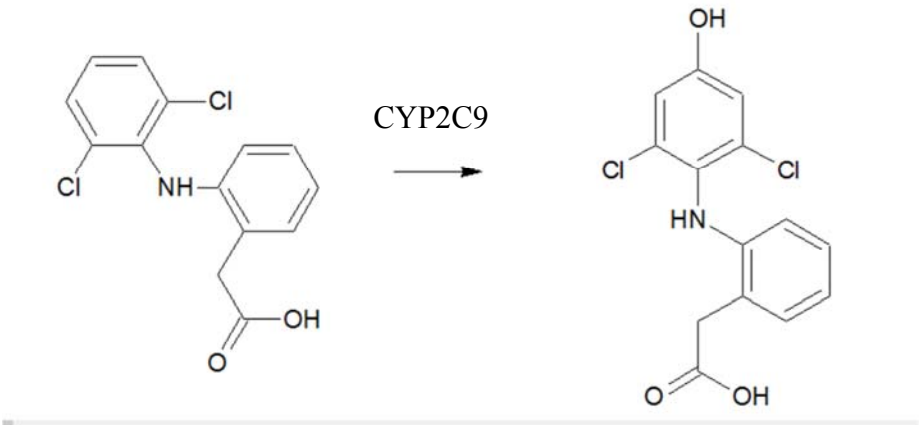


I.C. Cytochrome P450 2C9

Cytochrome P450 2C9 (CYP2C9) plays a key role in metabolizing xenobiotics and endogenous compounds. CYP2C9 constitutes approximately 18% of the cytochrome P450 enzymes found in the human liver. It has also been found that CYP2C9 is the second most common cytochrome P450 in the small intestine. Some of the drugs that are metabolized by CYP2C9 are ibuprofen, naproxen, warfarin, and hypoglycemic agents such as tolbutamide. Along with the other pharmaceuticals mentioned, CYP2C9 is also know to metabolize diclofenac. Diclofenac is a nonsteroidal anti-inflammatory drug that is typically prescribed for relieving moderate pain (13). After metabolism approximately

65% of the hydroxylated product is excreted in the urine after undergoing enterohepatic circulation (13). In the case of CYP2C9, diclofenac enters the active site of the enzyme and is hydroxylated in the four position, creating 4-hydroxydiclofenac. Figure 7 shows the reaction of diclofenac metabolism.

Figure 7. This Figure Shows the Metabolism of Diclofenac to 4-Hydroxydiclofenac by CYP2C9.

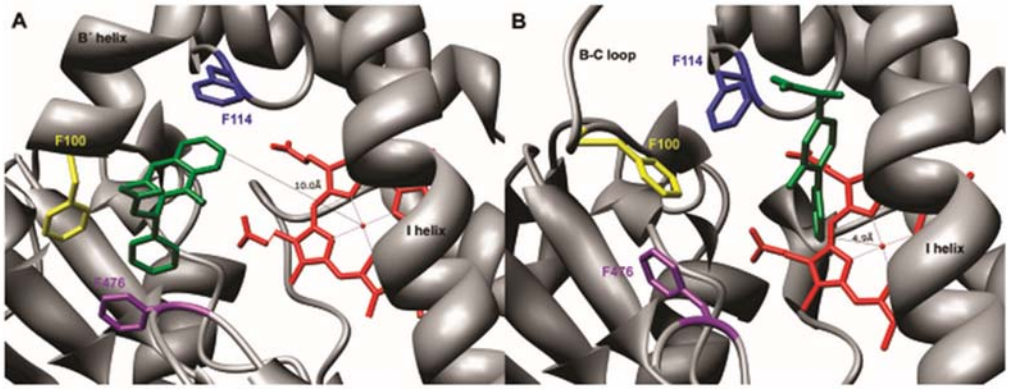


I.D. Cytochrome P450 2C9 Active Site

As mentioned before CYP2C9 is the second most prominent form of hepatic P450 and it has a preference for metabolizing weakly acidic substrates (14). Studies have been done in order to determine how substrates are arranged in the active site with relation to the heme center of the CYP450. In a study conducted by Mosher, the cytochrome P450 active site was mutated to see the relative effect on metabolism of warfarin and fluribprofen. The active site of most cytochrome P450's is very hydrophobic containing phenylalanine residues. In the study conducted by Mosher, site directed mutagenesis of

three key phenylalanine residues, F100, F114, and F476 was used to determine the functionality of the phenylalanine residues. This was done by single and double mutations of the three key residues previously mentioned to either lysine or tryptophan. Figure 8 shows the crystals structures of warfarin and fluribprofen bound to the CYP2C9 active site (wild type). It appears that the orientation of the substrate in the active site of CYP450 is related to the phenylalanine arrangement. The arrangement of the phenylalanine residues determined the distance that the substrate was in relation to the heme center, which is key for catalysis (14).

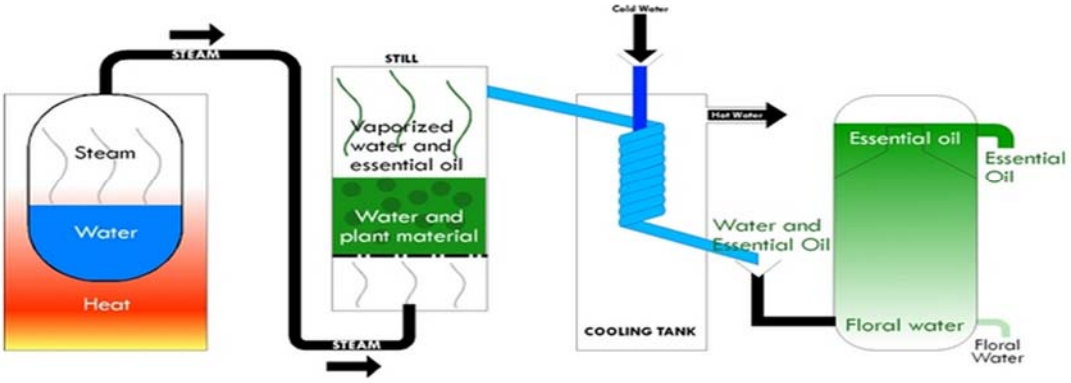
Figure 8. (S)-warfarin and Fluribprofen Bound in Crystal Structures. In panel A, the crystal structure for warfarin (1OG5) bound to the CYP2C9 active site. In Panel B, the crystal structure for fluribprofen bound to the CYP2C9 active site. The view is looking down the access channel to the heme center (red). The distances from the sites of oxidation to the heme iron are shown by dotted lines (14).



I.E. Essential Oils

Essential oils are plant derived oils used in the flavoring of foods and the cosmetics industries, in addition to their wide spread usage in natural therapies and supplements to treat or prevent a host of health conditions. Essential oils can be extracted by using one of seven methods: enfleurage, expressed oils, steam distillation, solvent extraction, fractional distillation and percolation, carbon dioxide extraction, and phytonic process (15). The most commonly used method is steam distillation. Figure 9 shows the process by which steam distillation is used to produce essential oils.

Figure 9. This Figure Depicts the Process by which Essential Oils are Produced via Steam Distillation.



First, the plant materials and water are mixed together. Then in a separate container, water is heated to produce steam which is shuttled into the container with the water and plant materials. The steam will eventually vaporize the water and the nonpolar organic molecules present in the plant materials. Once the nonpolar molecules and water are vaporized, they are sent through a series of condensing coils which facilitates the

formation of floral water and the essential oils. After condensing the mixture, they are transferred into a storage container which allows for the separation of the floral water and the essential oil. The essential oil mixture will be on top of the floral water since it is less dense. The essential oil mixture will be comprised mostly of volatile nonpolar compounds (15).

Essential oils have a wide variety of uses ranging from food flavoring to therapeutic treatments which allows for there to be many ways for them to be introduced into the body. There are four ways in which essential oils can be introduced into the body, inhalation, absorbing the oils the skin, absorbing the oils through orifices on the body, and through direct consumption of the oils (15). There are a variety of opinions on which way is best to administer the essential oils. Because essential oils are composed of a large number of mostly nonpolar molecules, they have the potential to act as inhibitors of human cytochrome P450's. The most common route for this to occur is via reversible binding of the inhibitor to the active site of the enzyme, taking the place of the substrate and thus competing for the active site (10). Depending on whether the P450 enzyme is taking part in a productive or nonproductive metabolic process, this inhibition could have a positive or negative health consequences. The broad goal of this study is to identify possible interactions between a large number of essential oils and the human CYP2C9.

CHAPTER II

MATERIALS AND METHODS

II.A. Reagents: Chemicals Purchased

The chemicals used during these experiments were purchased from different companies. The human liver microsomes were purchased from Xenotech. The diclofenac was purchased from Sigma. The p-Cymene was purchased from Aldrich. The limonene and eugenol (99%) were purchased from Acros Chemicals. All other essential oils were purchased from Birch Hill Happenings Aromatherapy. The monobasic and dibasic potassium phosphate were purchased from Carolina Biological Supply Company. The acetonitrile used for the mobile phase was purchased from Fischer. The NADPH was purchased from Research Products International.

II.B. Phosphate Buffer and NADPH Preparation

Potassium phosphate buffer was prepared by mixing monobasic potassium phosphate and dibasic potassium phosphate. A 1 M stock solution at pH 7.4 was used for all assays involved in this project. The NADPH was diluted with Nano-pure water and partitioned into 10 mM stock solution aliquots. The aliquots were stored in a freezer at -80^{o}C.

II.C. Human Liver Microsomes

Human liver microsomes were purchased from Molecular Toxcology Inc. They were put in 75 μL aliquots and stored in a freezer at -80^{0}C for usage.

II.D. Substrate Preparation

The reagent diclofenac was purchased from Sigma-Aldrich. 33.78 mg of diclofenac was weighed out and dissolved in 10 mL of deionized water to give 10 mM stock solution. This solution was then diluted 1:10, to give a final concentration of 1 mM stock solution of diclofenac, which was used as a substrate for all assays that involved CYP2C9 enzyme.

II.E. CYP2C9 Inhibition Assay Conditions

Enzyme kinetics studies were used to study the effects of essential oils on the activity of CYP2C9. The essential oils tested were: all spice, anise, bergamot, birch, camphor, cardamom, carrot seed, cedarwood, cinnamon leaf, citronella, clary sage, clove bud, p-Cymene, cypress, dill seed, douglas fir, elemi, eucalyptus lemon, eugenol, fennel, frankincense, geranium bourbon, ginger, grapefruit, hyssop, jasmine absolute, juniper berry, lavender, lemongrass, lime, mandarin, marjoram, Melissa pure, myrtle, myrrh, neroli, nutmeg, oregano vulgare, patchouli, pennyroyal, pepper (black), peppermint, petitgrain, pine needle, ravensara, roman chamomila, rose absolute, rosemary, rosemary verbenon, rosewood, sage, sandalwood, spearmint, spruce needle, tangerine, thyme borneal, vanilla absolute, wintergreen, and ylang ylang.

