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Increasing use of herbal products in recent years demand further studies relating to their safety and efficacy. Since cytochrome P450 enzymes play a major role in drug metabolism, studying their interactions with herbal extracts would be an important step in this direction. In the current study, *Echinacea purpurea* root and *Spilanthes acmella* whole flowering plant extracts were tested against CYP2C9, CYP1A2, CYP2A6 and CYP2E1. Both Echinacea and Spilanthes showed moderate inhibition against CYP2C9 while they both failed to show any inhibition towards CYP1A2. Echinacea showed moderate inhibition against both CYP2A6 and CYP2E1 but raw Spilanthes extract did not show any interaction with CYP2A6. It showed mild inhibition of CYP2E1 when used in high concentration. Isobutyl amides, known to be the only bioavailable components of Echinacea and Spilanthes were tested for their ability to inhibit CYP2E1. The four isobutyl amides present in major proportions in the ethanolic preparation of *Echinacea purpurea* root were isolated and shown to be potent inhibitors of CYP2E1. Spilanthol, the only major isobutyl amide found in the ethanolic preparation of *Spilanthes acmella* whole flowering plant was isolated and it showed strong inhibitory properties against CYP2E1.

INTERACTIONS OF ECHINACEA AND SPILANTHES WITH HUMAN
METABOLIZING SYSTEMS

by

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APPROVAL PAGE

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Committee Chair _____

Committee Members _____

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Date of Acceptance by Committee

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Date of Final Oral Examination

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

1.1 Increasing popularity of natural products: benefits and concerns

The use of herbal medicines for the treatment of human illness has existed in every culture throughout the world for thousands of years. They have eventually been replaced by scientific methodologies in most of the industrialized countries and conventional medicine has gained a position as a more effective method of health care. Interestingly recent years have seen a rapid growth in the use of the so-called alternative medicines, especially in the United States [1]. The nineteen nineties have witnessed a tremendous increase in the sale of herbal products, in fact between the years 1992 and 1998 there was an annual increase of 25% in the sale of herbal medications and in 1999 sales rose to over $3.3 billion [2,3].

Several factors may have contributed to the increasing popularity of herbal medicines. First, they are considered safe and without side effects by many people. In addition, lack of regulation allows producers to make unsubstantiated claims about the miraculous effects of their products. Finally, they can be purchased without a prescription, which may also contribute to their widespread use.

All this recent interest in these natural products is not absolutely without reason. Apart from the existing traditional beliefs, there has been reported evidence suggesting the efficacy of certain herbal products. Garlic has been shown to reduce cholesterol [4].

Gingko biloba, another popular herbal product has been reported for its effectiveness in delaying cognitive impairment [5,6]. Saw palmetto has proven helpful in the treatment of urinary tract symptom and flow rates [7] while St. John's wort has been found to be effective with patients undergoing depression [8,9].

An important point of difference between herbal products and conventional medicines is that these products are a complex mixture of several different compounds which may contain more than one biologically active reagent. While some of them may be useful, or at least safe, others may lead to dangerous side effects. These undesired effects may arise due to allergic reactions or due to contaminants sometimes added illegally to the herbs to produce desired effects. Another side effect, which is a matter of serious concern, is possible drug-herb interactions that occur when the herbal products are taken simultaneously with pharmaceutical drugs. The well-known example of women running the risk of pregnancy with concurrent use of St. John's wort and contraceptive pills may be cited as an example here [10].

Toxic effects are another concern regarding potential side effects of certain herbal products. For example, the herb, *Aristolochia fangchi*, used for reducing obesity has been shown to be nephrotoxic and also a potent carcinogen [11].

The term toxicity may also be linked with herbal products in a positive way. Some herbal products have been reported to have the potential of reducing the toxicity of other compounds in the body. For example *Glycyrrhizae radix* (licorice) has been shown to inhibit cell death caused by cadmium [12]. Further discussion regarding the possible

beneficial effects of herbal products with respect to reducing chemical toxicity will be presented later in the chapter.

All these observations lead towards a common solution: regulation of herbal products so that people can reap the benefit of these herbal medications without the fear of adverse side effects. Unfortunately herbal products are not regulated to the same extent as conventional medicines. The Dietary Supplements Health and Education Act was formulated in 1994 for the regulation of herbal products that are to be considered as dietary supplements [13]. By this law, manufacturers are not required to prove the safety or efficacy of a product before marketing it. Although they are required to make sure that the product is safe, there are no specific regulations for establishment of safety. As a result of the lack of regulation, there is no way to determine the actual amount of herbal extract present in a preparation and what an effective dosage should be. Neither is it possible to identify the active ingredient. Unless and until more research has been done to characterize the herbal products, based on their efficacy and safety issues like possible toxic effects or drug-herb interactions, people will have to abstain from availing of the good results of herbal medications or run the risk of falling prey to the possible adverse side effects. As has been mentioned in UC Berkeley Wellness Letter, July 1998, "the pity of it all is that herbs might have real value as medicine if we understood them better, could buy them in standardized form, and knew how much of them to take".

The current study deals with two very well known herbal products: *Echinacea purpurea* and *Spilanthes acmella*.

1.2 Spilanthes

Spilanthes spp. is generally found in the tropical regions of the world like India, Srilanka, Africa and South America. It is a perennial herb belonging to the family of Compositae. *Spilanthes acmella*, the species involved in the current study is an herbal product marketed in the US as a dietary supplement in the form of a tincture or extract. The flowers of this herb when chewed produce a numb sensation in the mouth and thus have been effectively used for the remedy of toothaches, sore mouth, itching, psoriasis, stammering problem and also to regulate the flow of saliva [14, 15]. Some of the efficacies of this herbal product have been scientifically proven. For example, larvicidal activity has been demonstrated in *Spilanthes acmella* against *Culex quinquefasciatus* [16]. The flowers have also been shown to have diuretic activity in rats [17].

A few bioactive compounds have been isolated from *Spilanthes acmella*. Spilanthol has been isolated and is regarded as very highly bioactive [18] due to its insecticidal activity. Other isobutylamides have also been reported which includes 2E-N-(2-methylbutyl)- 2-undecene-8,10-diynamide, 2E,7Z-N-isobutyl-2,7-tri- decadiene-10,12-diynamide, 7Z-N-isobutyl-7-tride- cene-10,12-diynamide [19], undeca- 2E,7Z,9E-trienoic acid isobutylamide and undeca-2E-en-8,10- diynoic acid isobutylamide [20]. Other than the N-isobutylamides, amino acids [21] and alkaloids [22] have also been reported in *Spilanthes acmella*. Figure 1.1. shows the structure of some of the N-isobutylamides isolated from *Spilanthes acmella*.

2 (Undeca-2E,7Z,9E-trienoic acid)

1 (Spilanthol)

3 (Undeca-2E-en-8,10-dynoic acid)
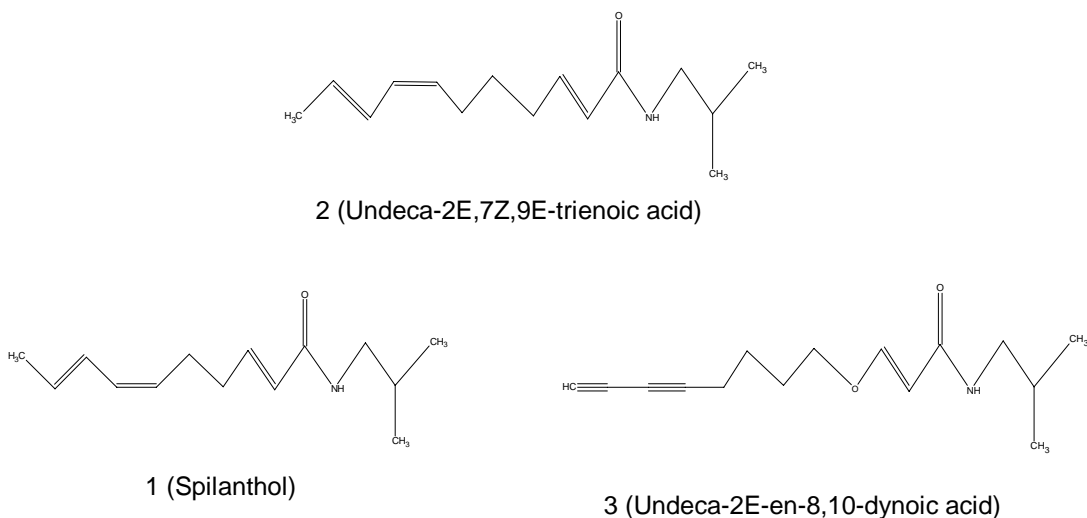
Figure 1.1. Some of the isobutyl amides present in *Spilanthes acmella*

Though some of the bioactive compounds present in *Spilanthes acmella* have been identified, research regarding the mechanisms involved in their biological effect or relative potency has been very limited. The isobutylamides found in *Spilanthes acmella* are very similar to the ones isolated from *Echinacea purpurea*. Thus it is likely that both herbs could display similar therapeutic properties and interact with other xenobiotic compounds through related mechanisms.

1.3 Echinacea

Echinacea has been an old name in the history of herbal medicines. Echinacea products are very well known in many parts of the world and can be rated among the best selling herbal products in North America as well as Europe [23, 24] with an annual average of about $300 million in US and $25 million in Canada [25, 26].

Echinacea, also known as black sampson, purple coneflower, red sunflower and sampson root, belongs to the Asteraceae family. This perennial plant is native to America and is grown in the central and eastern parts of United States. It is also cultivated in Europe. Nine kinds of Echinacea are known to grow in the United States, out of which three species have been identified to possess medicinal value. These are *Echinacea angustifolia*, *Echinacea purpurea* and *Echinacea pallida*. Herbal preparations are made separately from the roots or aerial parts of the plant. At times the whole plant are also used for such purposes. Though the German E Commission has approved of only the aerial part of *E purpurea* and underground part of *E pallida* to be used in the form of oral administration for treatment purposes [27], several combination of different parts of a single species or mixture of multiple ones of Echinacea are sold in US. Echinacea products are generally sold in the form of capsules, extracts, tinctures and tea.

Current study involves the species *Echinacea purpurea* or purple coneflower which is among the most commonly known species of *Echinacea spp*. Echinacea have been commonly used as a medicine for treatment of the common cold, cough, bronchitis, respiratory tract infections, inflammation of mouth and pharynx [28-30] and cancer [31] for a long time. But the most important pharmacological usefulness of the product lies in its claim to be an immunostimulant. Several studies have been done to confirm the efficacies of Echinacea and often with conflicting results. German Commission has only approved it for the treatment of colds, infection of respiratory tract, infection of urinary tract, and healing of wounds [27]. A clinical study done to observe the effect of *Echinacea purpurea* extract on cold or respiratory tract infection was inconclusive [32].

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Another study found *Echinacea purpurea* to be ineffective for treatment of upper respiratory tract infection in children [33], whereas compounds isolated from *Echinacea purpurea* have been shown to stimulate alveolar microphage function in healthy rats [34].

Many bioactive compounds have been isolated from *Echinacea purpurea* extract and some of them have also been quantitated. The five main types of constituents identified in Echinacea are caffeic acid derivatives [35], polysaccharides [37], alkamides [36], polyacetylenes [36] and glycoproteins [38], though only the first three are known to possess the immunostimulatory property [39]. The caffeic acid derivatives are polar and are present in the hydrophilic portion of the extract. These phenolic acids have gained importance in recent times as they have been shown to enhance the antioxidant capacity of a cell as a defense against cancer, cardiovascular disease, arthritis and aging [40]. The main caffeic acid derivatives found in *Echinacea purpurea* are caftaric acid and cichoric acid [41]. Caffeic acid is also shown to be present in trace amounts [41]. It may be mentioned here that, out of all the caffeic acid derivates isolated from *Echinacea purpurea*, only cichoric acid is known to possess several properties of pharmacological interest like immunostimulation [42]. Figure 1.2. shows the caffeic acid derivatives present in *Echinacea purpurea*.

Cichoric acid

Caftaric acid

Caffeic acid
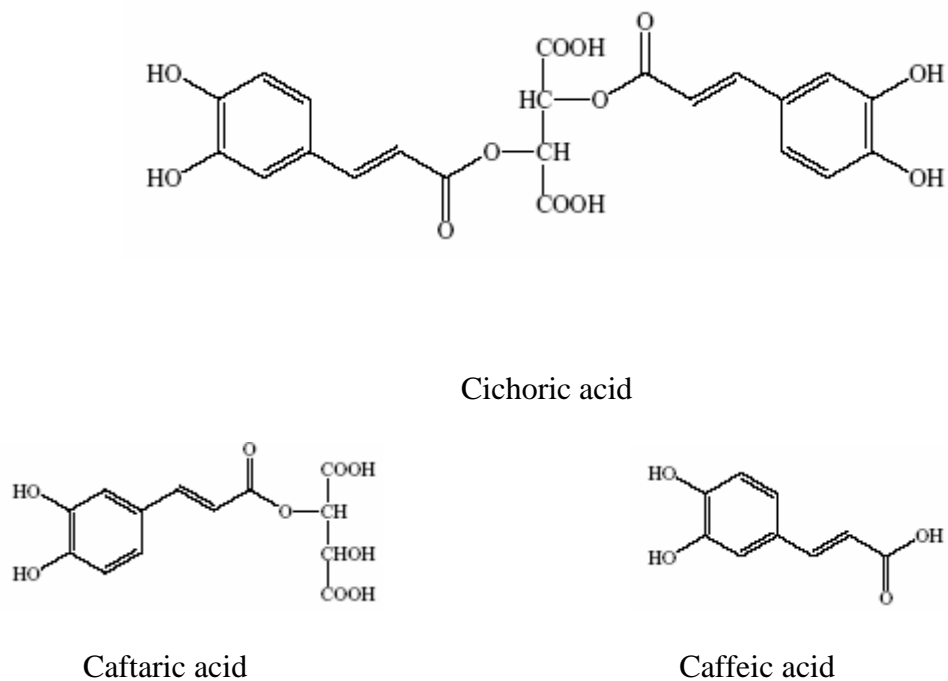
Figure 1.2. Main phenolic acid compounds in *Echinacea purpurea*