II.F. Initial Screening Assay for Inhibition of CYP2C9

The assay was used to measure the amount of inhibition of CYP2C9 in oxidizing diclofenac to 4-hydroxydiclofenac. The essential oil solutions were made by taking 1 µL of the oil being tested and dissolving it in 10 µL of deionized water. Conditions for the screening consisted of 8 µL of microsomes being incubated with 61 µL of deionized water, 100 mM phosphate buffer (7.4 pH), 50 µM of the substrate diclofenac (1mM stock solution), 1.0 mM NADPH, and 100 µL of the diluted essential oil being tested (actual concentrations reported based on density). Once all the components were mixed in a microcentrifuge tube, the reaction was incubated for 30 minutes at 37 ^{o}C. At the end of incubation, the reaction was quenched with 94%acetonitrile/6% glacial acetic acid solution and placed on ice for ten minutes to ensure that the reaction was stopped. Then the tubes were centrifuged at 13,000 rpm for 10 minutes. After centrifugation, 200 µL of the supernatant was placed in miniprep HPLC vials and analyzed by HPLC.

II.G. Michaelis-Menten Enzyme Kinetics Assay to Determine K_i for the Essential Oils

The Michaelis-Menten model of enzyme kinetics was used to determine the K_I for each oil, expressed as µg oil/ mL. Table 1 shows how the reactions were set up for each Michaelis-Menten study.

Table 1. Michaelis-Menten Enzyme Kinetics Assay. Reaction set up for the Michaelis-Menten enzyme kinetics.

Concentration of Substrate	DI water	Buffer (pH 7.4)	Microsome	NADPH	Inhibitor
0 μM	52 μL	100 mM	8 μL	1 mM	100 μL
25 μM	47 μL	100 mM	8 μL	1 mM	100 μL
55 μM	41 μL	100 mM	8 μL	1 mM	100 μL
125 μM	2 μL	100 mM	8 μL	1 mM	100 μL

The K_i values were found by plotting the relative activity (v) vs. the substrate concentration ([S]) at a range of increasing diclofenac concentration (0 μM to 125 μM) in the presence and absence of inhibitor. The concentration of the inhibitor was chosen based on the results of the screening experiments that gave >50% inhibition of CYP2C9. The Slide Write Plus program, by Advanced Graphics Software, Inc., was used to plot the relative activities vs. the concentration of diclofenac. The Michaelis-Menten equation (Equation 1) was used to determine the V_{max}, V_{max}^{app}, K_m, and K_m^{app}. The oils that showed >50% inhibition showed competitive inhibition and Equation 2 was used to solve for the K_i.

Equation 1. The Michaelis-Menten Equation.

$$v_o = \frac{V_{max}[S]}{K_m + [S]}$$

Equation 2. This equation was used to calculate the K_i by using the concentration of inhibitor used in the reaction

$$K_m{}^{app}/K_m = 1 + [I]/K_i$$

II.H. Two Stage Reversibility Studies for Binding to CYP2C9

Preliminary studies showed that there was definite inhibition of CYP2C9 in the presence of seventeen essential oils, the next phase was to look at how the enzyme was inhibited. A two phase method was used to determine if the inhibition present was reversible or irreversible. Table shows the parameters for the preincubation phase (stage 1). After the vials shown in Table are set up, they are preincubated at 37°C for ten minutes.

Table 2. Setup for the Preincubation Phase of the Reversibility Assay.

Vial 1	**Vial 2**	**Vial 3**
Microsomes 20 µL	Microsomes 20 µL	Microsomes 20 µL
Buffer 100 mM	Buffer 100 mM	Buffer 100mM
Inhibitor 40 µL	Inhibitor 40 µL	Inhibitor 40 µL
DI Water 20 µL	DI Water 40 µL	DI Water 70 µL
NADPH 1 mM		

After a ten minute preincubation, 40 µL from the stage 1 vials was transferred to the stage 2 vials of the experiment. In stage 2, the vials had 40 µL from each respective vial from stage 1, 11 µL of diclofenac, 20 µL of potassium phosphate buffer, 20 µL of NADPH, and 109 µL of deionized water, as shown in Table 3. Vial 1 in this experiment represented reaction conditions which would allow the inhibitors present in the essential oils to do damage to the cytochrome P450, if the reaction is irreversible then there would be no or a decreased amount of remaining activity toward the substrate when the 40 µL is transferred to stage 2, but if it happens to be reversible then there would be a fully activity with the substrate in stage 2 to the cytochrome P450. NADPH is added to vial 1 to see if the inhibition of the cytochrome P450 is dependent on NADPH. In vial 2, everything is the same as in vial 1, except that no NADPH is present. Vial 3 represents a control, with microsomes and buffer, when transferred to stage 2 the substrate should be able to bind to the cytochrome P450 and give full activity which can be compared to vial 1 and vial 2.

Table 3. This Table Shows How the Incubation Phase Reactions were setup for the Reversibility Studies. The stock solution consists of 1mM of NADPH, 100 mM of potassium phosphate buffer, 55 μM of diclofenac, and deionized water. The sock solution is made by taking the respective volumes mentioned and multiplying them by the number of vials for stage 2 of the reaction.

Vial 1a	Vial 1b	Vial 2a	Vial 2b	Vial 3a	Vial 3b
40 μL of preincub-ation solution 1	40 μL of preincub-ation solution 1	40 μL of preincub-ation solution 2	40 μL of preincub-ation solution 2	40 μL of preincub-ation solution 3	40 μL of preincub-ation solution 3
160 μL of the stock solution	160 μL of the stock solution	160 μL of the stock solution	160 μL of the stock solution	160 μL of the stock solution	160 μL of the stock solution

After the transfer of 40 μL of the preincubation reactions from stage 1 into the stage 2 vials, the reactions are incubated at 37^{o}C for ten minutes. After the ten minute incubation period, the reactions were quenched with 94% acetonitrile/6% glacial acetic acid solution. The quenched reactions were then put on ice for ten minutes to allow for any excess proteins and particulates to precipitate out of solution. Then the tubes were centrifuged at 13,000 rpm for 8 minutes. Then 200 μL of the supernatant was taken and analyzed by HPLC.

II.I. Rate of Inactivation Studies

Rate of inactivation studies were done to determine the rate at which the inhibitors present in the essential oils was deactivating the cytochrome P450 enzyme. Like the reversibility studies, there are two phases in which the reaction occurs: the preincubation phase (stage 1) and the incubation phase (stage 2). In stage 1 there are 2 vials, as shown is Table 4.

Table 4. This Table Shows How the Preincubation Phase (Stage 1) was setup for the Rate of Inactivation Reaction. Vial 1 contains NAPDH, inhibitor, microsomes, buffer, and water. Vial 2 contains buffer, deionized water, and microsomes.

Vial 1	Vial 2
Microsomes 80 µL	Microsomes 80 µL
Buffer 100 mM	Buffer 100 mM
Deionized water	Deionized water
NADPH 40 1 mM	
Inhibitor (varied based on inhibitor)	

The total reaction volume for the preincubation phase of the reaction. The vials in stage 1 were incubated for a total time of thirty minutes. At ten, twenty, and thirty minutes of the preincubation phase, 40 µL of the reaction was removed from the vial and placed in a vial for stage 2 (incubation phase). Table 5 shows the setup for the incubation phase of the rate of inactivation study. Once 40 µL of stage 1 were removed at the appropriate preincubation times and added to stage 2, the vials for stage 2 received 160 µL of stock solution added to them to bring the total reaction volume for the incubation phase to 200 µL. The stock solution consists of 20 µL of NADPH, 20 µL of potassium

phosphate buffer, 11 µL of diclofenac, and 109 µL of deionized water. The sock solution was made by taking the respective volumes mentioned and multiplying them by the number of vials for the incubation phase of the reaction. The vials were then incubated at 37^{o}C for thirty minutes. After the thirty minute incubation, the reactions were quenched with a 94% acetonitrile/ 6% glacial acetic acid solution and placed on ice for eight minutes. Then the vials were centrifuged at 13,000 rpm for 10 minutes. Then 200 µL of the supernatant was taken for HPLC analysis.

Table 5. This Table Shows How the Incubation Phase Reactions were setup for the Rate of Inactivation Studies. The stock solution consists of 1mM of NADPH, 100 mM of potassium phosphate buffer, 55 µM of diclofenac, and deionized water. The sock solution is made by taking the respective volumes mentioned and multiplying them by the number of vials for the incubation stage of the reaction.

Vial 1a	Vial 1b	Vial 2a	Vial 2b	Vial 3a	Vial 3b
40 µL of preincub-ation solution 1	40 µL of preincub-ation solution 1	40 µL of preincub-ation solution 2	40 µL of preincub-ation solution 2	40 µL of preincub-ation solution 3	40 µL of preincub-ation solution 3
160 µL of the stock solution	160 µL of the stock solution	160 µL of the stock solution	160 µL of the stock solution	160 µL of the stock solution	160 µL of the stock solution

II.J. HPLC Analysis

The reaction monitored in this study was the hydroxylation of diclofenac to 4-hydroxydiclofenac. This oxidation was monitored by HPLC (high-performance liquid chromatography). The analysis was conducted using a Shimadzu Automated liquid Chromatography system with UV detection set at 220 nm. The system was programed to inject 50 μL of each sample onto a C18 column (4.6x150 mm) with a flow rate of 1.0 mL/min. The mobile phase used for this method contained 20 mM phosphoric acid in a 50% acetonitrile/ 50% deionized water. The 4-hydroxydiclofenac peak for each reaction was integrated to give peak areas, where the area under the curve represents the relative activity of the CYP2C9 enzyme found in the human liver microsomes.

CHAPTER III

RESULTS AND DISCUSSION

III.A. Screening Assay Results

There were problems encountered in the initial screening of inhibitory activity caused by essential oils on CYP2C9 activity. Some oils contained constituents that has the same retention time as 4-hydroxydiclofenac. The original HPLC analysis used a mobile phase containing 50%acetonitrile/ 50% water with 0.1% trifluoracetic acid. While this mobile phase allowed for effective separation of the constituents, most of the essential oils had some constituents that had the same retention time as 4-hydroxydiclofenac. In order to increase the retention time of these essential oil constituents, the mobile phase was changed to be more acidic which would promote a longer retention time of the nonpolar molecules of the essential oils. Modifying the mobile phase allowed for better separation of the essential oil constituents and the product 4-hydroxydiclofenac. Figure 10 shows a chromatogram where the modified mobile phase was used. There is a distinct separation between the product 4-hydroxydiclofenac (shown at approximately 3.5 minutes) and the essential oil constituents of all spice oil (shown approximately 4.2 minutes).

The interaction of CYP2C9 with a wide range of essential oils led to the inhibition of this enzyme. This was shown by using an enzymatic assay in which the oxidation of diclofenac to 4'-hydroxydiclofenac was measured by HPLC analysis. The screening used to measure inhibition was successful in finding potent inhibitors which showed an inhibition of 50% or more when compared to a standard reaction. The essential oil solutions were prepared by taking 1 μL of oil and diluting it in 10 mL of DI water. Based on the measured density of each oil, this gave concentrations of the stock solutions ranging from 26 μg/mL to 88 μg/mL. In each inhibition reaction, 100 μL of the essential oil solutions were included in a 200 μL total reaction volume. Table 6 lists all of the essential oils and the amounts present in each inhibition reaction. Figures 11a, 11b, 11c, and 11d shows all the essential oils tested and their relative activity compared to an uninhibited reaction.

Figure 10. Chromatogram Showing the Separation when using the Modified Mobile Phase. An uninhibited reaction is shown in red and a reaction inhibited by all spice is shown in blue.

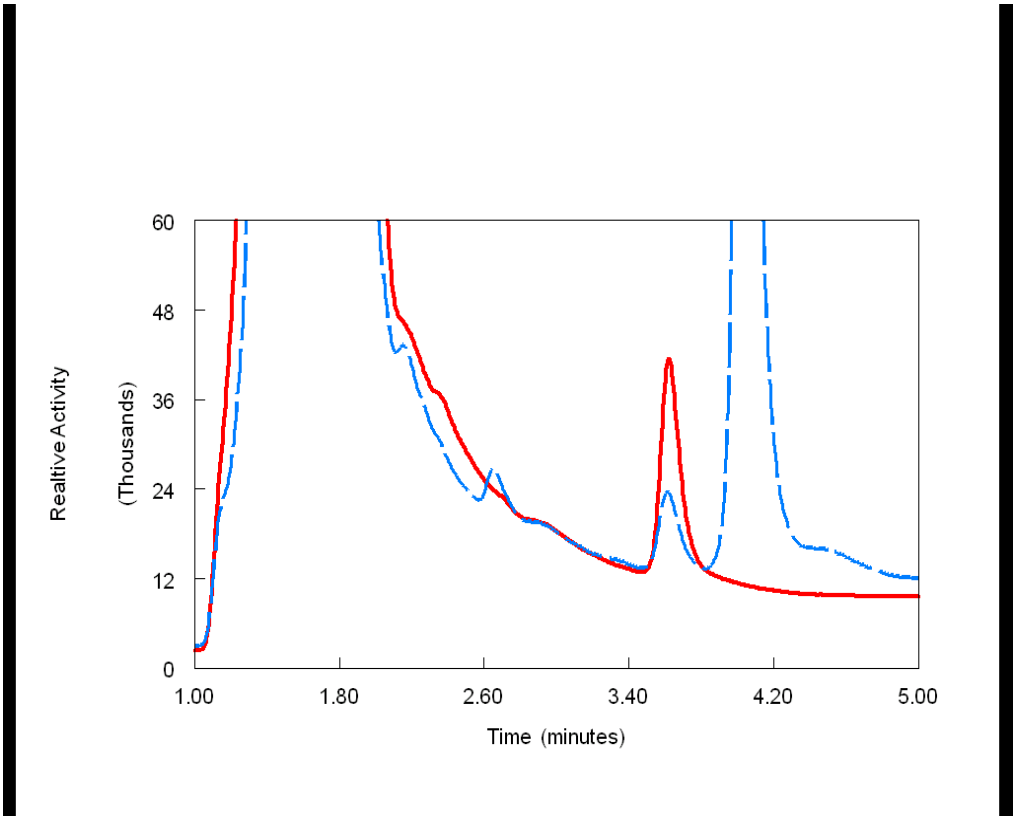


Table 6. This Table Lists all the Essential Oils tested and Their Respective Concentrations in each Reaction.

Essential Oil	Reaction Concentration (µg/mL)	Essential Oil	Reaction Concentration (µg/mL)
All Spice	51	Lime	88
Anise	61	Mandarin	42
Bergamot	37	Marjoram	50
Birch	66	Melissa Pure	63
Camphor	47	Myrtle	48
Cardamom	40	Myrrh	74
Carrot Seed	36	Neroli	39
Cedarwood	29	Nutmeg	46
Cinnamon Leaf	51	Oregano Vulgare	40
Citronella	42	Patchouli	36
Clary Sage	35	Pennyroyal	49
Clove Bud	45	Pepper (black)	33
Coriander	37	Peppermint	46
p-Cymene	36	Petitgrain	46
Cypress	36	Pine Needle	33
Dill Seed	33	Ravensara	46
Douglas Fir	36	Roman Chamomila	45
Elemi	50	Rose Absolute	50
Eucalyptus Lemon	48	Rosemary	61
Eugenol	60	Rosemary Verbenon	55
Fennel	43	Rosewood	36
Frankincense	36	Sage	33
Geranium Bourbon	36	Sandalwood	39
Ginger	45	Spearmint	41
Grapefruit	43	Spruce Needle	62
Hyssop	41	Tangerine	45
Jasmine Absolute	51	Thyme Borneal	78
Juniper Berry	52	Vanilla	53
Lavender	31	Wintergreen	68
Lemongrass	31	Ylang Ylang	44

Figure 11a. This Figure Shows the Essential Oils that Exhibited ≤20% Inhibition of CYP2C9 Activity. Those oils include nutmeg, lemongrass, marjoram, pine needle, jasmine absolute, hyssop and limonene.

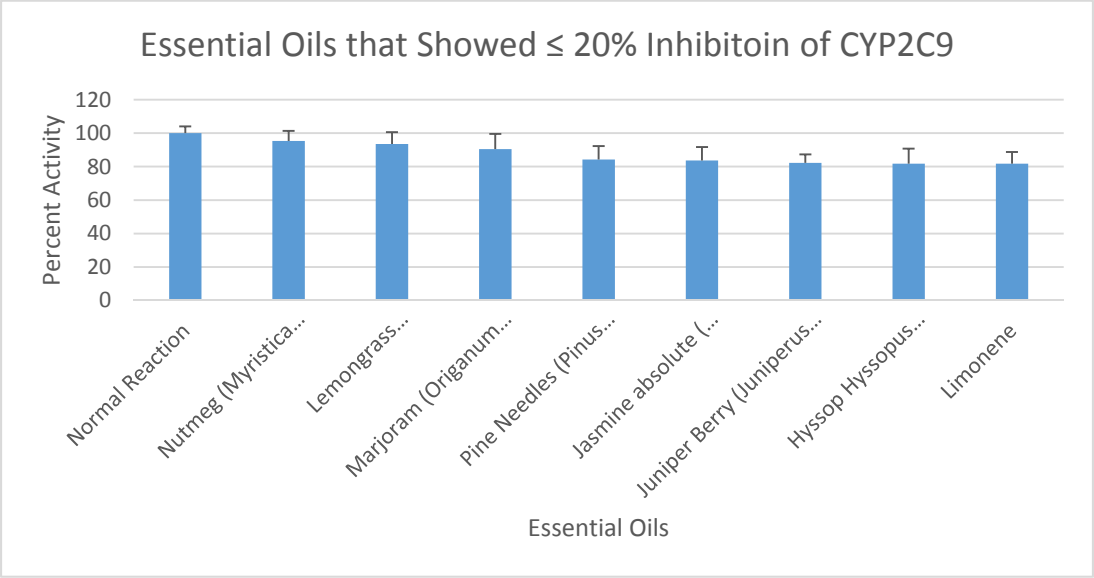


Figure 11b. This Figure Shows the Essential Oils that Exhibited ≤30% Inhibition of CYP2C9 Activity. Those oils include rosewood, frankincense, black pepper, tangerine, sage, patchouli, lavender, rose absolute, spearmint, wintergreen, cypress, cedarwood, birch, rosemary verbenon, and rosemary.

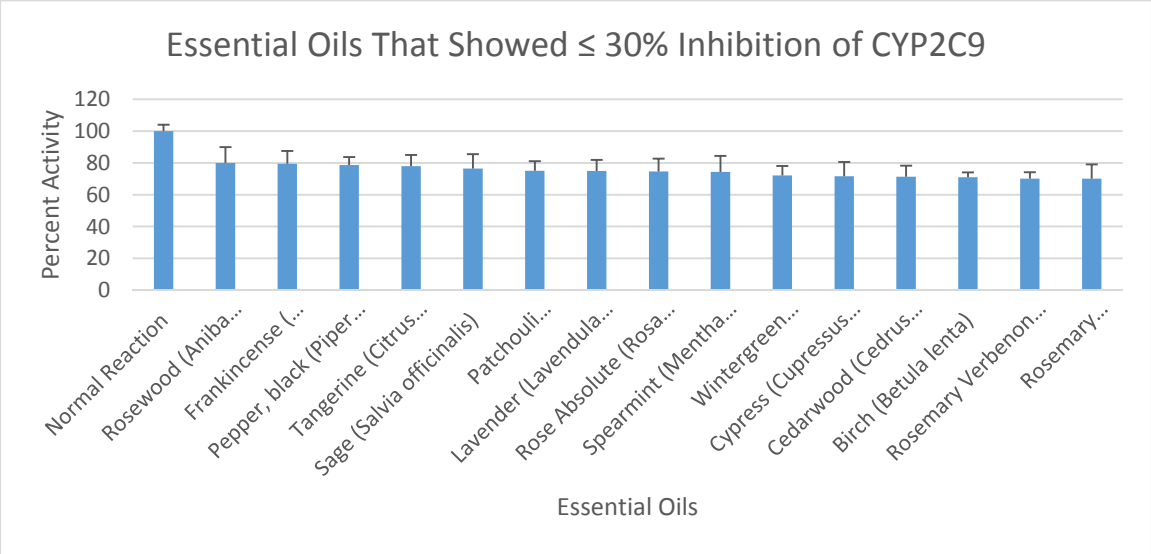


Figure 11c. This Figure Shows the Essential Oils that Exhibited ≤40% Inhibition of CYP2C9 Activity. Those oils include pennyroyal, ylang ylang, roman chamomile, p-cymene, fennel, thyme borneal, douglas fir, ravensara, peppermint, eucalyptus lemon, mandarin, melissa pure, and anise.