Alkamides, another class of bioactive constituents isolated from *Echinacea purpurea* are hydrophobic in nature and contains aliphatic acid residues linked to amine groups. The aliphatic acids present are mostly unsaturated. In fact the olefinic isobutyl amides are most abundant and appear to be the most active [43]. The content of different alkamides have been shown to vary in different parts of the plant [44]. Figure 1.3. shows the eleven alkamides identified in the roots of *Echinacea purpurea* [36]. The numbering system corresponds to the one used by Bauer and Remiger [36]. The isomeric pair dodeca- 2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamide (8/9) have been identified as the primary alkamide in the root extract[45].
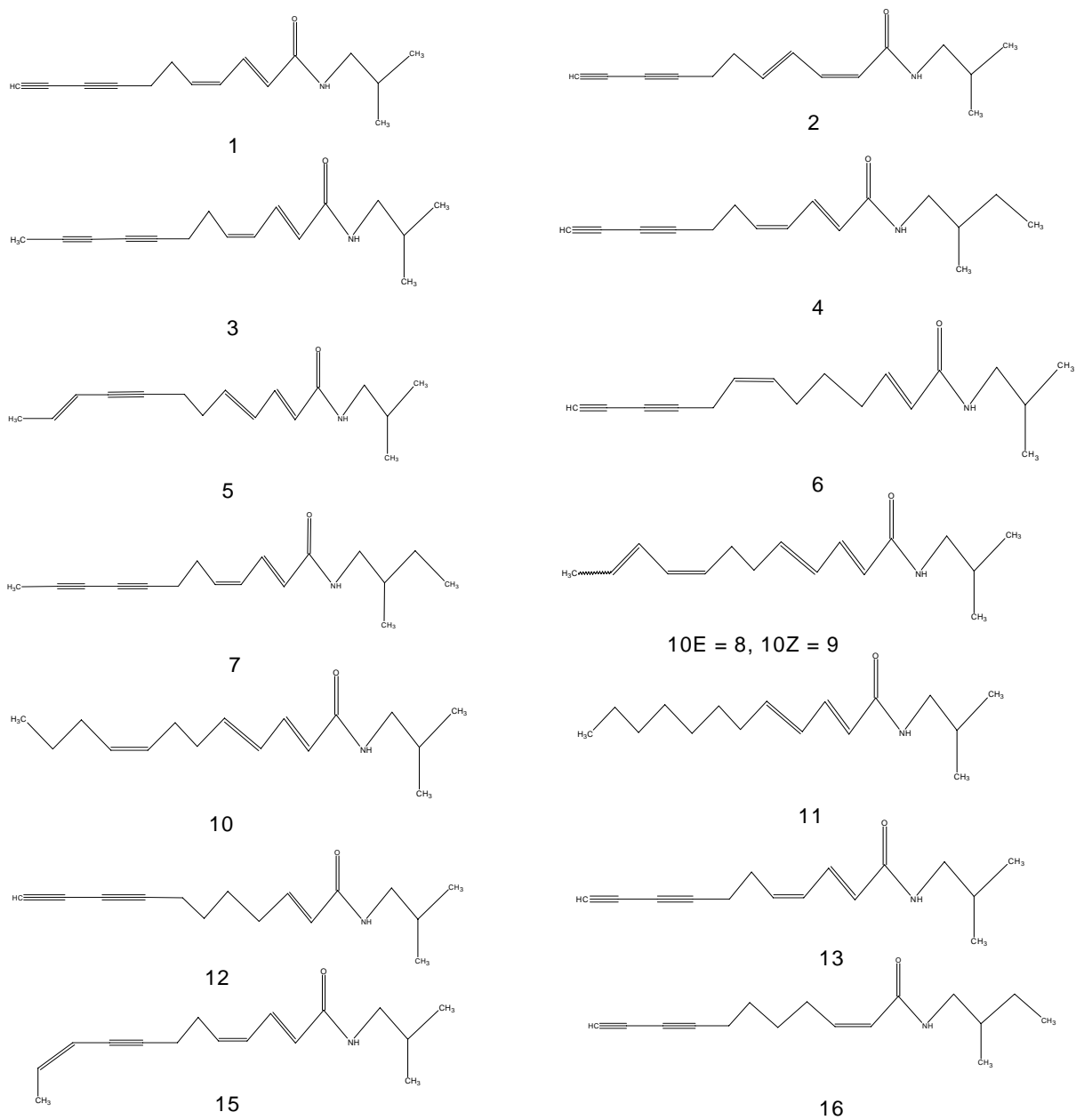
Figure 1.3. The alkamides isolated from the roots of *Echinacea purpurea*

Isobutylamides are a class of bioactive compounds found both in Echinacea and Spilanthes. A comparison of Figure 1.1. and Figure 1.3. shows similar structures of isobutylamides both in *Echinacea purpurea* root and *Spilanthes acmella*. For example Structure 4 in Figure 1.1. and structure 6 in Figure 1.3. are the same thus suggesting the possibility of similar interactions of the plant extracts with cytochrome P450.

Though a significant number of *in vivo* and *in vitro* studies have been done to identify the bioactive compounds present in *Echinacea purpurea* and to formulate their role in the immunostimulating activity displayed by Echinacea, little attention has been given to other potential physiological effects that may accompany the intake of these products. Further discussion regarding this matter would require some knowledge about how herbal products like Echinacea and Spilanthes are metabolized in humans. A major pathway for metabolism of foreign compounds in humans is oxidation by the cytochrome P450 class of enzymes. This family of enzymes is also responsible for metabolism of the majority of commercial drugs and play a substantial role in controlling toxicity of chemicals in the body. Thus there is a high possibility of interactions between compounds in these herbal products and pharmaceuticals or other xenobiotics. On one hand these interactions may lead to unwanted drug-herb interactions but it may also result in beneficial effects like attenuation of toxicity associated with chemical exposure in the human body.

1.4 Cytochrome P450

Cytochrome P450 has been studied extensively in the field of pharmacology due to its involvement in drug metabolism, but more recently it has gained considerable

attention for its role in activating or deactivating chemical toxicants. This class of enzymes is known to be present in nearly every type of organism and is involved in the oxidation of a variety of different exogenous and endogenous compounds. They take part in the metabolism of many xenobiotic compounds, like drugs, food additives and environmental chemicals. The main working principle of cytochrome P450 involves oxidation of the more lipophilic chemicals so that they become more polar and can be more easily excreted from the body or further metabolized by conjugating enzymes like glutathione transferases or sulfotransferases. Some of the endogenous compounds metabolized by cytochrome P450 are cholesterol and steroids.

While discussing cytochrome P450 it also needs to be kept in mind that the role played by cytochrome P450 is not always beneficial. They can produce highly carcinogenic compounds from benign ones via their oxidative action.

These membrane bound proteins are found primarily in the liver and small intestine where most of the drug and toxin metabolism takes place, however extrahepatic P450 expression has been observed in nearly every human tissue type examined, albeit at much lower levels than the liver. They are present mainly in the endoplasmic reticulum but may also be seen in the mitochondria.

1.4.1 Structure and spectral properties

Proteins of the cytochrome P450 family have a mass in the range of 50 kDa. The active site contains an iron protoporphyrin IX prosthetic group, where oxygen activation and substrate oxidation occur. Figure 1.4. shows the structure of the heme prosthetic group. Here an iron atom is present in the center of a protoporphyrin ring bound to four

nitrogen atoms from the four pyrrole rings. It can also accomodate two axial ligands, one

of which is a cysteine residue which serves as the link to the P450 protein.
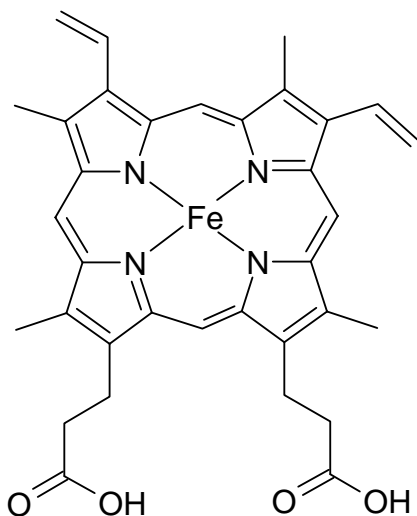
Figure 1.4. Active site of P450 showing the heme group

The cytochromes P450s can be identified by the formation of a characteristic strong

absorption peak at 450 nm when complexed with exogenous carbon monoxide in their

reduced state.

1.4.2   General reaction and catalytic cycle

Cytochrome P450s are the terminal oxidase of a mixed function oxidase systems

that bring about oxidation of the otherwise unreactive hydrocarbons. The reactions

catalyzed by cytochrome P450s include hydroxylation, N- or O- dealkylation,

epoxidation and heteroatom oxidation. The reaction requires NADPH and O_2 where one

of the oxygen atoms may get incorporated into the hydrocarbon while the other one is

released as water. The general reaction is as follows:

RH + O$_2$ + NADPH + H$^+$→ ROH + H$_2$O +NADP$^+$
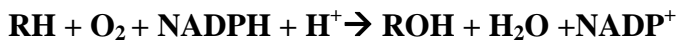
The reaction takes place by initial activation of the oxygen molecule by the heme

group of cytochrome P450. The first step in the process involves two electrons from

NADPH being passed to the heme one at a time via a reductase. These electrons are used

to activate oxygen, thus forming a molecule of water. The resulting highly activated

oxygen is used to oxidize the substrate. Figure 1.5. shows the proposed catalytic cycle of
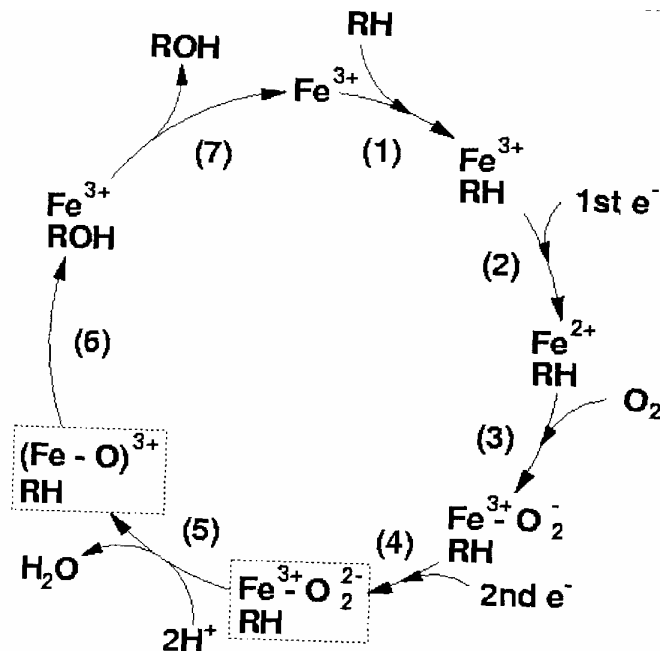
cytochrome P450.

Figure 1.5. Catalytic cycle of cytochrome P450

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1.4.3   Nomenclature and isoforms

Cytochrome P450 enzymes are generally designated by CYP followed by letters and numbers to signify different isoforms. Cytochrome P450 consists of a number of isoforms, an isoform being an enzyme variant derived from a particular gene. CYP isoforms are classified into families, subfamilies and individual genes based on their amino acid sequences. Numbers like CYP1, CYP2, represents families. About seventeen CYP families are known in man. Subfamilies are identified by a letter, giving rise to CYP2E, CYP 1A. Individual genes are also represented by numbers, for example CYP2E1. Each of these isoforms has their own specific substrates. Some of these are well known names in oxidative metabolism like CYP1A1/2, CYP3A4, CYP2D6, CYP2C9 and CYP2E1.

Before considering how the activities of some of these isoforms have the possibility of getting suppressed or enhanced with the herbal products discussed at the beginning of the chapter and see how that may be of importance to pharmacology and toxicology it will be beneficial to discuss the fundamentals of enzyme kinetics, induction and inhibition.

1.5 Enzyme kinetics and inhibition

Enzymes are biological catalysts that help a reaction to proceed at a faster rate. The working principle of enzymes involve the lowering of activation energy, generally by binding to the substrate, and making the reaction more kinetically favorable. These biocatalyzed reactions generally involve two steps which are represented by Equation 1. In the first step, the substrate S interacts reversibly with the enzyme to form an enzyme-

substrate complex, ES. In the figure, k_1 and k_{-1} are the rate constants for the forward and backward reaction respectively. In the second step, ES forms the product P and releases the enzyme. This step is irreversible and here the rate constant is represented by k_2.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$
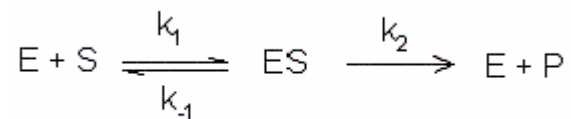
(Equation 1)

With the steady-state assumption for the enzyme-substrate concentration, [ES] and the initial velocity assumption where possibility of backward reaction leading to the formation of ES from E + P is ignored for initial velocity, we get Equation 2.

$$[ES] = \frac{[E_T]\ [S]}{K_M\ +\ [S]}$$

(Equation 2)

Here $[E_T]$ is the total enzyme concentration and K_M is the Michaelis constant given by $k_1 + k_{-1} / k_2$.

Finally the rate of product formation is given as shown in Equation 3. This expression is called the Michaelis-Menten equation.

$$V = \frac{V_{max}\ [S]}{K_M\ +\ [S]}$$

(Equation 3)

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Here V_{max} is the maximum velocity reached when the substrate concentration, [S] is high enough to saturate total enzyme, $[E_T]$ and is equal to $k_2 [E_T]$.

Michaelis-Menten equation when plotted using V/ [S] gives a rectangular hyperbola as shown in Figure 1.6.
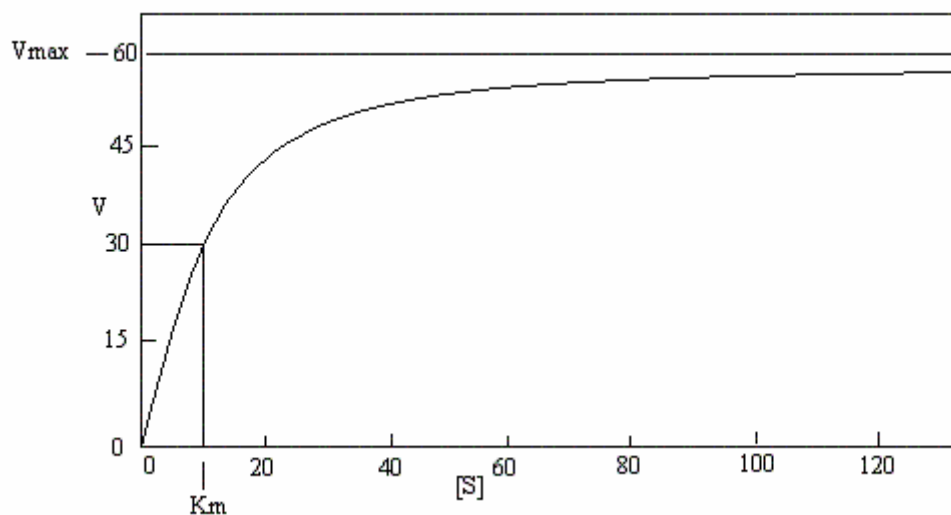
Figure 1.6. Michaelis –Menten plot showing the V_{max} and K_m values

It can be derived from Equation 4, that when K_M is equal to the substrate concentration [S], the velocity of product formation V, can be given as V_{max} / 2. Thus from the plot in Figure 1.6., the approximate value of V_{max} can be calculated at saturating substrate concentration and K_M can be calculated using that value.