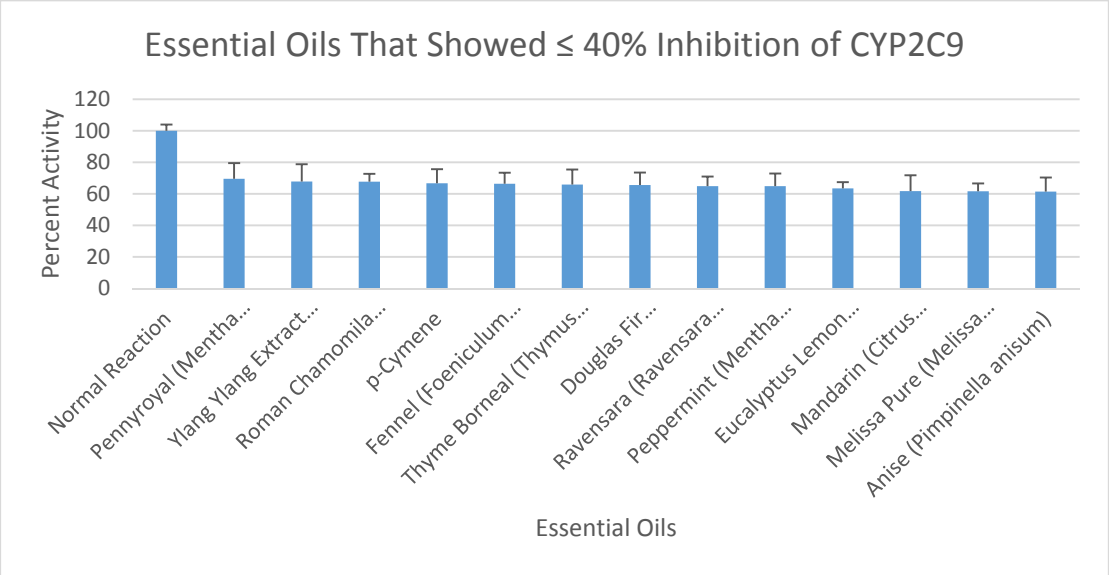
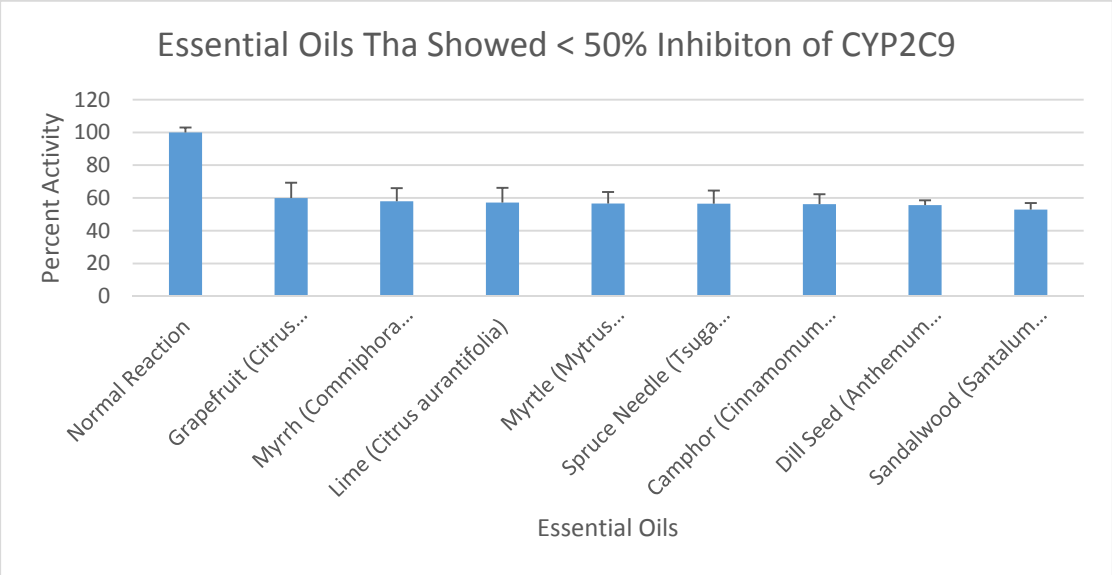
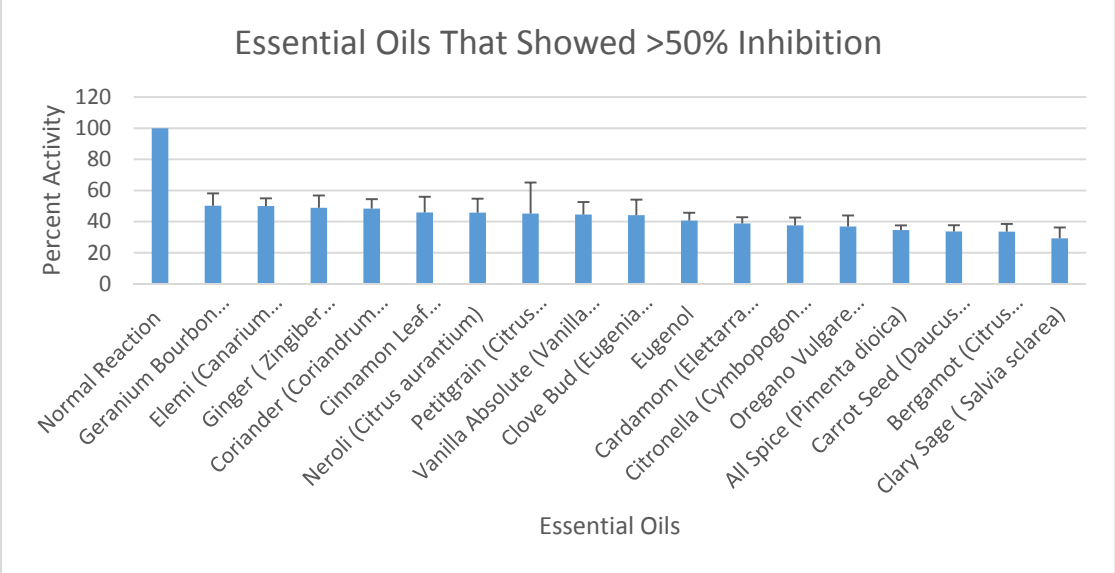


Figure 11d. This Figure Shows the Essential Oils that Exhibited <50% Inhibition of CYP2C9 Activity. Those oils include grapefruit, myrrh, lime, myrtle, spruce needle, camphor, dill seed, and sandalwood.



Of the 60 essential oils tested, there were several different oils that had >50% inhibition when compared to an uninhibited reaction. The essential oils that showed inhibition >50% were all spice, bergamot, cardamom, carrot seed, cinnamon leaf, citronella, clary sage, clove bud, coriander, elemi, ginger, eugenol, geranium bourbon, oregano vulgare, neroli, petitgrain, and vanilla (shown in Figure 12).

Figure 12. This Graph Shows the Seventeen Essential Oils that Showed >50% Inhibition of CYP2C9 Activity when Compared to an Uninhibited Reaction.



As seen in figure 12, clary sage showed >50% inhibition of CYP2C9 activity. Clary sage reduced the activity of CYP2C9 from 100% to 29%. After the screening process determined that these seventeen essential oils showed >50% inhibition, the type of inhibition needed to be determined. This was done by using Michaelis-Menten enzyme kinetics.

III.B. Enzyme Kinetics Analysis

A more precise characterization of the inhibitory effects of the essential oils on the activity of CYP2C9 was done via kinetic analysis using the Michaelis-Menten model of enzyme kinetics and inhibition. The kinetic experiments were carried out using 3 different concentrations of diclofenac ranging from 25 μM to 125 μM in the presence and absence of inhibitor in duplicate to ensure reproducibility. The concentration of oil was selected for each experiment was based on the results of the initial screening studies. A concentration was selected where ≥50% inhibition was observed under the standard conditions used in the screening process. Human liver microsomes were incubated with one of the seventeen oils examined and diclofenac using difference concentrations of the substrate ranging from 25 μM to 125 μM. The relative activities (determined by integrating the peak area for the product 4-hydroxydiclofenac) were plotted against the concentration of diclofenac as in a Michaelis-Menten model. Based on the observation that the Vmax for the control experiments and those that had inhibitor present were roughly the same, a competitive model of inhibition was used to calculate the K_i for each of the seventeen oils examined, in order to obtain a quantitative measure of the inhibition. Table 7 shows the K_m, K_m^{app}, V_{max}, V_{max}^{app}, and the K_i for each of the seventeen essential oils that showed >50% inhibition of CYP2C9 activity

Table 7. This Shows the Michaelis-Menten Calculations for the Seventeen Oils that Showed >50% Inhibition of CYP2C9 Activity. Averaged K_i values and error values calculated for CYP2C9 inhibition activity of the seventeen oils tested. The K_m, K_m^{app}, V_{max}, and V_{max}^{app} are included for all of the oils.

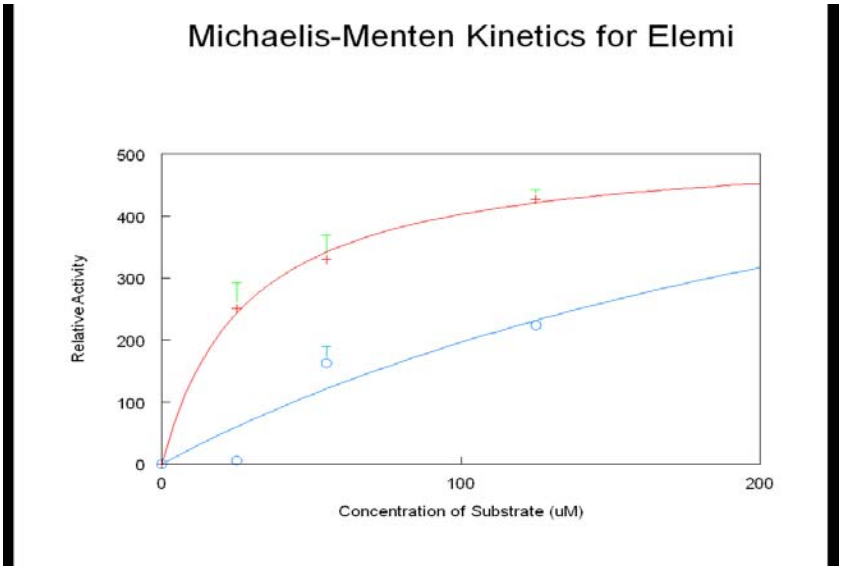
Essential Oil	K_i (µg/mL)	Error (µg/mL)	K_m	K_m^{app}	V_{max} (µmol/min)	V_{max}^{app} (µmol/min)
Geranium Bourbon	26	±0.51	48	114	781	816
Elemi	4.7	±2	27	316	516	516
Ginger	250	±0.19	27	32	516	516
Coriander	28	±0.22	27	63	516	516
Cinnamon Leaf	28	±1	88	250	452	367
Neroli	88	±0.40	48	73	781	781
Petitgrain	43	±0.43	48	154	781	781
Vanilla	141	±0.44	48	66	781	781
Clove Bud	24	±0.44	27	78	516	516
Eugenol	34	±0.42	48	132	781	781
Cardamom	87	±0.96	88	129	452	403
Citronella	26	±0.62	27	71	516	462
Oregano Vulgare	24	±0.40	48	177	781	781
All Spice	106	±1.2	88	131	452	357
Carrot Seed	18	±1.1	88	146	452	263
Bergamot	20	±0.89	88	250	452	452
Clary Sage	16	±0.29	27	86	516	516

Of the seventeen oils tested using the Michaelis-Menten model, elemi was found to be the most potent inhibitor based on its calculated K_i. To show how the values in Table 7 were obtained, sample kinetic plot for each of the seventeen oils are shown in Figure 13 through Figure 29. Figure 13 shows a Michaelis-Menten plot for elemi. Based on the data shown in the Michaelis-Menten plot (Figure 13), a V_{max} and a K_m were calculated in the presences and absence of elemi. Based on the observation that the Vmax was relatively unaffected, the competitive model of inhibition was used to calculate a K_i of 4.7 µg/mL for elemi. The K_m was 27 and the K_m^{app} was 316. The ratio of K_m^{app} to K_m was set equal to α and substitute in to equation 3.

Equation 3. Ratio of K_m^{app} to K_m for competitive inhibition.

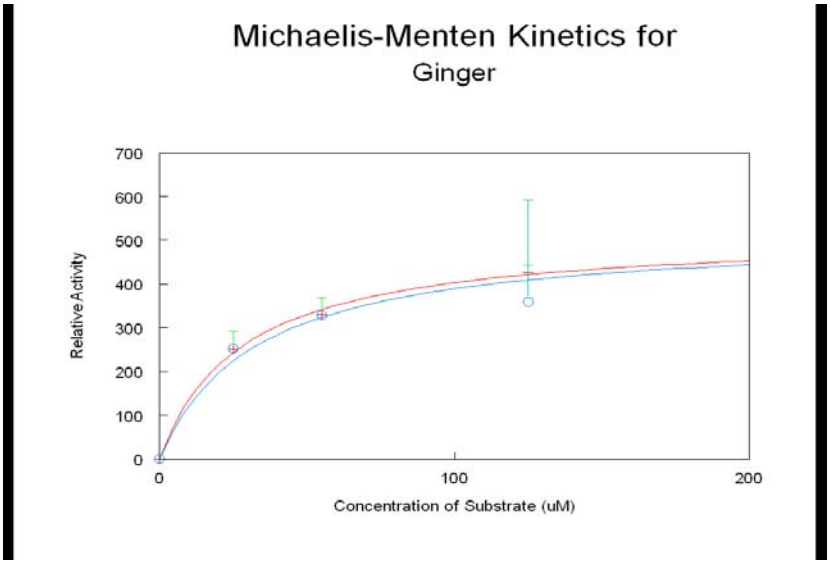
$$\alpha = 1 + [I] / K_i$$

Figure 13. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Elemi. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.



Of the seventeen oils tested, ginger oil was found to be the least potent inhibitor based on Michaelis-Menten experiments. The corresponding data for α of ginger oil is included. Based on the Michaelis-Menten plot (Figure 14), a V_{max}, K_m were calculated in the presence of ginger oil. The Vmax was relatively unaffected by the presence of the oil and thus a competitive model of inhibition was used to calculate a K_i of 250 µg/mL. This is in contradictory to the results of the screening process where >50% inhibition was observed which could be due to the citral present in the ginger oil. Another explanation for the effects of ginger oil not being as potent when compared to the secreting process, is that aldehydes that are present in small amounts in essential oils, which can be inhibitors of CYP450's, can form hydrates over time and lose the ability to inhibit activity.

Figure 14. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Ginger Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.



The other fifteen essential oil had moderate to low inhibitiory activity. The oils that showed moderate inhibitory activity were geranium bourbon, coriander, cinnamon leaf, clove bud, eugenol, citronella, oregano vulgare, carrot seed, bergamot, and clary sage. The value of the Ki for these oils ranged from 16 µg/mL to 34 µg/mL. Based on the Michaelis-Menten plots for these moderate inhibitors (Figure 15-24), a V_{max} and K_m were calculated in the presence and absence of inhibitor. The Vmax was unaffected by the presence of the oil and thus a competitive model of inhibition was used to calculate the K_i of the oils. The oils that showed low inhibitory activity were neroli, petitgrain, vanilla, cardamom, and all spice. The value of the Ki for these oils ranged from 43 µg/mL to 141 µg/mL. Based on the Michaelis-Menten plots for these moderate inhibitors (Figure 24-28), a V_{max} and K_m were calculated in the presence and absence of inhibitor.

The Vmax was unaffected by the presence of the oil and thus a competitive model of inhibition was used to calculate the K_i of the oils.

Figure 15. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Geranium Bourbon oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

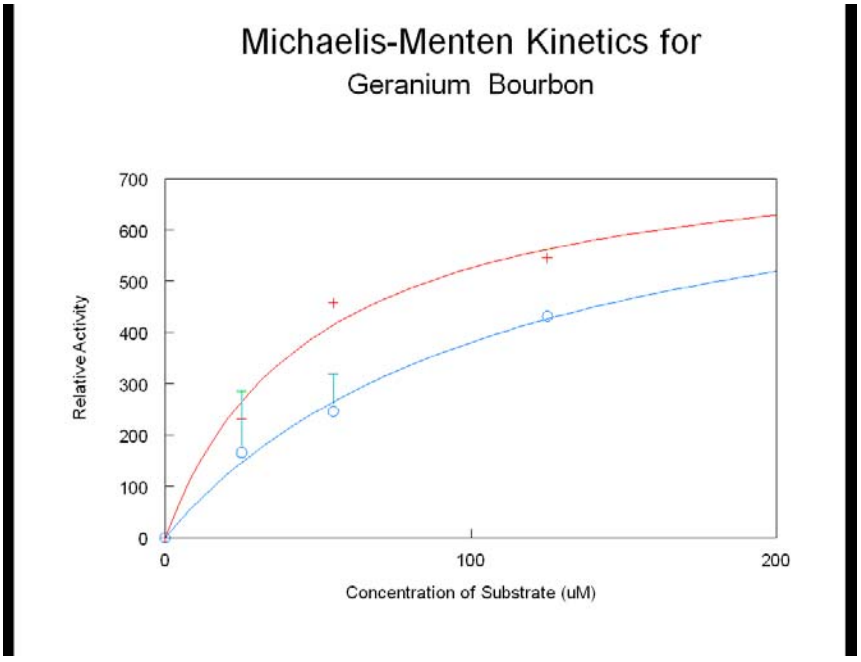


Figure 16. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Coriander Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

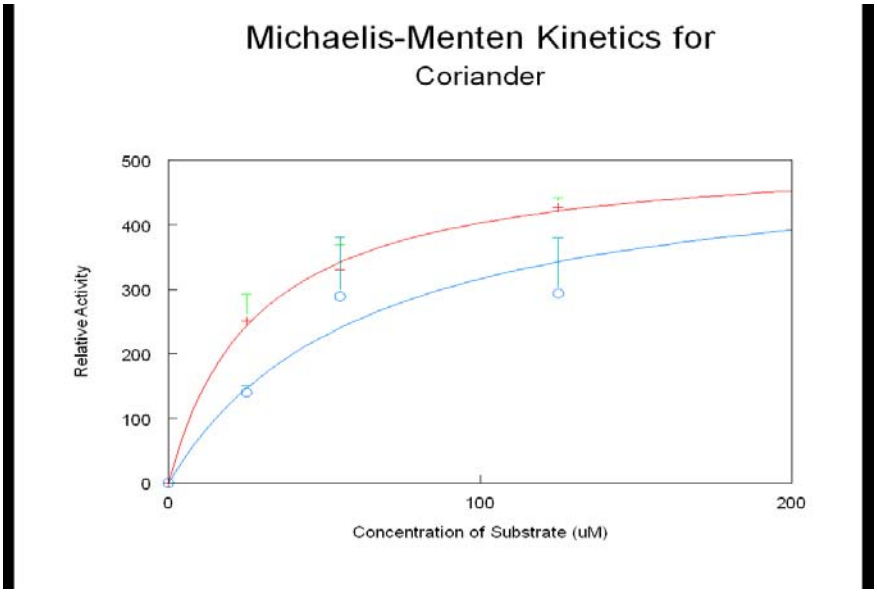


Figure 17. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Cinnamon Leaf Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

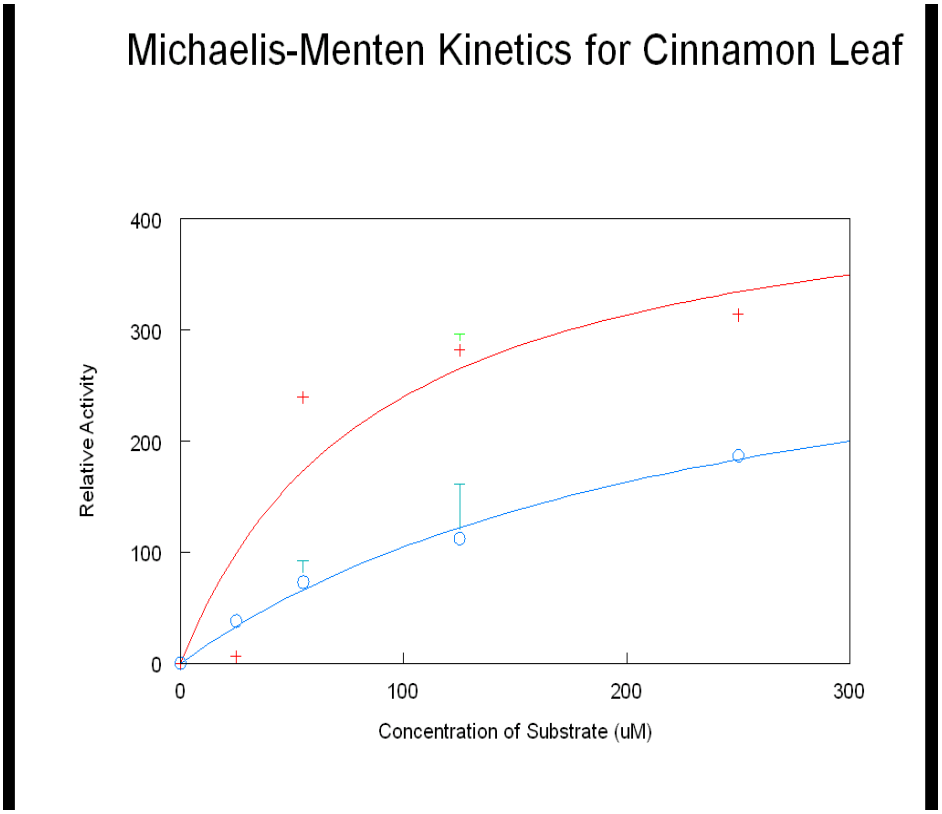


Figure 18. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Clove Bud Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

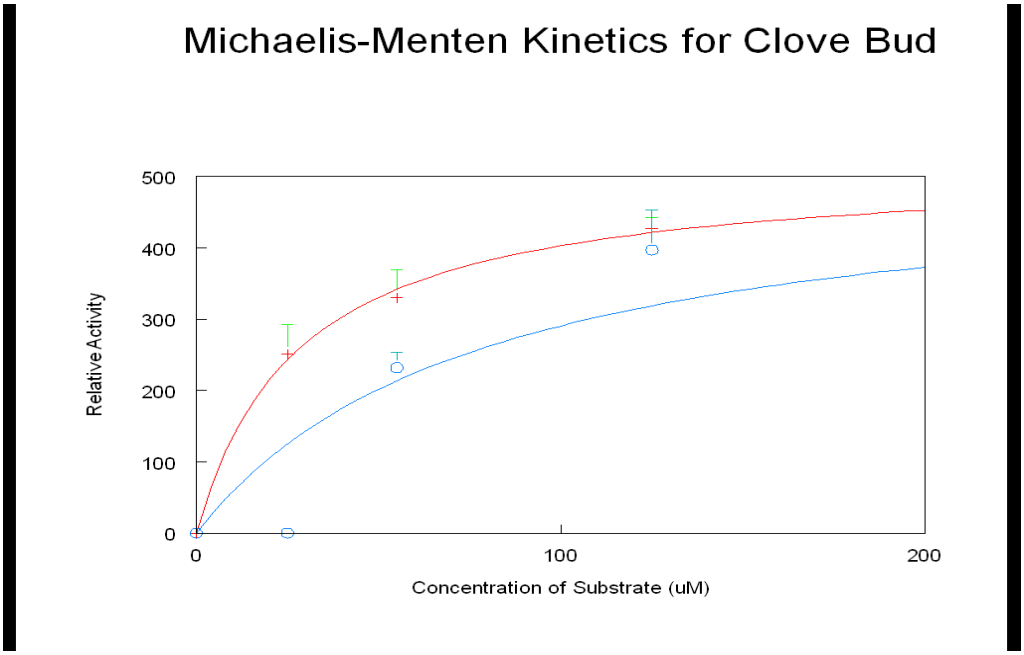


Figure 19. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Eugenol Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