The value of V_{max} and K_M in a reaction can be calculated more easily using Lineweaver-Burk plot which is a modification of the Michaelis-Menten plot. Here the reciprocal of the Michaelis-Menten equation is taken which gives Equation 4.

$$\frac{1}{V} = \left(\frac{K_M}{V_{max}}\right) \frac{1}{[S]} + \frac{1}{V_{max}}$$

(Equation 4)

In the Lineweaver-Burk plot, 1/V is plotted against 1/[S] resulting in a linear graph with a slope equal to K_M / V_{max}. Therefore the x-intercept is equal to $-1/ K_M$ and the y-intercept is equal to 1/ V_{max}. This plot is used very commonly in kinetic studies. Figure 1.7. shows the Lineweaver-Burk plot.
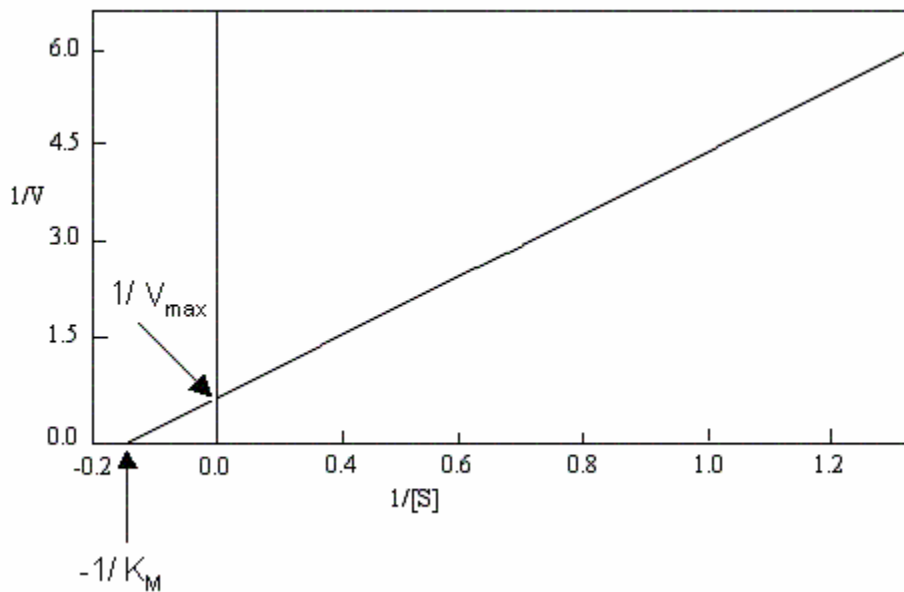
Figure 1.7. Lineweaver-Burk plot showing K_m and V_{max}

At times, the activity of the enzyme gets inhibited due to interaction with other compounds. When the enzyme is inhibited, the velocity of the reaction decreases. Kinetic studies related to inhibition are generally done by using several fixed

concentration of the inhibitor and varying the concentration of the substrate. The chief parameters studied are V_{max} and K_m values obtained from the Lineweaver-Burk plot thus generated. Inhibitors may be primarily classified as reversible and irreversible.

Irreversible inhibitors typically associate with the enzyme through covalent interaction. Here the inhibiting reagent permanently modifies the enzyme, usually by changing a side chain crucial for the enzyme activity. Reversible inhibitors on the other hand, interact with the enzyme noncovalently. This class of inhibitors can be divided into four categories. They are competitive inhibitors, uncompetitive inhibitors, noncompetitive inhibitors and mixed inhibitors. Lineweaver-Burk plots can be used to show how the K_m and V_{max} values differ with the different types of reversible inhibitors. Figure 1.8. shows the Lineweaver-Burk plot for competitive inhibition. Here all the lines representing plots both with and without inhibitors are seen to intersect each other at the y-axis. Since y-intercept gives the V_{max} value for that plot, it can be concluded that for competitive inhibition, the V_{max} value remains the same for all the lines. But the K_m values determined from the x-intercept are seen to differ and it increases with the addition of inhibitor. The Lineweaver-Burk plot for uncompetitive inhibition is shown in Figure 1.9. Here the lines are not seen to intersect with each other. The lines with inhibitor are seen to have lower V_{max}, as well as lower K_m than the line without inhibitor. In fact, both the values decrease at a similar rate, giving rise to parallel lines. In mixed inhibition, the Lineweaver-Burk plot, which is shown in Figure 1.10., indicates a decrease in V_{max} values with the addition of inhibitor, whereas the K_m values increase. And lastly in noncompetitive inhibition all the lines are seen to meet each other at the x-intercept

showing the same K_m values, while the V_{max} values decrease with the addition of inhibitor.

The Lineweaver-Burk plot for noncompetitive inhibition is shown in Figure 1.11.
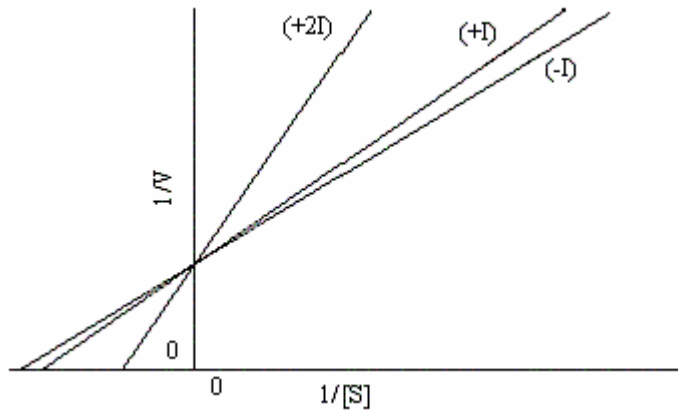
Figure 1.8. Lineweaver-Burk plot showing competitive inhibition
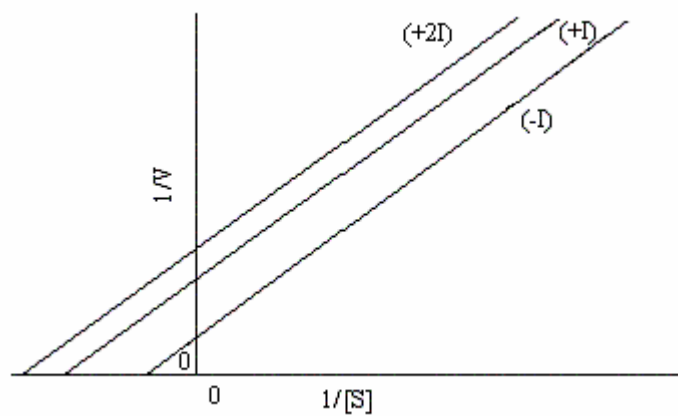
Figure 1.9. Lineweaver-Burk plot showing uncompetitive inhibition
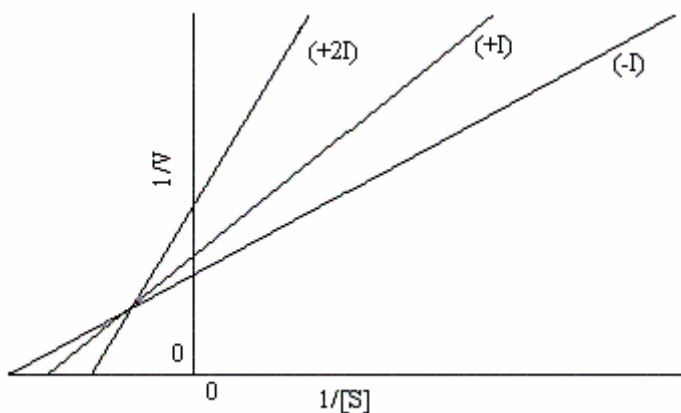
Figure 1.10. Lineweaver-Burk plot showing mixed inhibition
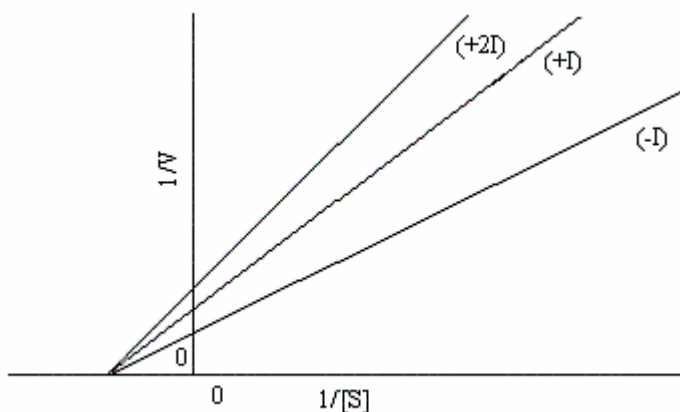
Figure 1.11. Lineweaver-Burk plot showing noncompetitive inhibition

Further understanding of the reasons behind the difference in the nature of the

Lineweaver-Burk plots seen above requires an evaluation of the kinetic models

associated with the different types of reversible inhibition. In competitive inhibition, the

substrate and the inhibitor usually resemble each other structurally and they both compete

for the same binding site in an enzyme, so they cannot bind to the enzyme

simultaneously. Figure 1.12. shows the model for competitive inhibition. Here the enzyme binds reversibly with the inhibitor to form the enzyme-inhibitor complex. The dissociation constant for this reaction, known as the enzyme-inhibitor dissociation constant is given by K_I.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$+ \atop I$$

$$K_I \updownarrow$$

$$EI$$
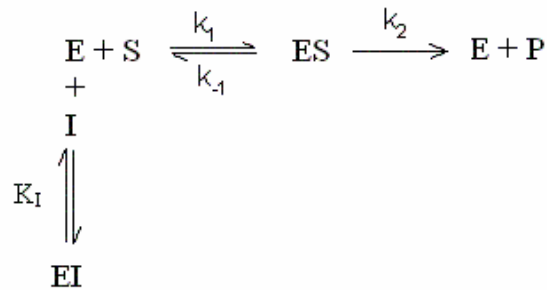
Figure 1.12. Mechanism for Competitive inhibition

Due to the reversibility of the reaction, when the substrate concentration is very high, the equilibrium shifts toward ES, and the inhibition can be overcome. Therefore the V_{max} in this type of inhibition remain unchanged with the addition of inhibitor. But due to simultaneous binding of the enzyme with the inhibitor, the dissociation constant for the enzyme substrate complex K_M decreases. Change in the velocity of product formation in the presence of inhibitor can be given as shown in Equation 5.

$$V = \frac{V_{max}[S]}{K_M \left(1 + \frac{[I]}{K_I}\right) + [S]}$$

(Equation 5)

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Therefore the apparent enzyme-substrate dissociation constant, K_M^{app} is equal to $K_M(1 + I/K_I)$, which is reflected in the Lineweaver-Burk plot associated with this inhibition as has been shown in Figure 1.8.

In uncompetitive inhibition, the inhibitor binds only to the enzyme-substrate complex and not to the free enzyme. This may be due to a change in conformation in the enzyme, which takes place when the enzyme binds to the substrate. The mechanism of this reaction is shown in Figure 1.13. Here the inhibitor I, binds reversibly with the enzyme-substrate complex ES, to form the enzyme-substrate-inhibitor complex, EIS. The EIS cannot form product. The dissociation constant for this reaction is known as the enzyme substrate complex-inhibitor dissociation constant, K_I'.
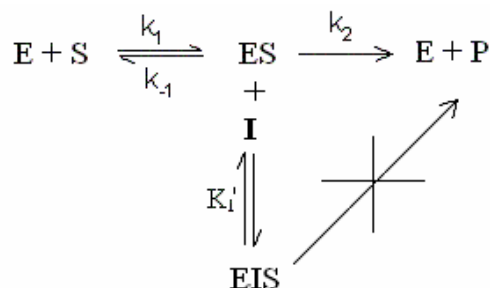
Figure 1.13. Mechanism for Uncompetitive inhibition

In uncompetitive inhibition, due to the presence of ESI, even when the substrate concentration is increased the whole of E is not converted to ES. Some of it goes in the formation of ESI. Therefore the V_{max} value with inhibitor will be less. In case of K_M, since I binds to ES, the equilibrium of the reaction shifts toward ES thus effectively

reducing the value of K_M, which is related to k_{-1}/k_1. The rate of product formation in uncompetitive inhibition is given as shown in Equation 6.

$$V = \frac{V_{max}[S]}{[S]\left(1 + \frac{[I]}{K_I'}\right) + K_M}$$

(Equation 6)

Thus in uncompetitive inhibition the $K_M^{app} = K_m/(1 + I/K_I')$ and $V_{max}^{app} = V_{max}/(1 + I/K_I')$ which is what is expected from the Lineweaver-Burk plot.

In mixed and noncompetitive inhibition, the inhibitor binds to both enzyme and enzyme substrate complex. Thus I bind both with E and ES reversibly to form EI and EIS respectively. EI may also react with the substrate to form EIS, but EIS cannot form product. The dissociation constants are same as discussed in the previous two cases of inhibition. The only difference between mixed and noncompetitive inhibition is that in the later one the value of K_I and K_I' are the same. Figure 1.14. shows the mechanism for mixed and noncompetitive inhibition.
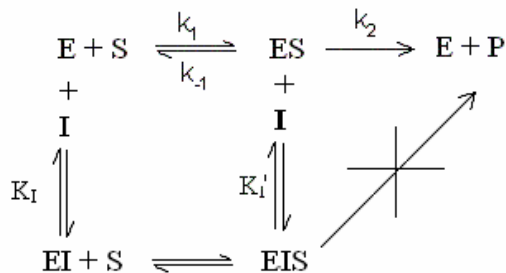
Figure 1.14. Mechanism for Mixed and Noncompetitive inhibition

It is apparent from Figure 1.9. that even at higher substrate concentrations there will be some ESI present and that E will not be totally converted to ES. Therefore V_{max} for both mixed and noncompetitive inhibition will be lowered. The value of K_M in noncompetitive inhibition will remain unchanged as I binds with both E and ES at the same rate. In the case of mixed inhibition, the value of K_M will change and the change will be governed by whether the value of K_I is greater or less than K_I'. The velocity of product formation for mixed inhibition is given in Equation 7.

$$V = \frac{V_{max}[S]}{[S]\left(1 + \frac{[I]}{K_I'}\right) + K_M\left(1 + \frac{[I]}{K_I}\right)}$$

(Equation 7)

Thus both for mixed and noncompetitive inhibition, $V_{max}^{app} = V_{max}/(1 + I/K_I')$. For noncompetitive inhibition, the K_M^{app} will remain be the same as without inhibitor and for mixed inhibition it will be $K_M^{app} = K_M (1 + I/K_I) / (1 + I/K_I')$.

1.6 Drug metabolism, cytochrome P450 and Echinacea

As already mentioned cytochrome P450 is involved in the metabolism of most of the drugs in market today. Though the isoforms are substrate specific, with so many drugs and other xenobiotic components to be metabolized by the same enzyme, interactions seem inevitable. Interactions of drug or herbal products including Echinacea and Spilanthes with drug metabolism by cytochrome P450 can be manifested through

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either inhibition or induction. In many substances inhibition of drug metabolism is competitive in nature, where both the drug and the chemical from the herbal product compete for the same binding site, thus slowing the metabolism of the drug. With induction the metabolizing capacity of the enzyme in increased when the product is administered as a result of an increase in functional P450. As we have seen, each isoform of cytochrome P450 has a long list of drugs and other compounds that it will act on and there are certain known inhibitors and inducers of each activity as well. Table 1.1. shows the list of some of the known drug substrates, inhibitors and inducers specific to individual isoforms that are relevant to the current study [46, 47].

Table 1.1. Some drugs, inducers and inhibitors affecting P450 isoforms of interest

Isoform	Substrate	Inhibitor	Inducer
1A2	Propanolol, theophylline, F-Warfarin	Amiodarone, cimetidine	Omeprazole, Phenobarbital
2E1	Enfluren, chlorzoxazone, theophyline	Diethyldithiocarbamate, disulfiram	Ethanol, isoniazid
2C9	Diclofenac, ibuprofen, tolbutamide.	Fluconazole, lovastatin	Rifampin, secobarbital
2A6	Aflatoxin B_1, clozapine, dexamethasone	Amiodarone hydrochloride, ketokonazole	Phenobarbital sodium

Echinacea has been the focus of many studies, however the primary emphasis of most of these studies has been the immunostimulatory activity and identification of compounds in Echinacea that may contribute to it. The possibility of unwanted drug-herb interaction with the intake of Echinacea is a field which still needs to be explored. In a recent study, the effects of *Echinacea purpurea* root extract on select cytochrome P450 activities were studied *in vivo* [48]. Echinacea was shown to inhibit the activity of CYP1A2 to a considerable extent whereas it failed to cause a significant effect on CYP2C9 and CYP2D6. The effect on Echinacea extract was found to be selective on intestinal and hepatic CYP3A4 which means that it was predicted to induce the activity of hepatic CYP3A but inhibit the activity of intestinal CYP3A. *Echinacea purpurea* root extracts were also shown to inhibit the activity of c-DNA-expressed CYP3A4 in another study [49]. The first part of our study deals with the study of interaction of Echinacea with the CYP1A2, CYP2A6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 isoforms of cytochrome P450. After obtaining a general picture, the activity of 2E1 has been studied with a greater detail. The reason for choosing CYP2E1 lies in the significant role played by this isoform in controlling toxicity in the body and evidence that a variety of natural products appear to modulate its activity.

1.7 Toxicology, CYP2E1 and *Echinacea purpurea*

Although the main function of cytochrome P450 seems to be to help the body get rid of xenobiotic substances, many times it actually causes xenobiotic bioactivation instead. This often leads to severe toxicologic effects like cellular dysfunction and destruction. CYP2E1 is considered to be very important in this regard. This is primarily

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due to its ability to activate many xenobiotics into toxic compounds. For example, CYP2E1 leads to the production of a highly toxic metabolite from the drug acetaminophen [50]. It is known to be involved in the bioactivation of a number of procarcinogens into either or both cytotoxic and carcinogenic compounds [51, 52].

CYP2E1 does perform some physiological functions like protecting the body against toxic xenobiotics, especially airborne ones, by detoxifying them [50]. It also takes part in fatty acid and acetone metabolism [50]. However, the harm it is capable of doing to the human body seems to be of much more significance. Moreover these effects are magnified in presence of ethanol which is both a substrate and inducer for CYP2E1. The process of ethanol metabolism itself leads to production of oxygen radicals which when accumulated beyond a certain level cause oxidative stress and finally lipid peroxidation and membrane damage [50]. Also acetaldehyde produced in the process is highly toxic [50]. CYP 2E1 may also be linked with breast cancer due to its involvement in the conversion of N-nitrosamines, derived from tobacco smoke, into breast carcinogens as a part of their metabolizing procedure [53]. Apart from N-nitrosamines, CYP2E1 also activates other procarcinigens like benzene known to promote leukemia [54], urethane and styrene [55, 56]. It is also known to metabolize solvents, out of which compounds like carbon tetrachloride and halothen generally produce cytotoxicity, whereas chloroform, vinyl chloride and others are carcinogenic. As mentioned above alcohol being an active inducer of CYP2E1, increases the level of the active isoform and thereby effectively facilitates harmful bioactivations. For example, females who comsume alcohols have a higher risk of breast cancer. Thus it may be observed that a compound

which can inhibit the activity of CYP2E1 so that its level can be controlled, may prove to be beneficial to mankind. Therefore in the final part of our study we have tried to characterize the 2E1 reaction based on its interactions with *Echinacea purpurea* and specific components isolated from Echinacea extract.

1.8 Proposal

In our research we are making an attempt to evaluate the effects of *Echinacea purpurea* and *Spilanthes acmella* on the catalytic activity of a variety of drug metabolizing cytochrome P450 enzymes present in human and rabbit liver. The objective of this study is to identify potential interactions between *Echinacea purpurea/ Spilanthes acmella* extracts and cytochrome P450 enzymes in the endoplasmic reticulum of rabbit and human liver microsomes to address the potential mechanisms involved in the process. This may be carried out using isoform specific substrates as probes and determining the effect of the plant extracts on the catalytic activity of P450. Further, isolated components from the whole plant extracts may be used so that a comparative study may be carried out to determine which constituents are responsible for the observed inhibitory effects on the 2E1 isoform of cytochrome P450.

CHAPTER II

EXPERIMENTAL

2.1 Materials

Rabbit livers, human livers and supersomes were purchased from Pel-Freez (Rogers, Arkansas), Moltox and BD Gentest respectively. Echinacea and Spilanthes was obtained from Horizon Herbs (Williams, OR). NADPH, p-nitrophenol, p-nitrocatechol, 7-ethoxycoumarin, 7-hydroxycoumarin, diclofenac and potassium phosphate were all obtained from Sigma Chemical Co. Sodium borate, methanol, sodium hydroxide and acetonitrile were obtained from Fisher Scientific. Perchloric acid and hydrochloric acid were obtained from Acros Organic Chemicals and chloroform was obtained from Lab Guard. All other reagents were of highest quality and were from common commercial sources.

Liver microsomes were prepared by members of Dr. Raner's lab following a previously published procedure and the amount of microsomal protein was determined to be 29mg/mL using the biuret method devised by Lowery et al. [57]. The protein content of the human liver was 33mg/mL according to the literature provided with the samples.

2.2 Preparation of Echinacea and Spilanthes extract

The Echinacea and Spilanthes extracts were prepared by a research group under Dr. Nadja Cech. Fresh *Echinacea purpurea* plants were separated into roots or aerial portions and each part was washed with deionized water. After that it was weighed.

Extracts were made using a ratio of 2mL menstruum (solvent)/1 g plant material. The extracts studied in this project were made of 33% ethanol for the hydrophilic interactions and 100% ethanol for hydrophobic interactions.

The first step in the preparation of the extract involved blending of the plant material thoroughly with menstruum and storing it for two weeks. The aqueous extracts were stored at 0°C while the ethanolic extracts were stored at room temperature. During this period, the extracts were shaken from time to time. After that the extract was removed from the plant material. A hydraulic press was used for this purpose. It was then filtered, and stored in clean jars at -20 °C for future analysis.

2.3 Detection of Caffeic acid derivatives

Caffeic acid derivatives were also detected, isolated and quantitated by Dr. Nadja Cech's research group. The instrument used for analysis was HPLC/ESI-MS. In the sample preparation procedure, 1 ml aliquot of each extract was used. It was first centrifuged to remove particulate matter. After that it was filtered using a 0.2μM filter. The analysis involved separation of the extracts using an HPLC (HP1100, Agilent) with a C18 column (50 x 2.1mM, 3.0 μM particle size). Any particulate caused was filtered using a 4μM precolumn filter (MacMod Analytical) prior to the column. An injection volume of 10 μL was used and the flow rate was set to 0.2 mL/min.

For detection purpose an ion trap mass spectrometer with electrospray ionization source (LCQ Advantage, Thermo Finnigan) was used to which the HPLC was interfaced. The samples were analyzed in the negative ion mode. The scan range used was 150 to

1500 m/z. The spray voltage was set at -4.5 kV and the tube lens offset was -50V. The capillary temperature and voltage were 275 °C and -10V respectively.

Next step involved preparation of Calibration curves. Here a mixture of standard compounds prepared in ethanol having a concentration of 1×10^{-3} M (Chromadex, Santa Ana, CA) was used which consisted of caftaric acid, chlorogenic acid, cichoric acid, and cynarin. This mixture was diluted to a concentration of 1×10^{-4} M and run using the same conditions as the extracts. Calibration curves were then plotted, the parameters used being peak area of the selected ion chromatogram for caftaric acid and chlorogenic acid versus concentration, caftaric acid and chlorogenic acid being the compounds of interest in the current study.

2.4 Detection of Isobutylamides

The isolation and quantitation of isobutylamides from Echinacea and Spilanthes involved three steps. The raw extracts were first subjected to large scale purification as described in the following section.

2.4.1 Large scale purification

The large scale purification was done by Ashey Mortenson and Dr. Gregory Raner in the lab headed by Dr. Gregory Raner. In this process the extract was separated into several fractions based on the solubility of the components in ethanol. The apparatus used consisted of a Waters Sep-Pak Vac 35cc C18 -10g tube attached to a Spectra/ Chrom MP-1 Pump. For this purpose the raw extract was first diluted with water to a 1:1 ratio. Next the C18 column being used was wetted with 100% ethanol and flushed several times with 50% ethanol and 50% water. After that the C18 tube was loaded with the

diluted extract prepared earlier. Solvent mixtures with varying concentration of ethanol and water ranging from 50%-50% to 90%-10% combination were then fed to the tube using the pump and fractions coming out of the column through the exit tube were collected.

The fractions thus collected were then analyzed using HPLC where 250μL of the fractions were injected into a HAISIL 100 C18 column (5 micron, 150 x 4.6mM) from Higgins Analytical Inc. The flow rate was 1.5mL per minute and the mobile phase for this analysis consisted of 50% of acetonitrile with 0.1% trifluoroacetic acid and 50% of water with 0.1% of trifluoroacetic acid. The HPLC system consisted of an SCL-10A VP Shimadzu system controller, a SIL-10AD VP Shimadzu autoinjector, a LC-10AT VP Shimadzu liquid chromatogram, a FCV-10AL VP quarternary mixing chamber, a DGU-14A degasser and a SPD-10AV VP Shimadzu UV-VIS detector. Let this system be named as HPLC 1 for future references. The system was interfaced to a personal computer operating with a CLASS-VP automated software system. The lamp used was a deuterium lamp.

2.4.2 Isolation of isobutylamides

The first fraction collected using a solvent combination of 50% ethanol and 50% water and the same using 60%-40% combination were relevant to the study and were used as a raw material for further isolation of compounds. The fractions were first diluted with water to a 1:1 ratio. These fractions were then injected into the HAISIL 100 C18 column mentioned earlier and fractions were collected which had significant absorbance at a particular wavelength. A deuterium lamp was used for the purpose. The injection

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volume was 4mL and the flow rate was 3mL per minute. The mobile phase was 50% acetonitrile with 0.1% trifluoroacetic acid and 50% water with 0.1% trifluoroacetic acid. The HPLC system consisted of a LC-10AT VP Shimadzu liquid chromatogram, a SCL-10A VP Shimadzu system controller, a FCV-10AL quarternary mixing chamber, a DGU-14A in-line degassing unit, a SPD-M10A VP Shimadzu diode array detector and a SIL-10A Shimadzu autoinjector. The system was interfaced to an AST Bravo LC 5166M computer operating with a CLASS-VP automated software system. Let this HPLC system be named as HPLC 2 for future references.

2.4.3 Determination of the concentrations of the isobutylamides

The samples collected using the previous procedure were tested for concentration using a HPLC (HP1100, Agilent) with a C18 column (50 x 2.1mM, 3.0μM particle size). An ion trap mass spectrometer with electrospray ionization source (LCQ Advantage, Thermo Finnigan) detector was interfaced with the HPLC system for detection purpose. Detection was done using the same conditions as was used for the quantitation of caffeic acid derivatives mentioned in section 2.3. Finally, concentration was calculated by running a standard sample under same conditions and comparing the concentration of the standard with that of the samples.

2.5 Oxidation of p-nitrophenol (2E1 activity) by herbal extracts

Initially the microsomes (rabbit liver/ human liver/ supersomes) and the NADPH were taken out of refrigeration and thawed. Next, a series of reaction mixtures were prepared. To each of these mixtures 10μL of microsomes were first added followed by 50μL of 1M potassium phosphate buffer having a pH of 7.4. Next various amounts of p-

nitrophenol was added to make concentrations ranging from (10μM- 100μM). Finally

deionized water was added to the reaction mixture to make up a final volume of 500μL.

The reaction was started with the addition of 25μL of 20mM NADPH. The samples were

incubated for 30 minutes in a water bath at a temperature of 30°C. The reaction was

quenched with the addition of 200μL of 6% (v/v) perchloric acid and after that placed on

ice for 15 minutes. The samples were then centrifuged for 10 minutes at 8000 RPM and

analyzed using a Shimadzu high performance liquid chromatograph (HPLC 1). The

mobile phase for the analysis was 30% acetonitrile (with 0.1% trifluoroacetic acid) and

70% of deionized water (with 0.1% trifluoroacetic acid) having a flow rate of 1.0

mL/min. The HPLC column used in the analysis was a Higgins Analytical Inc, HAISIL

100 C18 (150 x 4.6mM, 5micron) column. The product had an absorbance at 350nM.

The peak areas were integrated and then plotted against concentrations using the

SlideWrite software and the V_{max} and K_M values were determined.

Table 2.1. shows the pNP assay used for 2E1 activity. Total volume of the reaction

mixture is 500μL.