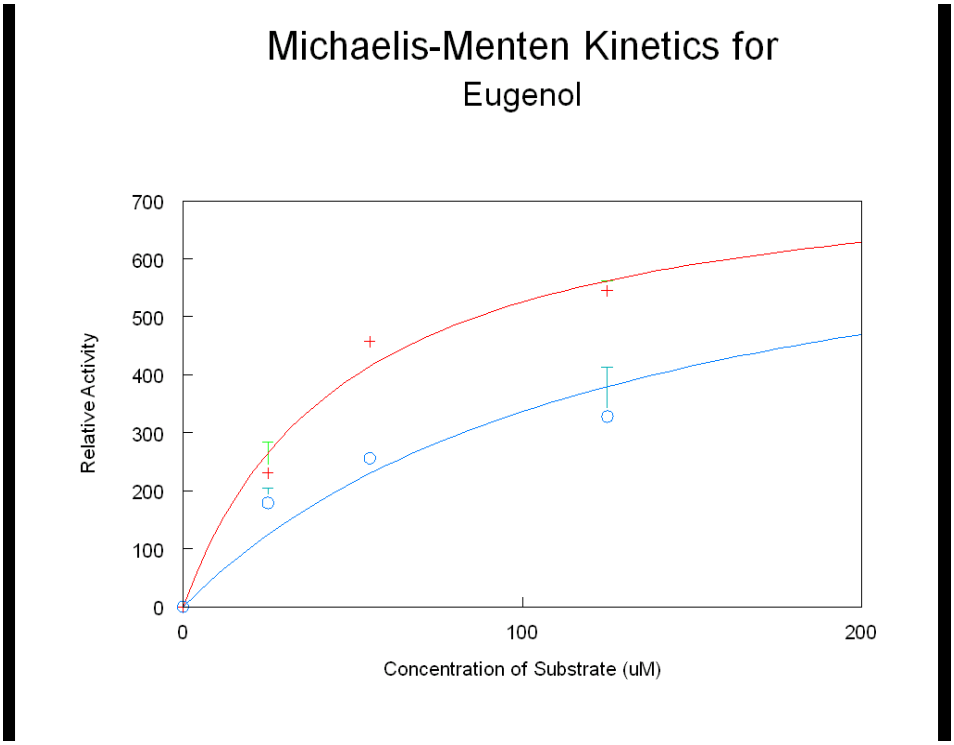


Figure 20. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Citronella Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

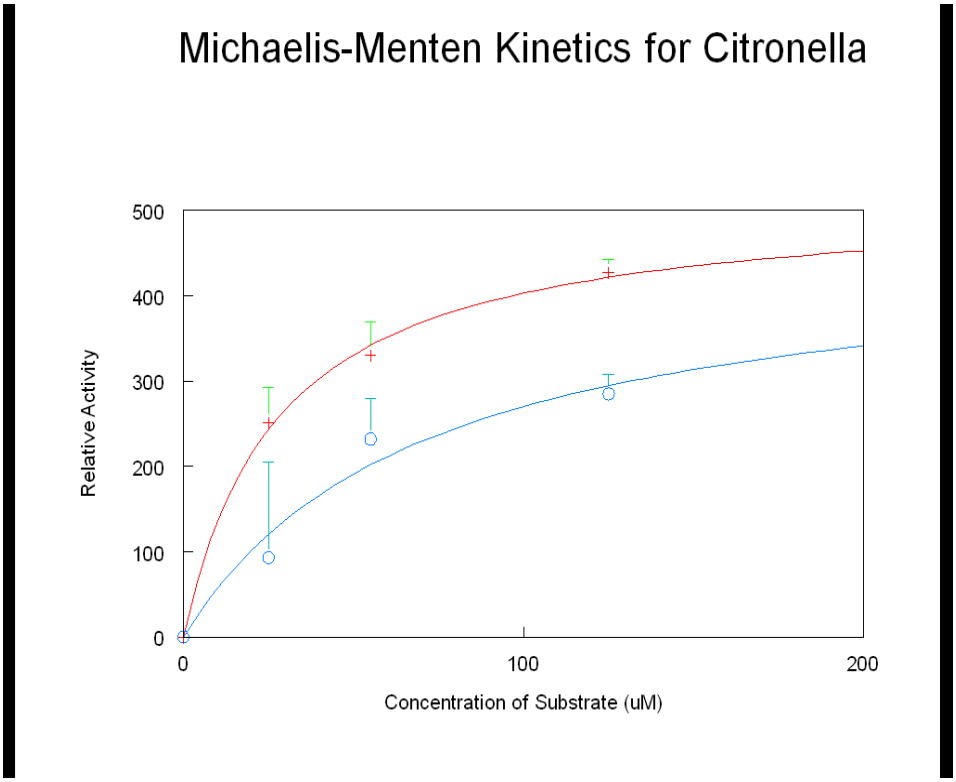


Figure 21. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Oregano Vulgare Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

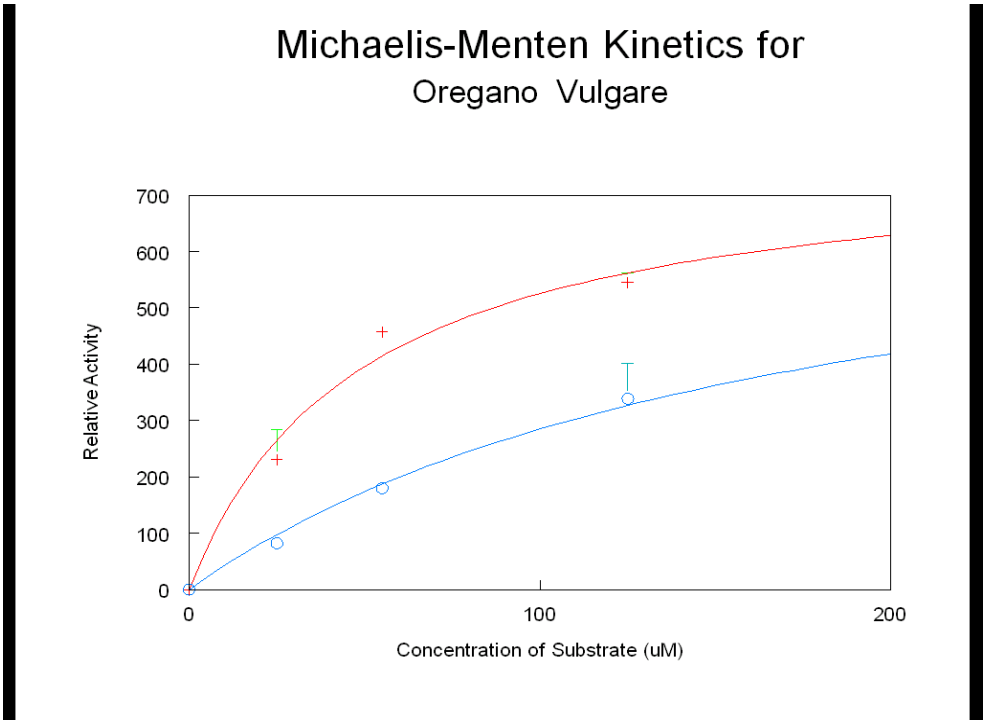


Figure 22. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Carrot Seed Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

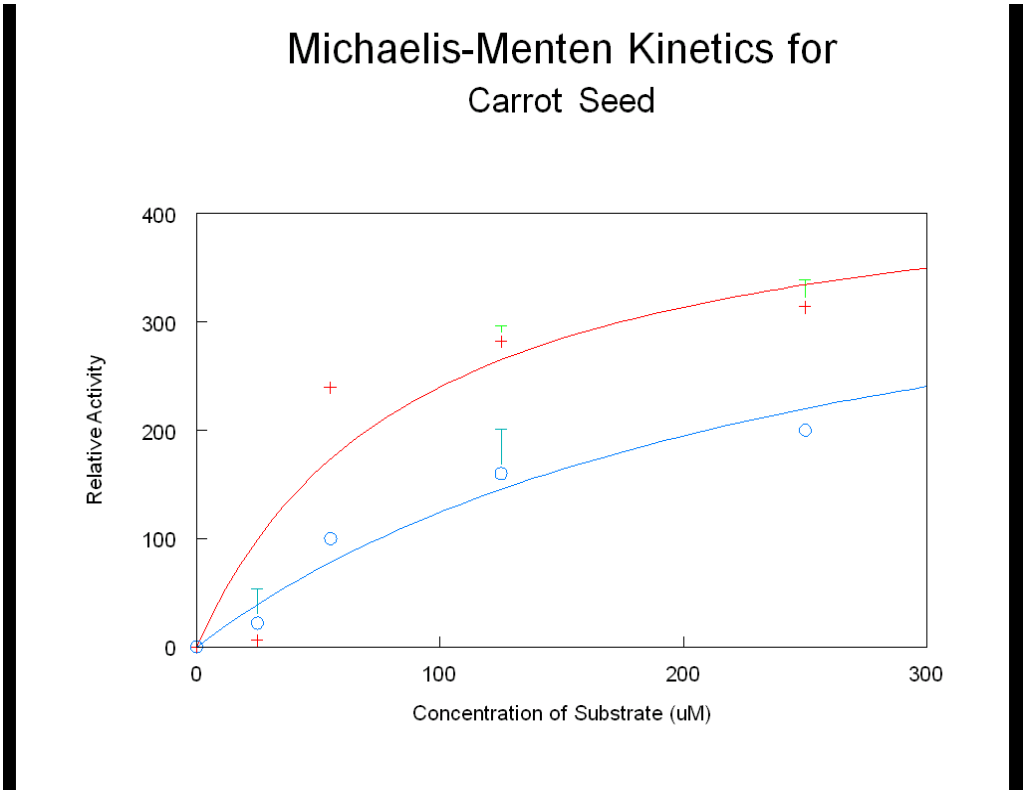


Figure 23. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Bergamot Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

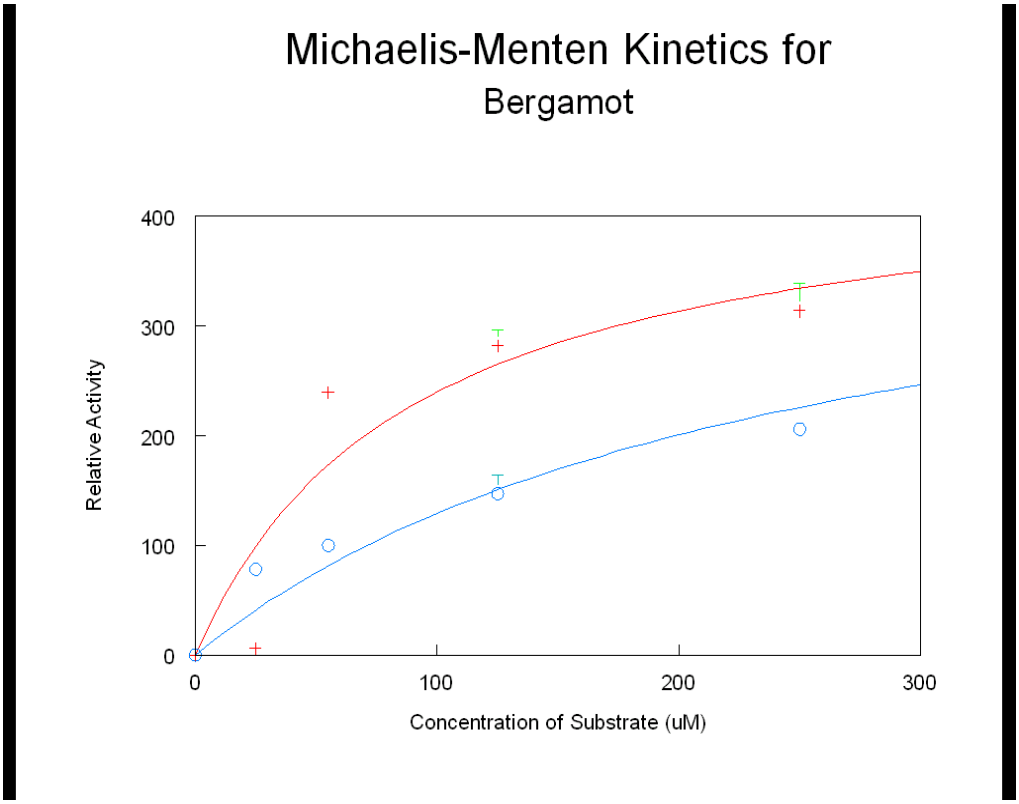


Figure 24. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Clary Sage Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

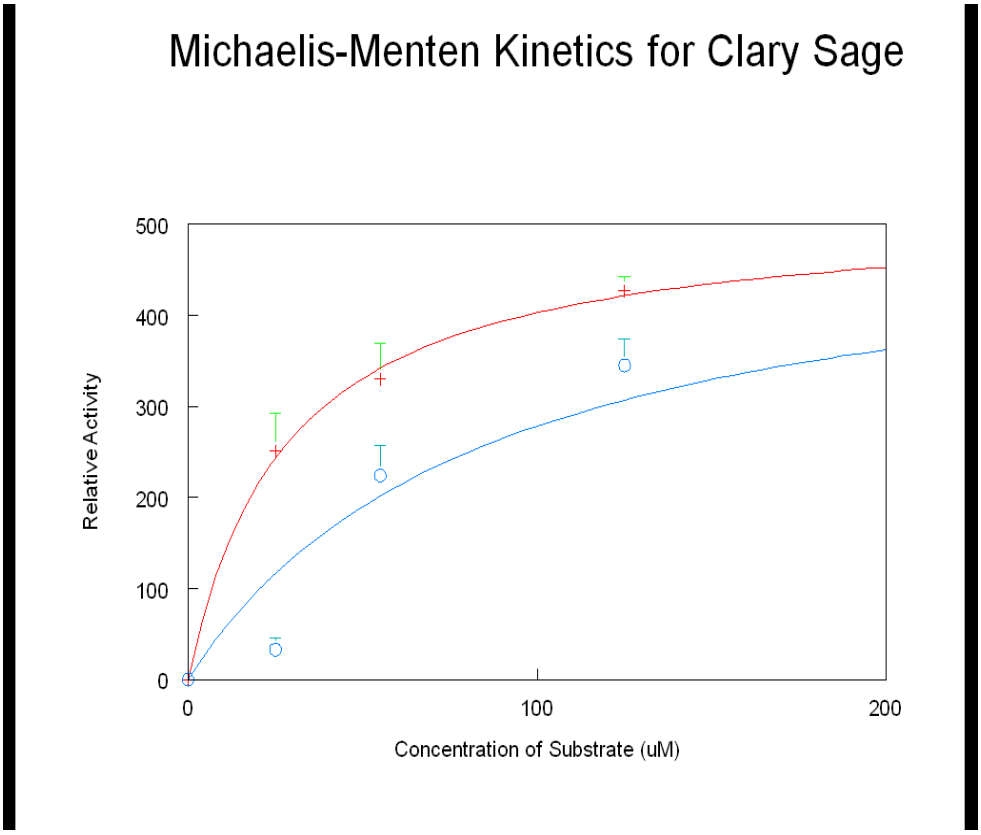


Figure 25. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Neroli Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

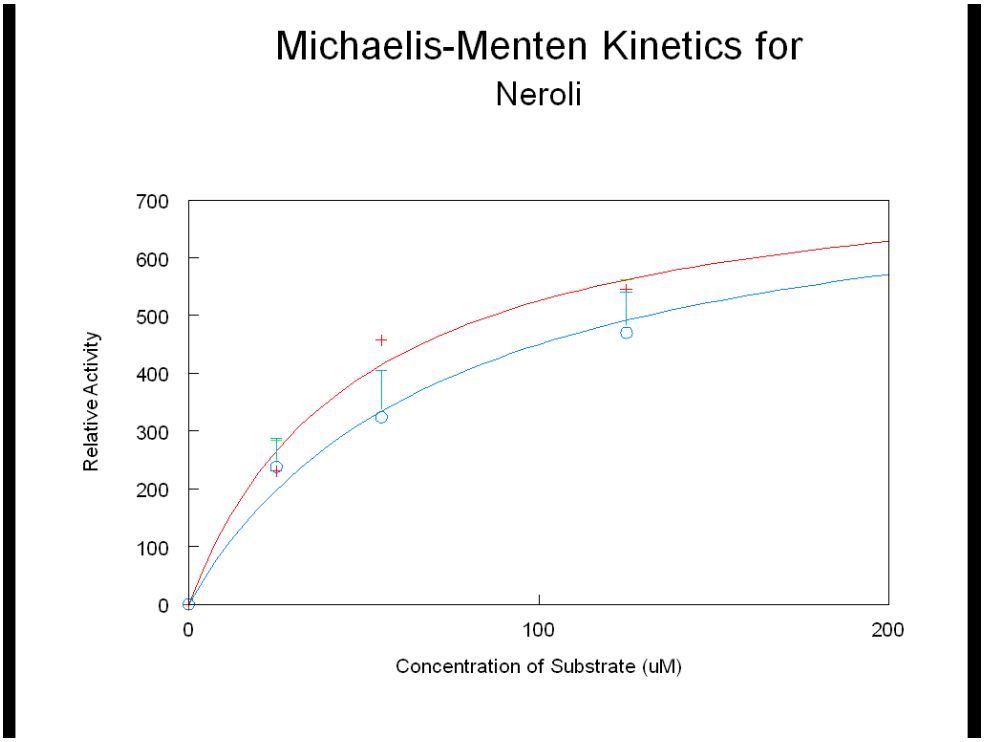


Figure 26. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Petitgrain Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

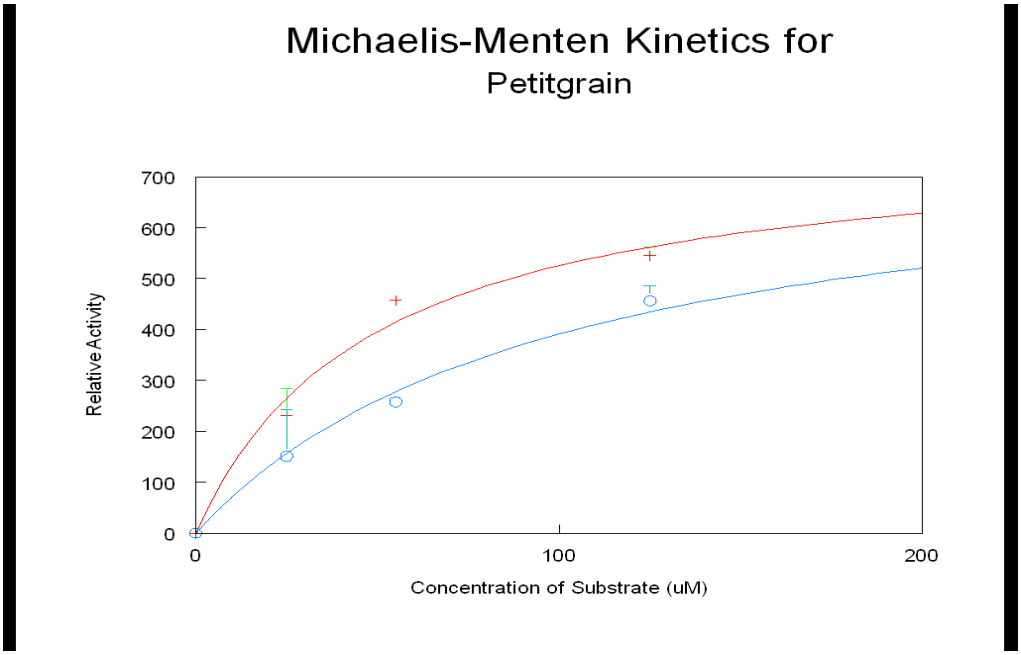


Figure 27. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Vanilla Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

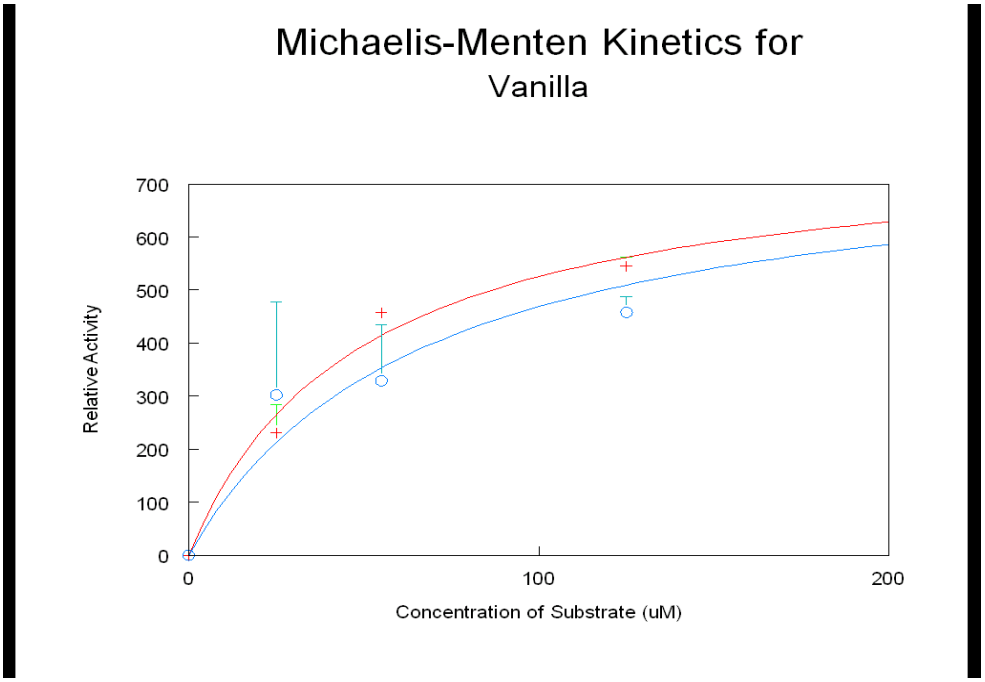


Figure 28. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of Cardamom Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.