Table 2.1. Reaction mixture for p-nitrophenol hydroxylation assay

Microsomes	Phosphate buffer (pH 7.4) 1M	PNP 1mM	H$_2$O	NADPH 20mM
10μL	50μL	5μL	410μL	25μL
10μL	50μL	5μL	435μL	--
10μL	50μL	10μL	405μL	25μL
10μL	50μL	10μL	425μL	--
10μL	50μL	20μL	395μL	25μL
10μL	50μL	20μL	420μL	--
10μL	50μL	30μL	385μL	25μL
10μL	50μL	30μL	410μL	--
10μL	50μL	40μL	375μL	25μL
10μL	50μL	40μL	400μL	--
10μL	50μL	50μL	365μL	25μL
10μL	50μL	50μL	390μL	--

Inhibition of 2E1 activity by *Echinacea purpurea* and *Spilanthes acmella* was studied by repeating the assay as described in the previous section with addition of Echinacea and Spilanthes extract. The extracts were first added to the test tubes. Then the contents of the test tubes were evaporated using a centravap concentrator (Labconco)

attached to a Duoseal vacuum pump (Welch) to get rid of the ethanol. Next, microsomes, buffers, p-nitrophenol and water were added as mentioned before and the samples were analyzed again with the HPLC system. Similar assays were performed with different fractions of the extracts and also with the individual compounds.

2.6 Inhibition of hydroxylation of diclofenac (2C9 activity) by herbal extracts

Here the experimental procedure involved preparation of a 0.50mL reaction mixture containing 20pmole CYP2C9 supersome, 50μL of 100mM phosphate buffer (pH 7.4), different concentrations of diclofenac solution ranging between (0.01mM and 0.2mM) and water to make up the final volume. The reaction was initiated using 25μL of 20mM NADPH. The reaction mixture was incubated at 37° C for 20 minutes. The reaction was stopped by the addition of 100μL of 94% acetonitrile and 6% glacial acetic acid and centrifuged at (10,000 x g) for 3 minutes. Next 100μL of the supernatant was injected into a Higgins HAISIL C18 (250 x 4.6mM, 5 micron) column with a mobile phase initially of 20% acetonitrile, 30% methanol with 1mM perchloric acid in water changing to 100% methanol over 20 minutes and at a flow rate of 1.0mL/min. The product had an absorbance at 254nM. The HPLC system used was HPLC 1.

Inhibition of 2C9 activity by the herbal extracts were studied by repeating the assay described above with addition of the plant extracts into the test tubes. The test tubes were first placed in a centravap concentrator (Labconco) attached to a Duoseal vacuum pump (Welch) to evaporate the ethanol before adding the rest of the contents. The samples were treated in the same way as mentioned in the previous section and subjected to HPLC analysis using HPLC 1 system. The peak area corresponding to the product in

chromatogram thus obtained were integrated and then plotted against the substrate concentration to give Michaelis-Menten plots. The reciprocals gave Lineweaver-Burk plots from which the V_{max} and K_M values were determined.

2.7 Inhibition of hydroxylation of coumarin (2A6 activity) by herbal extracts

Similar procedure as mentioned in the previous sections was followed in the study of 2A6 activity for the preparation of the reaction mixtures. Coumarin solution having concentrations ranging between (0.001mM and 0.04mM) was added to the reaction mixtures containing 3μL of 2A6 supersomes and 50μL of 100mM phosphate buffer (pH 7.4). The reaction was started using 25μL of 20mM NADPH and the reaction mixture was incubated at 37° C for 20 minutes. After incubation, the reaction was stopped by the addition of 0.1 ml 20% trichloroacetic acid and centrifuged at (10,000 x g) for 10 minutes. Next, 100μL of the supernatant is added to 1.9mL of 100mM Tris buffer having a pH of 9. Finally the samples were analyzed using a spectrofluorometer (FluoroMax2) with a 150W continuous ozone free xenon lamp (Isa Jobin Yvon, Spex Horiba Group) where the fluorescence was determined with excitation at 368nM and emission at 456nM. The peaks were integrated and the activity was quantitated by subtracting the fluorescence of the blank.

Inhibition of 2A6 activity by the herbal extracts was studied by repeating the assay with addition of Echinacea and Spilanthes extract. The extracts were first added to the test tubes and the ethanol was evaporated using a centravap attached to a pump. The rest of the procedure was same as above and the samples were finally analyzed with the

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FluoroMax2 and quantitated by subtracting the integrated value of the fluorescent peak generated by the blank.

2.8 Inhibition of 7-Ethoxycoumarin O-deethylation (1A1/1A2 activity) by herbal extracts

For the analysis of 7-ethoxycoumarin O-deethylation, all procedures were carried out in dim light, due to the fluorescent property of the product. For these analyses 5μL of microsomes were placed in test tubes with 1M phosphate buffer of pH 7.4 and different concentrations of 7-ethoxycoumarin, ranging from 0.01μM to 0.7μM. Deionized water was then added to bring the volume 500μL. Incubations were initiated with 25μL of 20mM NADPH and incubated for 20 minutes at a temperature of 37°C. The reaction was quenched with 100μL of 2.0N HCl. Next, 2.0mL of chloroform was added to the test tubes. The reaction mixtures were then vortexed for 30 seconds and centrifuged for 5 minutes. After that the lower phase was taken in another test tube and 3.0mL of sodium borate buffer was added to it. Next the tubes were centrifuged and the upper phase containing the product was transferred to a cuvette and fluorescence was measured with excitation wavelength set at 338nM and emission wavelength set at 450nM. The instrument used was a FluoroMax-2 with DataMax having a 150W continuous ozone free xenon lamp (Isa Jobin Yvon, Spex Horiba Group).

The peaks thus obtained were integrated and the area was determined by subtracting the area of the peak in the blank from that in the samples.

Inhibition of 1A1/1A2 activity by *Echinacea purpurea* and *Spilanthes acmella* was studied by repeating the assay with addition of Echinacea and Spilanthes extract.

Here like the previous methods the extracts after being added to the test tubes were first subjected to a centravap condenser to evaporate the alcohol and the rest of the procedure was carried out in the same way as the mentioned above.

CHAPTER III

RESULTS

As has been discussed earlier drug-herb interactions are a widely studied phenomenon and more so these days as the increasing demand for herbal products in the market has made it essential to learn how these products interact when concurrently taken with conventional drugs. Most known drug-herb interactions involve the activity of cytochrome P450, an important drug-metabolizing enzyme. Hence in the first part of the current study an attempt has been made to investigate the interactions of two popular herbal products, Echinacea and Spilanthes with different isoforms of cytochrome P450. The method employed for this purpose has been to observe how raw Echinacea and Spilanthes extracts inhibit the activity of various isoforms of cytochrome P450. Reactions have been carried out using known substrates for a specific isoform and inhibitory activity of the herbal products has been studied by adding the herbal product into the reaction medium to observe a change in the amount of product formed. The extracts used in this part of the study were *Ehinacea purpurea* root in 100% ethanol and *Spilanthes acmella* whole flowering plant in 100% ethanol. The nature of inhibition has been determined by plotting Michaelis-Menten graph followed by a Lineweaver-Burk modification.

3.1 Inhibition of 2C9 activity by Echinacea and Spilanthes

The first activity studied for this purpose was the 2C9 activity. This isoform catalyzes the hydroxylation of diclofenac to form 4-hydroxydiclofenac, diclofenac being a well-known substrate for CYP2C9. The general reaction is as follows:
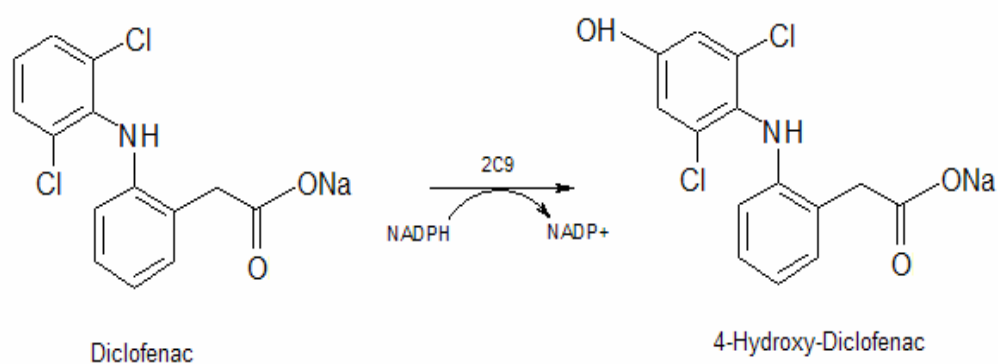
Reaction in presence and absence of the herbal extracts were carried out as described in the previous chapter and the resulting chromatogram showed clear peaks of the substrate and the product based on their absorbances. The CYP2C9 supersomes were used in the reactions. The HPLC analysis was monitored at 254 nm. Diclofenac had a retention time of 11.7 minutes whereas 4-hydroxydiclofenac had a retention time of 9.6 minutes. Initially inhibition was observed as a decrease in product peak area in the presence of inhibitor using a single substrate concentration. Inhibition was noticed with the addition of only 5μL of extract in the case of both the herbal products. The percentage of inhibition at 0.1mM substrate concentration was found to be 36.2% for Echinacea extract and 51.7% for Spilanthes extract. Reactions were then carried out using different concentrations of the substrate both in presence and in absence of the herbal extracts and

the peak areas of the product were plotted against concentration of the substrate used. The resulting Michaelis-Menten plot is shown in Figure 3.1. for inhibition by Echinacea. The reciprocal of the data set from the Michaelis-Menten plot gave the Lineweaver-Burk plot, which is shown in Figure 3.2.
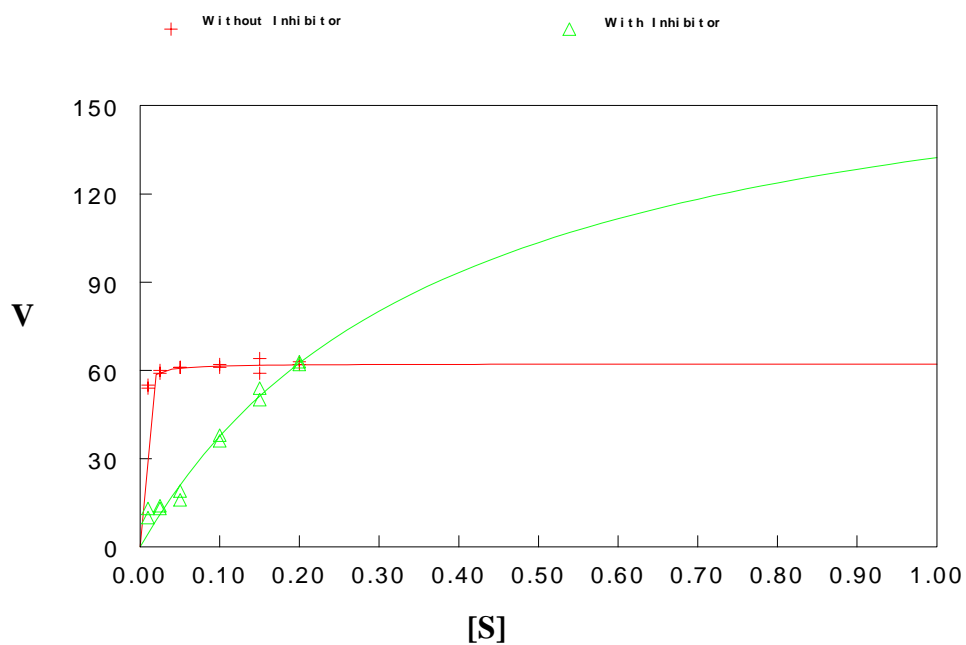
Figure 3.1. Michaelis-Menten plot showing inhibition of CYP2C9 with *Echinacea purpurea* root extract
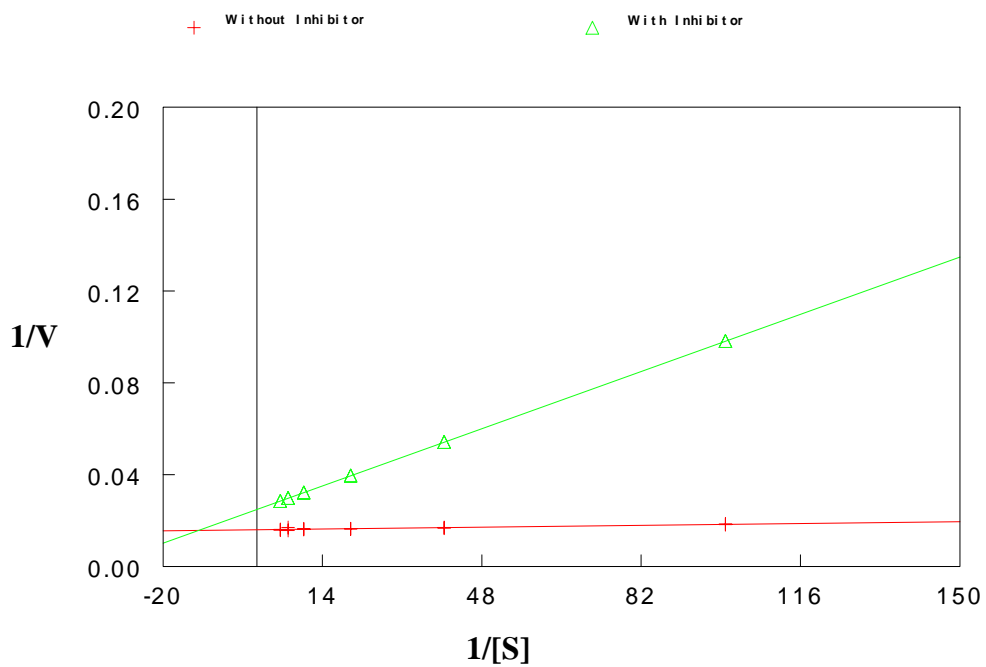
Figure 3.2. Lineweaver-Burk plot showing inhibition of CYP2C9 with *Echinacea purpurea* root extract

The nature of the inhibition was determined from the Lineweaver-Burk plot to be reversible and mixed.