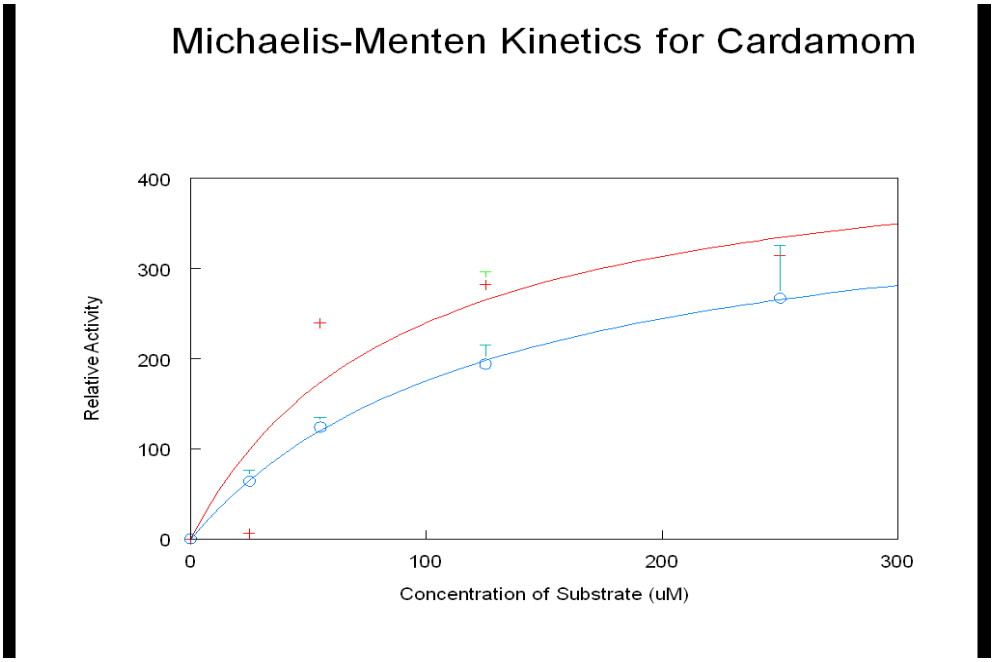
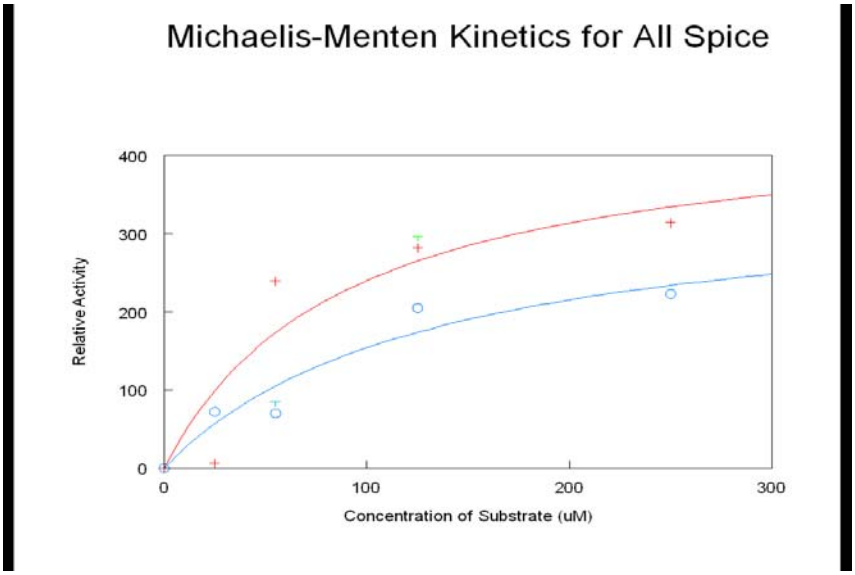


Figure 29. Plot Area vs. Diclofenac (µM) Concentration in the Presence (blue circles) and Absence (red triangles) of All Spice Oil. Reactions contained inhibitor, human liver microsomes, a range of substrate concentrations from 25 µM to 125 µM, and NADPH.



III.C. Reversibility Studies Results

To determine if the inhibitor was reversible or irreversible, a two stage reversibility study was done. Table 8 shows the results of the reversibility studies. Vial 1 in this experiment represented reaction conditions which would allow the inhibitors present in the essential oils to do damage to the cytochrome P450, if the reaction is irreversible then there would be no or a decreased amount of remaining activity toward the substrate when the 40 µL is transferred to stage 2, but if it happens to be reversible then there would be a full recovery of activity with the substrate in stage 2 to the cytochrome P450. NADPH is added to vial 1 to see if the inhibition of the cytochrome P450 is dependent on NADPH. In vial 2, everything is the same as in vial 1, except that no NADPH is present. Vial 3 represents a control, with microsomes and buffer, when transferred to stage 2 the substrate should be able to bind to the cytochrome P450 and give full activity which can be compared to vial 1 and vial 2.

Table 8. Results of Reversibility Studies. Vial 1 corresponds with the reaction that was preincubated with the inhibitor and NADPH. Vial 2 corresponds with the reaction that was just preincubated with just inhibitor. Vial 3 corresponds to a control reaction in which no inhibitor was added or substrate was added to the preincubation phase.

Essential Oil	Vial 1	Vial 2	Vial 3
All Spice	85±8	102±12	100±9
Bergamot	100±8	93±9	100±9
Cardamom	78±12	80±10	100±10
Carrot Seed	78±13	76±10	100±7
Cinnamon Leaf	105±4	106±6	100±3
Citronella	63±7	74±6	100±6
Clary Sage	73±5	74±13	100±10
Clove Bud	106±8	117±8	100±10
Coriander	89±5	98±6	100±6
Elemi	91±8	84±9	100±6
Eugenol	82±10	82±8	100±10
Geranium Bourbon	110±6	100±6	100±5
Ginger	104±2	102±2	100±4
Neroli	81±12	102±10	100±8
Oregano Vulgare	55±4	61±4	100±6
Petitgrain	105±2	96±3	100±3
Vanilla	105±4	96±3	100±4

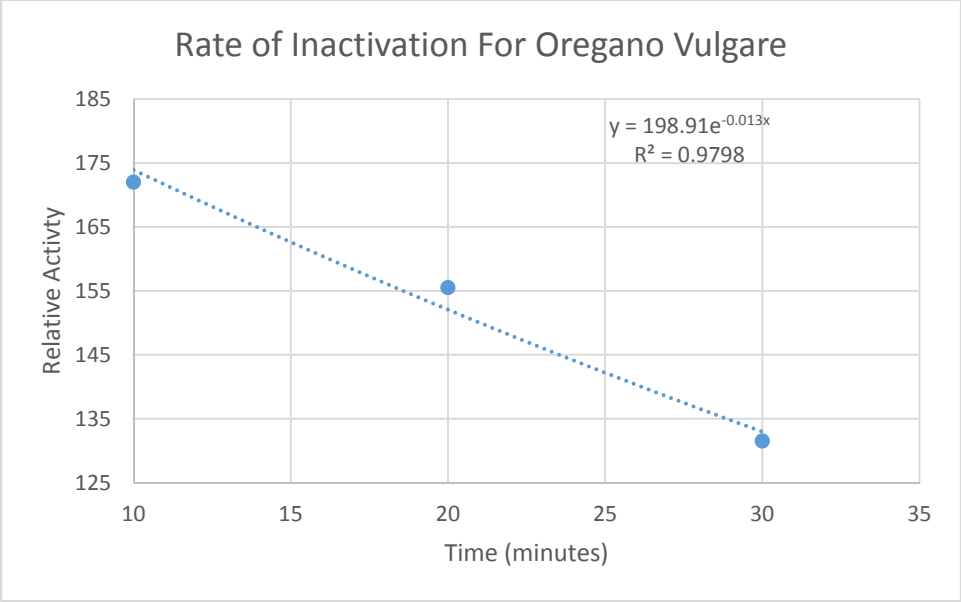
One oil was of particular interest, oregano vulgare oil. When looking at the results of the reversibility study, sixteen of the seventeen essential oils fell within the parameters of reversible inhibition. This can be seen when comparing the activity of vial 1 and vial 2. When looking at the results of vial 1 (with vial 3), if a oils is causing inhibition there should be a significant decrease in activity and when that is compared to vial 3. A decrease in activity in vial 1 relative to vial 2 signifies that there is a mechanism based inactivation of the enzyme even after the incubation phase which contains substrate. This is one type or irreversible inhibition that can occur and it is

dependent on enzyme turnover rate. For example, when looking at oregano oil the activity for vial 1, the oil significantly decreased the activity of the enzyme which was not able to be restored when substrate was introduced into the system. This means that there is potentially mechanism based inhibition occurring. When looking at vial 2, the same conditions are being looked at except there was no NADPH present in the preincubation phase. The inhibition occurring in this reaction does not require enzyme activation. This can be referred to as direct inactivation rather than mechanism based inactivation. Of the oils tested, oregano vulgare was the only oil that showed significantly decreased enzyme activity. The rate of inactivation was further studied for oregano vulgare.

III.D. Rate of Inactivation Studies

This study was done to see the rate at which the inhibitor was deactivation the enzyme. Like the reversibility studies, there are two phases in which the reaction occurs: the preincubation phase (stage 1) and the incubation phase (stage 2). Figure 30 show the graph depicting the rate of inactivation. The calculated rate of inactivation was 0.78 min^{-1}.

Figure 30. Rate of Inactivation Assay Plot for Oregano Vulgare. The time intervals for the preincubation solutions was 10, 20, and 30 minutes.



This study can be compared to the rate of inactivation of CYP3A4 with the component of grapefruit, bergamottin. Bergamottin is a known inhibitor of CYP3A4 with a rate of inactivation of 0.3 min^{-1} (16). This means that the bergamottin is decreasing the activity of the enzymes present in solution at a rate of 0.3 per minute. In the case of this study, oregano oils is destroying the enzyme at a rate of 0.78 min^{-1} which results in a loss of overall activity which was not able to be restored.

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