The same procedure has been followed with Spilanthes which is represented by the Michaelis-Menten plot in given in Figure 3.3. Figure 3.4. shows the corresponding Lineweaver-Burk plot.
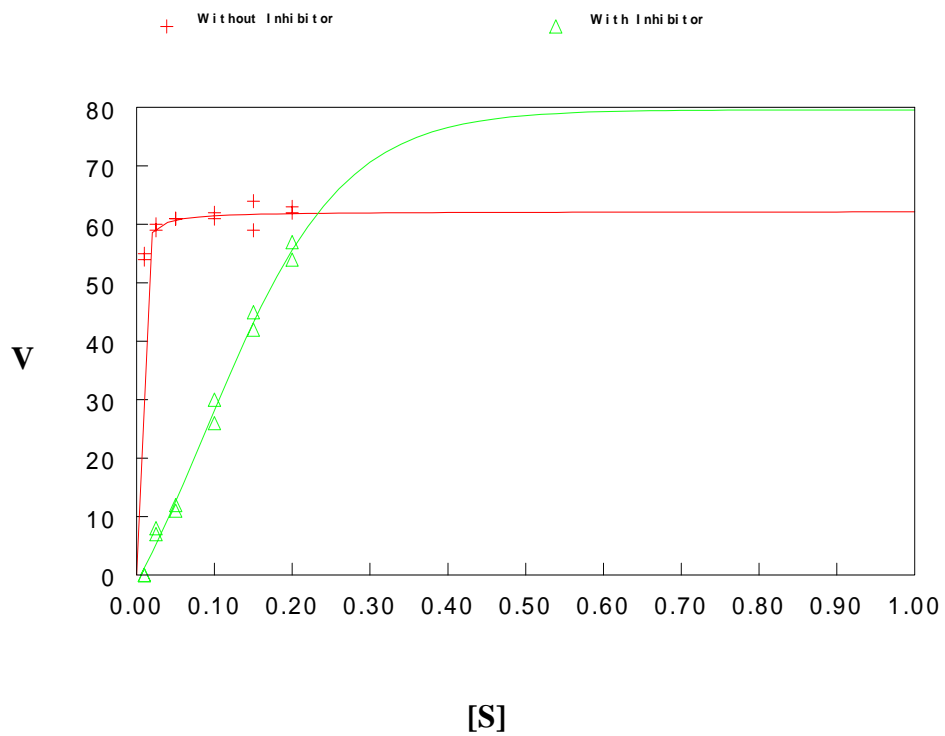
Figure 3.3. Michaelis-Menten plot showing inhibition of CYP2C9 with *Spilanthes acmella* root extract
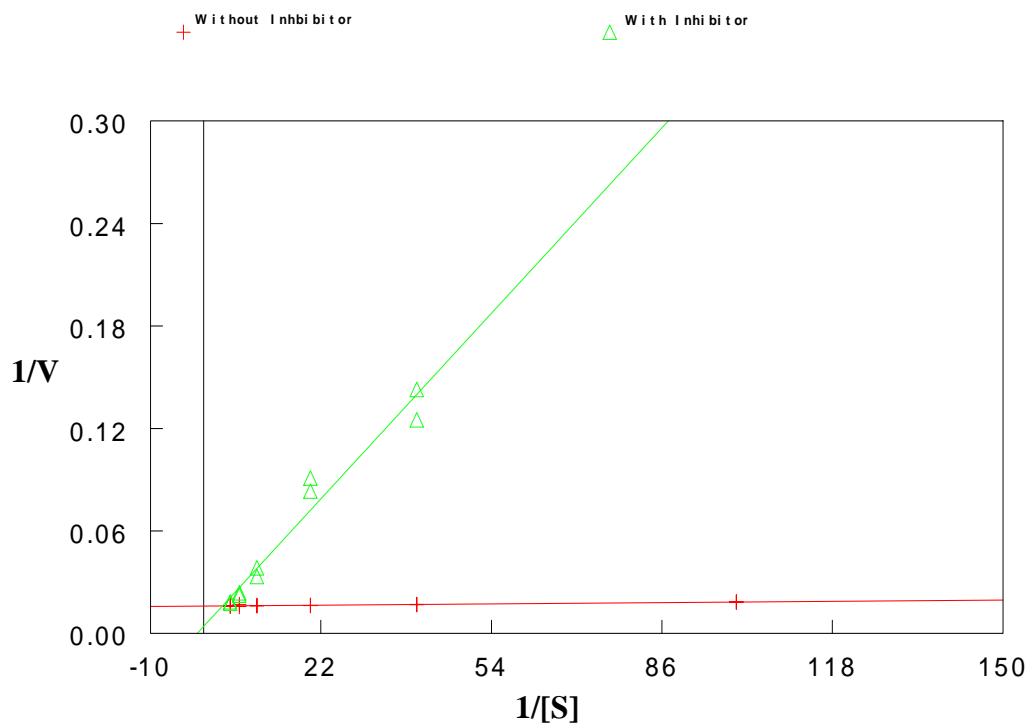
Figure 3.4. Lineweaver-Burk plot showing inhibition of CYP2C9 with *Spilanthes acmella* root extract

It may be concluded from the nature of the graph in figure 3.4. that the inhibition is mixed, although the range of [S] should be shifted to slightly lower values for a more reliable interpretation. It may be noted that the activity of the enzyme is nearly saturated at the lowest [S] concentration. It also needs to be determined whether this inhibition is reversible or irreversible.

3.2 Inhibition of 2A6 activity by Echinacea and Spilanthes

The 7-hydroxylation of coumarin to produce 7-hydroxycoumarin is catalyzed by CYP2A6 and this reaction has been used in the project to study the inhibitory activity of the herbal extracts of interest on the 2A6 isoform of the P450 enzymes. The general reaction is as follows:

Coumarin   7-Hydroxycoumarin
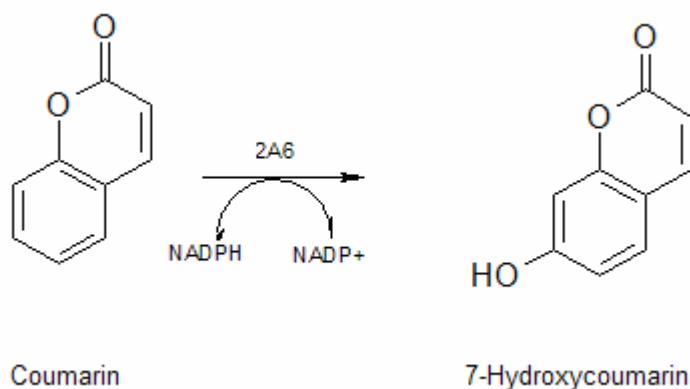
This reaction is light sensitive and the product formed is fluorescent in nature. The product formation was analyzed with a spectrofluorometer, Fluoromax 2. Initially a calibration curve was generated using different known concentration of the product. The product formation was monitored by generating a peak at an excitation wavelength of 338 nm and emission wavelength range of 410-500nM. Reactions were then carried out using several substrate concentrations ranging from 0.005mM to 0.05mM. The peak areas were integrated and plotted against substrate concentration. Considerable background was generated in the assay specially with the addition of herbal extracts. Care was taken to filter the substrate and the buffer solutions before use. Background produced with the

addition of herbal products could be due to many unknown fluorescent compounds which may have been present there. The background was quite high in presence of Echinacea. This factor was handled by running blank reaction and subtracting the peak area of the background from the rest. With the addition of 10μL of Echinacea extract, inhibition in product formation at 0.04mM substrate concentration was calculated to be 80%. With Spilanthes inhibition of about 75% was observed when 10μL of the extract was added at 0.04mM substrate concentration. The Michaelis-Menten plot and the Lineweaver-Burk plot demonstrating the inhibition of 2A6 activity caused by Echinacea and Spilanthes extract is shown in the Figures 3.5., 3.6., 3.7. and 3.8. respectively.
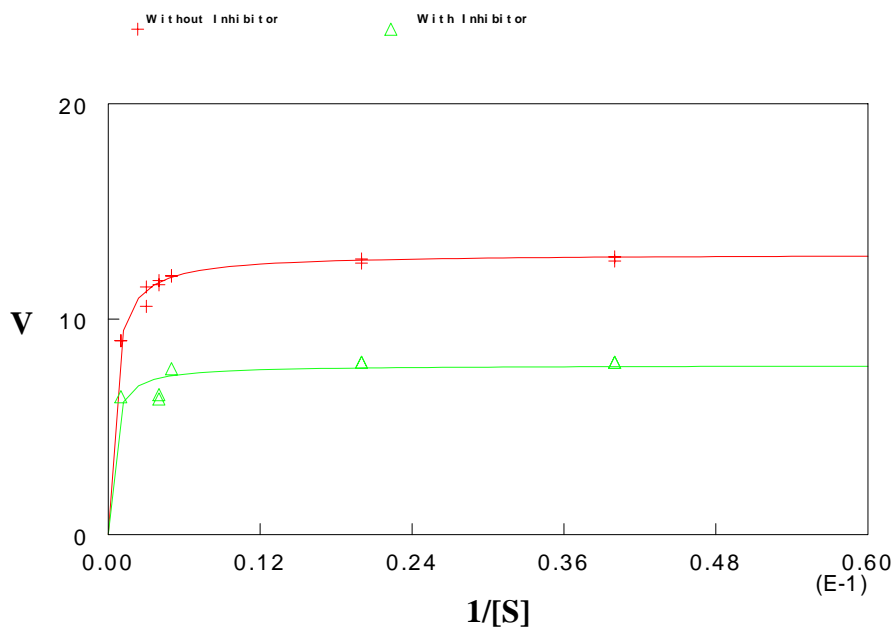
Figure 3.5. Michaelis-Menten plot showing inhibition of CYP2A6 activity with the addition of 10μL of raw *Echinacea purpurea* root extract in 100% ethanol

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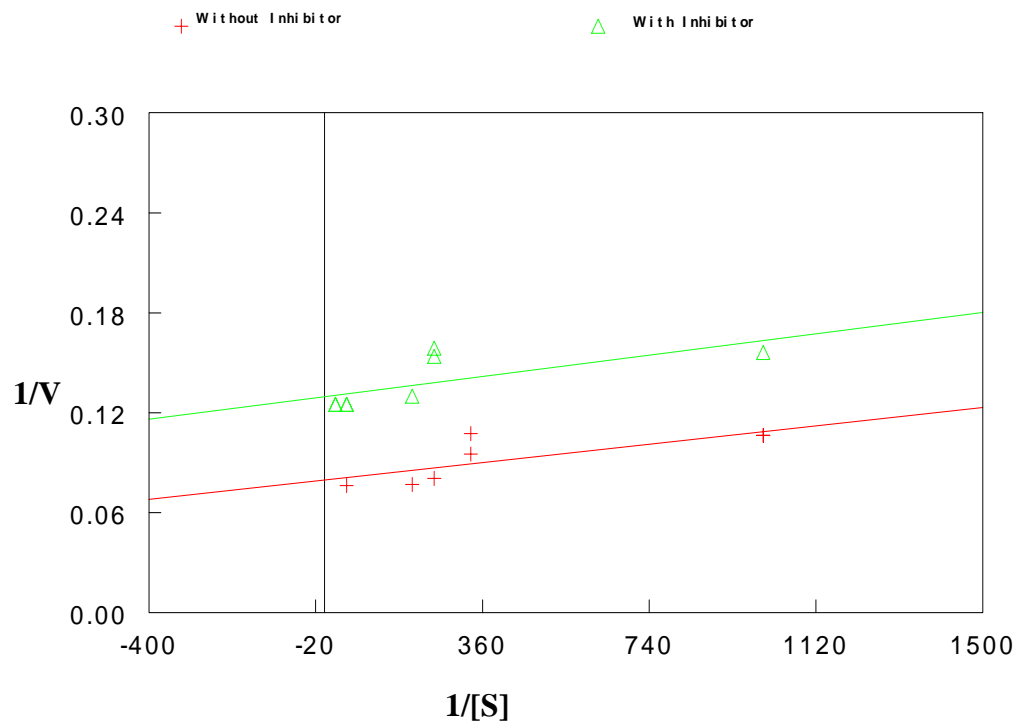
Figure 3.6. Lineweaver-Burk plot showing the nature of inhibition of CYP2A6 with the addition of 10μL of raw *Echinacea purpurea* root extract in 100% ethanol

Figure 3.7. Michaelis-Menten plot showing inhibition of CYP2A6 activity with the

addition of 10μL of raw *Spilanthes acmella* flowering plant extract in 100% ethanol
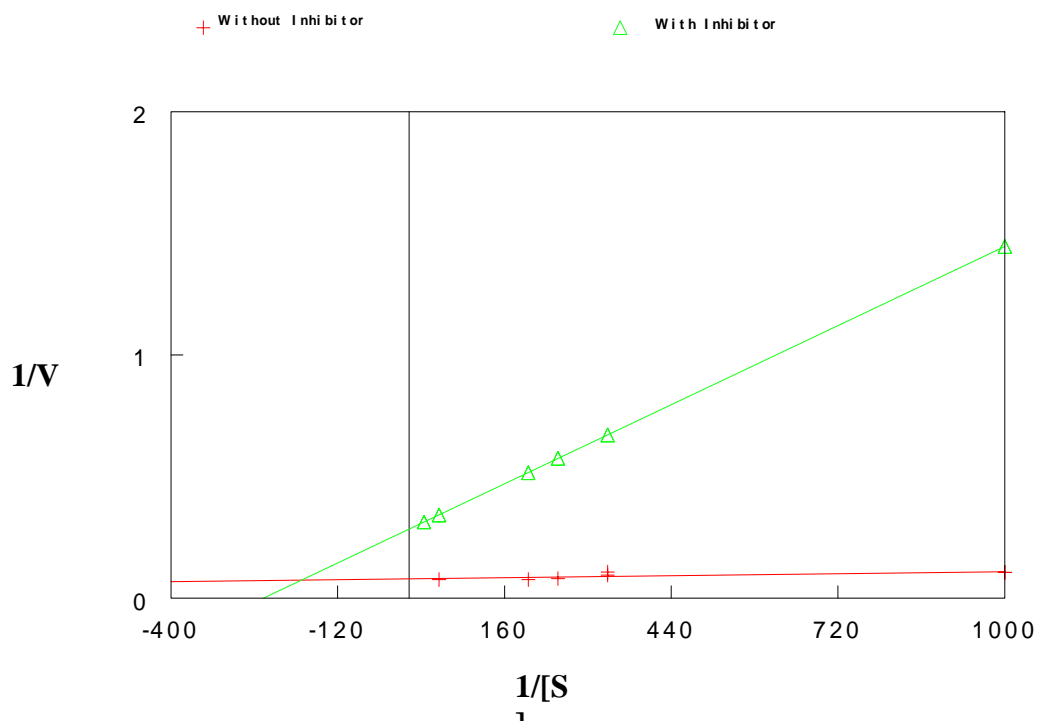
Figure 3.8. Lineweaver-Burk plot showing the nature of inhibition of CYP2A6 with the addition of 10μL of raw *Spilanthes acmella* flowering plant extract in 100% ethanol

The Lineweaver-Burk plots corresponding to Echinacea shows the nature of inhibition to be uncompetitive whereas the nature of inhibition with Spilanthes extract as determined from the Lineweaver-burk plot appears to be mixed.

3.3 Inhibition of 1A2 activity by Echinacea and Spilanthes

The deethylation of 7-ethoxycoumarin to produce 7-hydroxycoumarin is catalyzed by CYP1A2. The general reaction is given below:

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7-Ethoxycoumarin → (1A2, NADPH → NADP+) → 7-Hydroxycoumarin
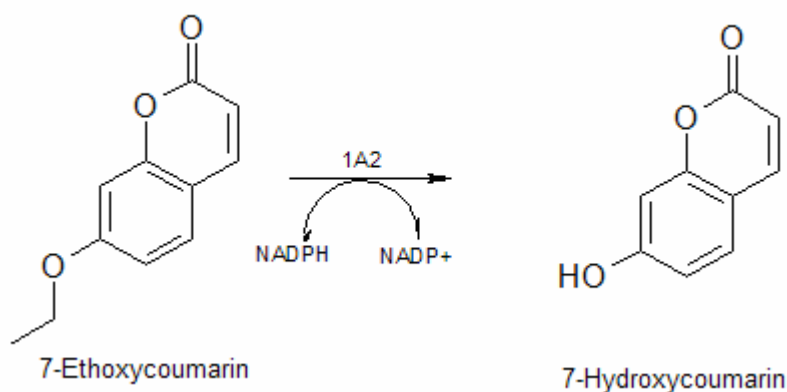
The product formation in this reaction was also monitored using the spectroflourimeter, Flouromax 2, due to the fluorescent nature of the product. The study of inhibition of this activity by the herbal extracts was however rendered difficult due to high background produced by other fluorescent compounds present in both the extracts and also the solutions used for extraction procedure. Addition of up to 10μL of Echinacea extract and 40μL of Spilanthes extract failed to show any inhibition of CYP1A2 with a substrate concentration of 1mM.

3.4 Inhibition of 2E1 activity by Echinacea and Spilanthes

Para-nitrophenol is hydroxylated in the presence of CYP2E1 to form para-nitrocatechol. This reaction is unique to CYP2E1 at low substrate concentration and has been used here to study the interaction of herbal products with CYP2E1. The general reaction is given as follows:

p-Nitrophenol   4-Nitrocatechol
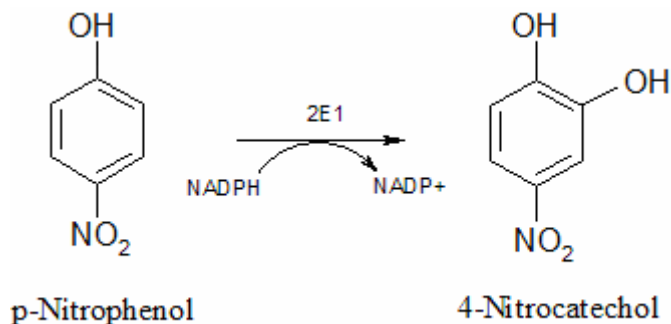
Initially, inhibition of 2E1 activity by Echinacea was determined to be reversible. Next, further analysis of the product formed in the reaction was carried out using HPLC 1 system. Inhibition with Echinacea was observed with the addition of even 2μL of raw extract whereas 40μL of raw Spilanthes extract was required to produce significant inhibition of CYP2E1 activity. For this activity the K_M and V_{max} values have also been calculated for use in the second part of the project. The experiments regarding inhibition were performed using both human liver microsomes and human CYP2E1 containing supersomes.

3.4.1 Inhibition on 2E1 by Echinacea using liver microsomes

The Michaelis-Menten plot that shows inhibition of 2E1 with the addition of 2μL(0.4%) and 5uL(1%) of raw *Echinacea purpurea* root extract in human liver microsome is shown in Figure 3.9. and the corresponding Lineweaver-Burk plot is shown in Figure 3.10. The percentage of inhibition at 0.02mM substrate concentration for 0.4% Echinacea extract is 27.0% while that using 1% Echinacea extract is 42.3%.
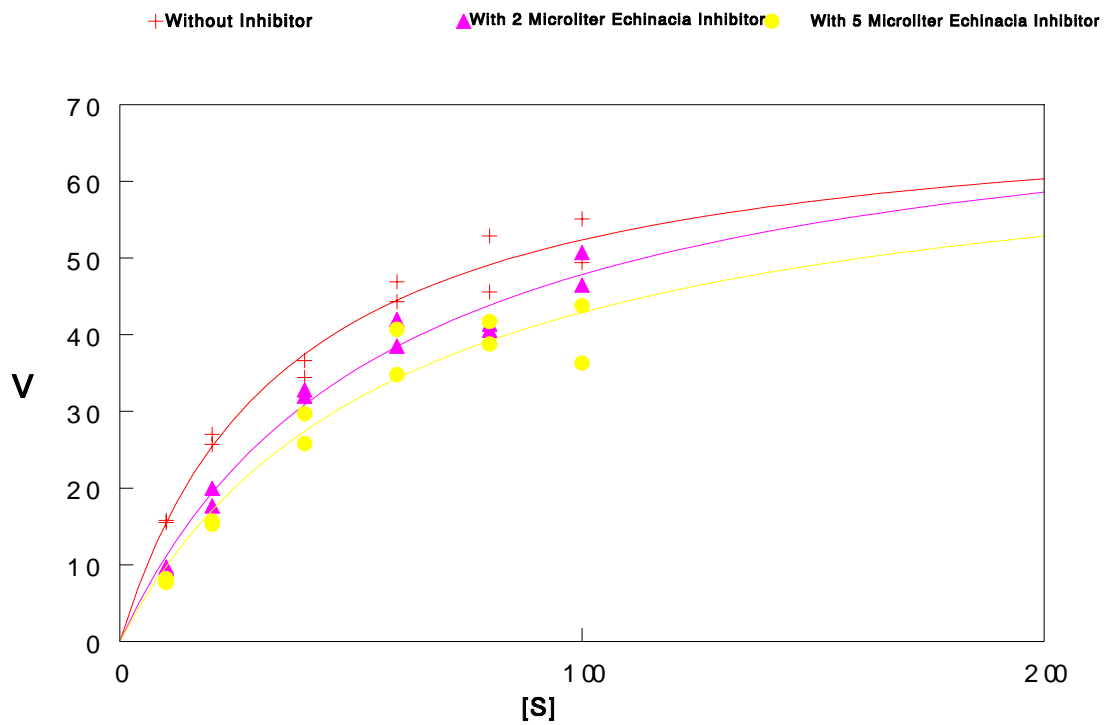
Figure 3.9. Michaelis-Menten plot showing inhibition of CYP2E1 with 2μL and 5μL raw *Echinacea purpurea* root extract using human liver microsomes
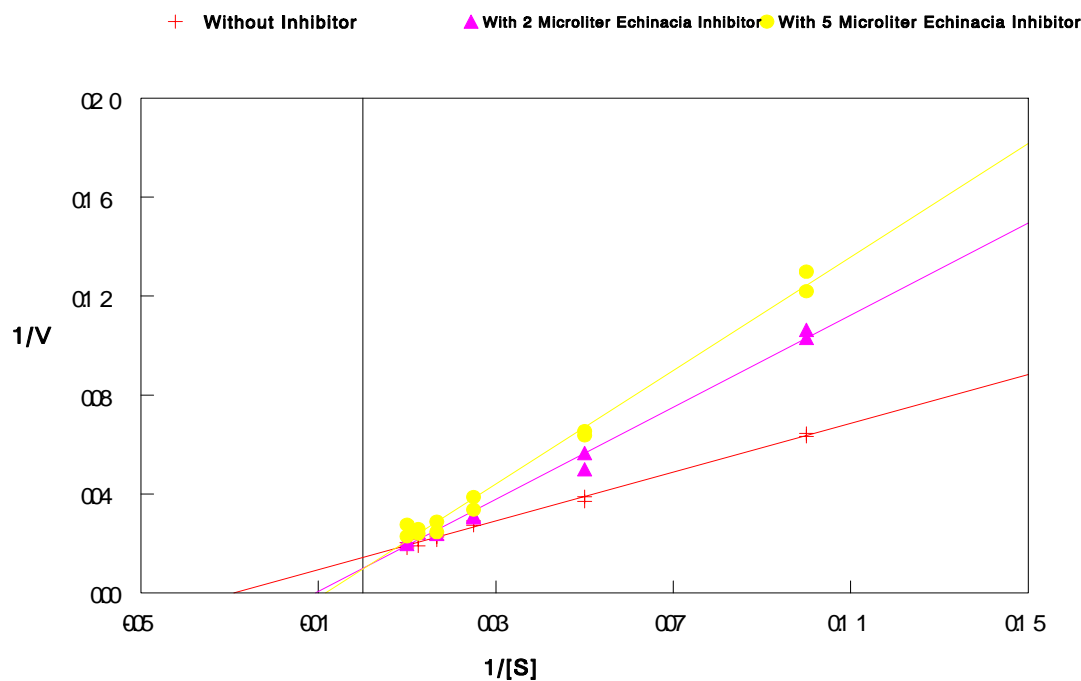
Figure 3.10. Lineweaver-Burk plot showing inhibition of CYP2E1 with 2μL and 5μL raw *Echinacea purpurea* root extract using human liver microsomes

The nature of inhibition as observed from the Lineweaver-Burk plot appears to be competitive with some mixed element as well. The V_{max} value is nearly the same for both the inhibited and noninhibited conditions and the values were determined to be 71μM/min, 75μM/min and 69μM/min for the control, addition of 0.4% Echinacea extract and 1% Echinacea extract respectively. The K_M value in absence of the inhibitor was found to be 36μM which increased to 57.8μM with the addition of 0.4% inhibitor. There

was further increase in the K_M value to 60.3μM when 1% Echinacea extract was added to the reaction.

3.4.2 Inhibition on 2E1 by Echinacea using human 2E1 containing supersomes

The Michaelis–Menten plot generated with the addition of 2μL and 5μL of raw Echinacea extract using CYP2E1 supersomes and the corresponding Lineweaver-burk plot is given in Figures 3.11. and 3.12. respectively. Here the percentage of inhibition at 0.02mM substrate concentration with 0.4% and 1% extract is 29.4% and 50% respectively.
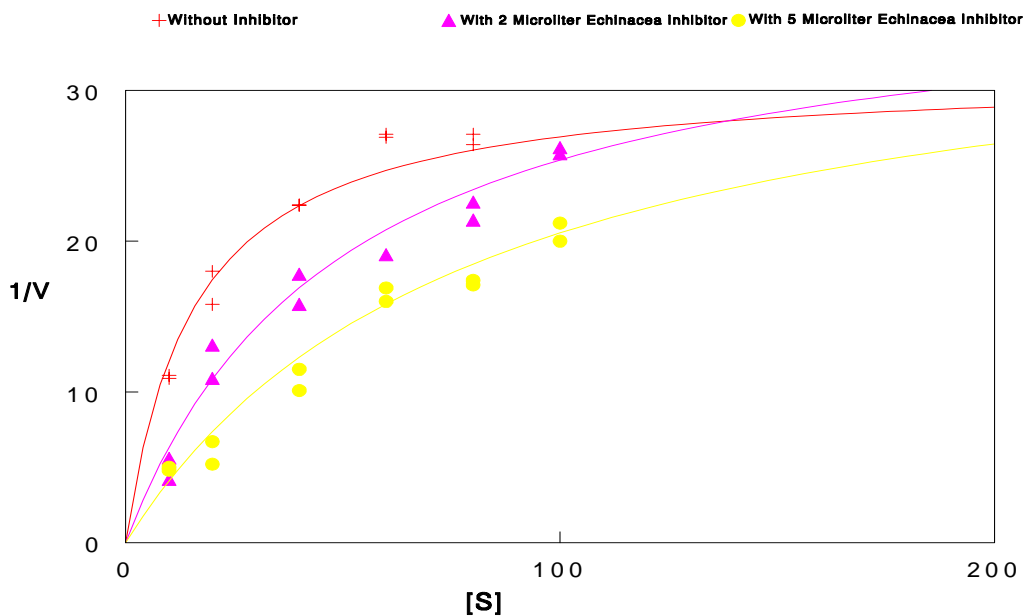
Figure 3.11. Michaelis-Menten plot showing inhibition of CYP2E1 with 2μL raw *Echinacea purpurea* root extract using CYP2E1 supersomes
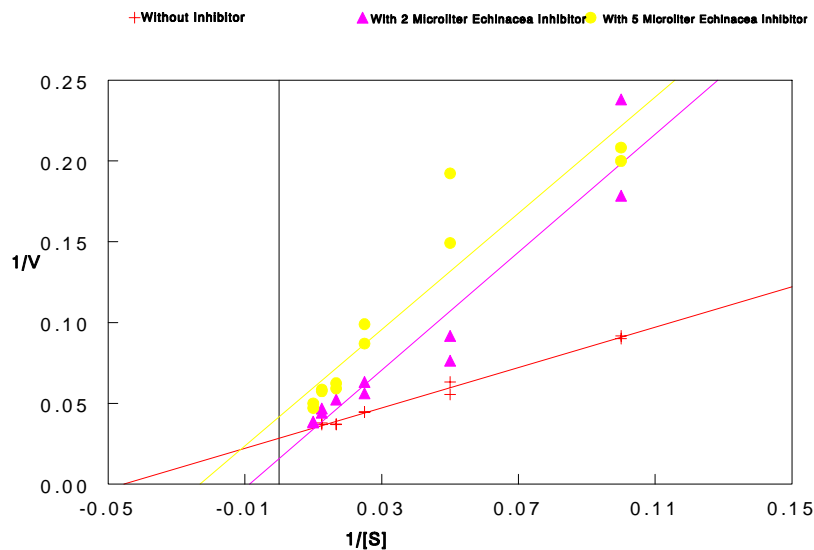
Figure 3.12. Lineweaver-Burk plot showing inhibition of CYP2E1 with 2μL raw *Echinacea purpurea* root extract using CYP2E1 supersomes

The nature of inhibition here appears mixed as seen in Figure 3.12. The V_{max} value is nearly the same both in presence and in absence of inhibitor and is 38μM/min in presence of extract at both concentrations and 35μM/min without inhibitor. The K_M value in absence of inhibitor is 21μM while that in presence of 0.4% inhibitor is 50μM. Addition of 1% extract caused increase in the K_M value to 51μM.

3.4.3 Inhibition on 2E1 by Spilanthes using liver microsomes

With spilanthes, 40μL of the raw extract was used to determine inhibition. Figures 3.13. and 3.14. displays the Michaelis-Menten plot and the corresponding Lineweaver-Burk plots showing inhibition of CYP2E1 using human liver microsomes. At

0.02mM substrate concentration, 29.1% inhibition was observed with 8% raw Spilanthes extract.

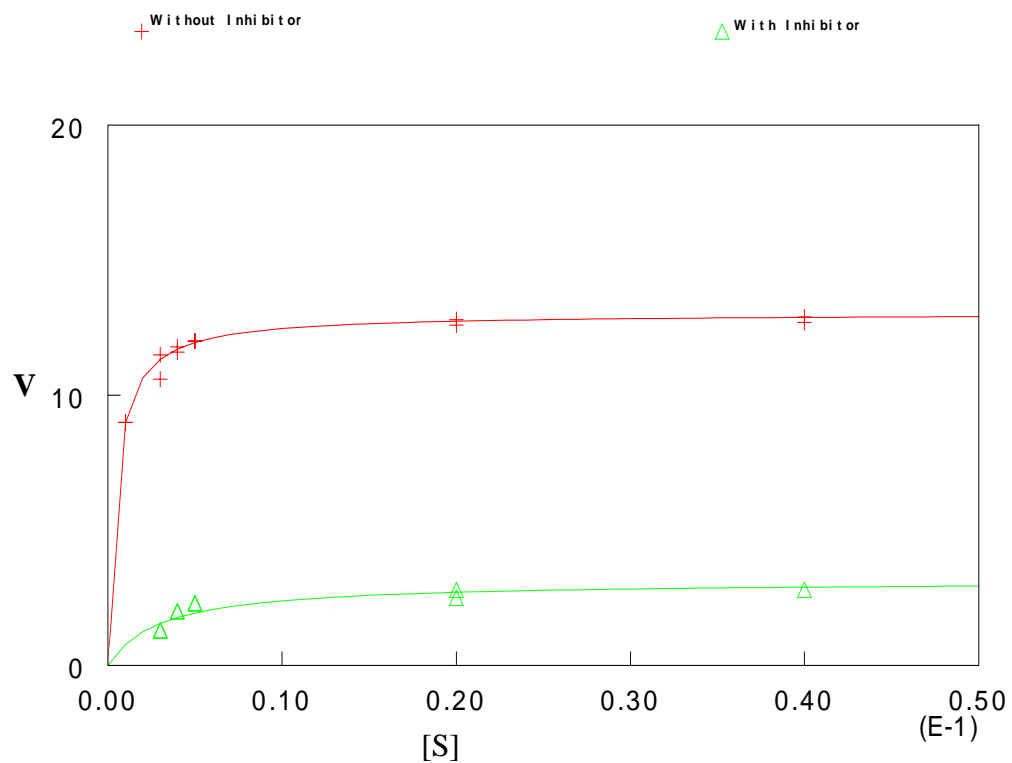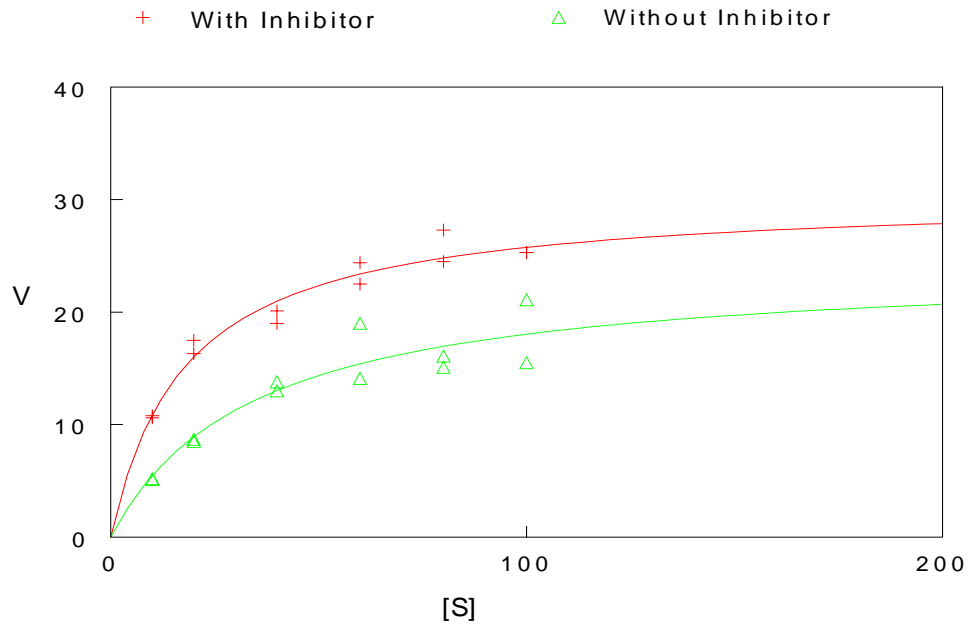**Figure 3.13. Michaelis-Menten plot showing inhibition of CYP2E1 with 40μL raw *Spilanthes acmella* flowering plant extract using human liver microsomes**
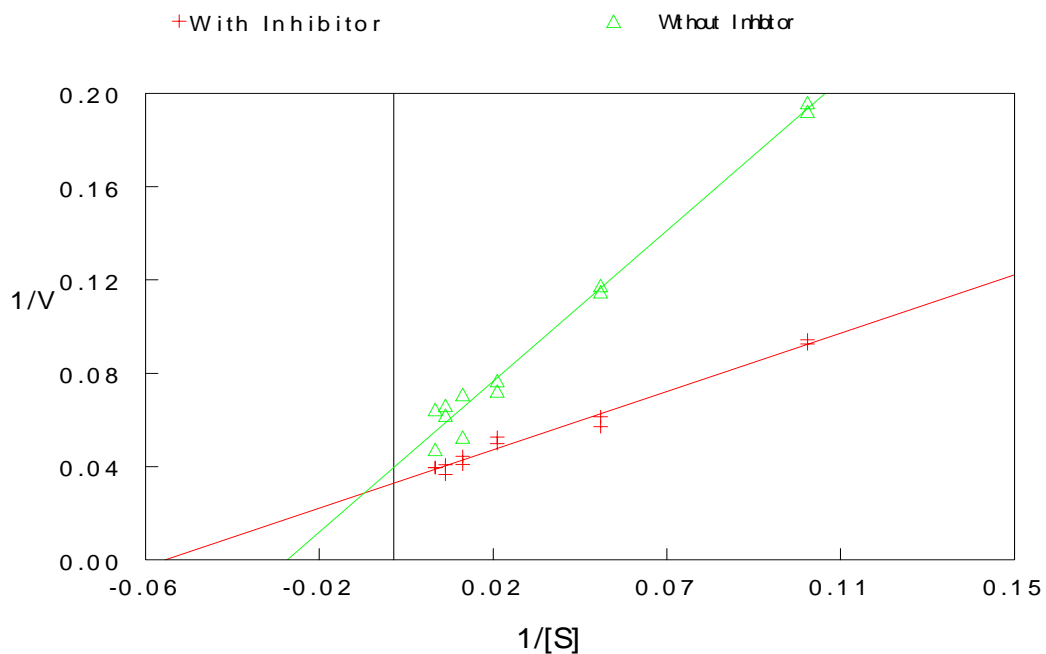
Figure 3.14. Lineweaver-Burk plot showing inhibition of CYP2E1 with 40μL raw
Spilanthes acmella **flowering plant extract using human liver microsomes**

The nature of the inhibition of the 2E1 acitivity of cytochrome P450 with the addition of the Spilanthes extract appears to be competitive with some mixed characteristics from the Lineweaver-Burk plot in Figure 3.14. The V_{max} value in absence of inhibitor is 30μM/min while that in presence of inhibitor is 24μM/min. The K_M value in absence of inhibitor is 18μM while that in presence of inhibitor is 34μM.

3.4.4 Inhibition on 2E1 by Spilanthes using human 2E1 containing supersomes

Michaelis-Menten plot generated using CYP2E1 supersomes and raw Spilanthes extract is shown in Figure 3.15. and Figure 3.16. shows the corresponding Lineweaver-Burk plot.
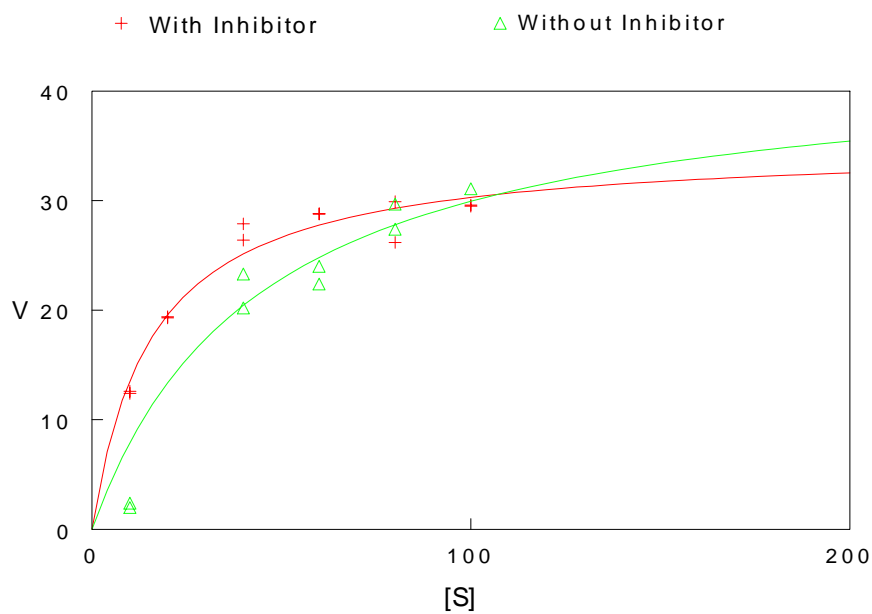
Figure 3.15. Michaelis-Menten plot showing inhibition of CYP2E1 with 40μL raw *Spilanthes acmella* flowering plant extract using CYP2E1 supersomes
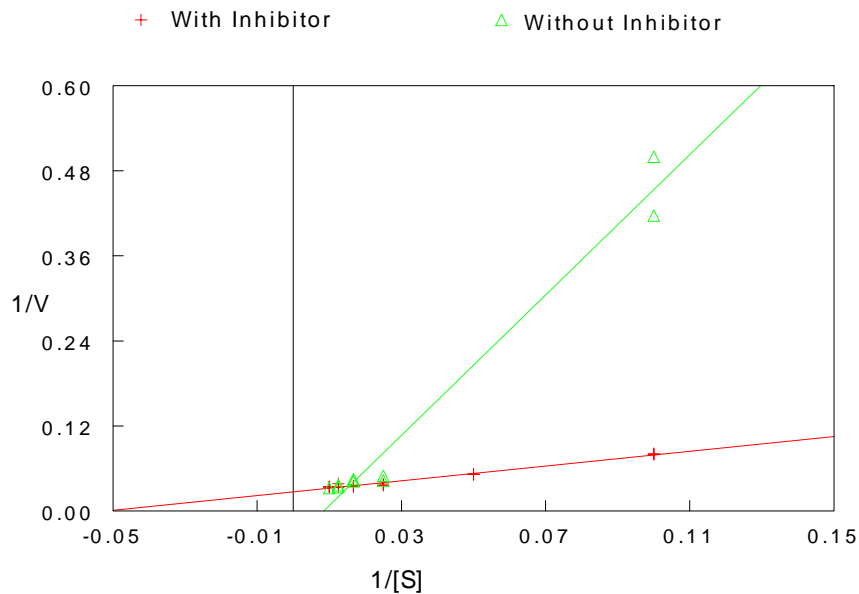
Figure 3.16. Lineweaver-Burk plot showing inhibition of CYP2E1 with 40μL raw

***Spilanthes acmella* flowering plant extract using CYP2E1 supersomes**

The Lineweaver-Burk plot in Figure 3.16. shows the nature of the inhibition to be mixed. Ideally the V_{max} value should be same both in presence and in absence of the inhibitor. Here an assumption has been made as the V_{max} value in both cases appears to be similar. The V_{max} value here without inhibitor is 35μM while that with inhibitor is 60μM. The K_M value in without and with the inhibitor are 16μM/min and 91μM/min respectively.

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3.5 Summary of inhibition of cytochrome p450 activities by raw Echinacea and Spilanthes extracts

Table 3.1. shows the nature and percentage of inhibition by raw Echinacea extract observed in the study for different isoforms used.

Table 3.1. Inhibition of different activities of cytochrome P450 with supersomes by raw *Echinacea purpurea* root extract showing amount of extract added, substrate concentration used, percentage of inhibition at that concentration and nature of inhibition.

Activity	Amount of Raw extract added (μL)	Substrate conc. (mM)	Percentage inhibition at that concentration	Nature of inhibition
2C9	5	0.1	36.2%	Reversible(mixed)
2A6	10	0.04	80%	(uncompetitive)
1A2	5	Undetermined	Undetermined	Undetermined
2E1	1. 2	0.02	29.4%	Reversible(mixed)
	2. 5	0.02	50%	Reversible(mixed)

Table 3.2. shows the nature and percentage of inhibition by raw Spilanthes extract observed in the study for different isoforms used.

Table 3.2. Inhibition of different activities of cytochrome P450 with supersomes by raw *Spilanthes acmella* flowering plant extract showing amount of extract added, substrate concentration used, percentage of inhibition at that concentration and nature of inhibition.

Activity	Amount of Raw extract added (μL)	Substrate concentration (mM)	Percentage inhibition at that concentration	Nature of inhibition
2C9	5	0.1	51.7%	Reversible(mixed)
2A6	10	0.04	20%	(mixed)
1A2	5	Undetermined	Undetermined	Undetermined
2E1	40	0.02	15.4%	Reversible(competitive)

3.6 Inhibitory properties of individual constituents of Echinacea and Spilanthes

In the second part of the project several active compounds have been isolated from the raw Echinacea and Spilanthes extracts and studies have been performed to see how significant each of those are in inhibiting the activity of CYP2E1. The K_M and V_{max}

values have been determined for this purpose and K_I values have been calculated using that data.

3.6.1 Caffeic acid derivatives

The hydrophilic compounds like the caffeic acid derivatives present in *Echinacea purpurea* were first studied for inhibitory activity of CYP2E1. The concentration of caffeic acid derivatives were found to be the highest in the raw *Echinacea purpurea* extract with 33%-77% ethanol/water solvent composition and was thus used for the analysis of the hydrophilic compounds. Also the extract of the aerial portion of the plant was seen to have a higher concentration of the phenolic acid compounds than the root extract, the concentrations being about 1.2E-03M and 2.0E-04M respectively. The two main caffeic acid derivatives found in *Echinacea purpurea* are caftaric acid and cichoric acid. The concentration of caftaric acid and cichoric acid in the extracts were determined to be 4.3E-04M and 3.74E-04 M in the aerial extract and 9.4E-05M and 8.95E-05M in the root extracts respectively.

Initially the raw aerial and root extracts were separately tested for inhibition of CYP2E1 with a single substrate concentration. The root extract was seen to inhibit the activity of CYP2E1 more than the aerial extract though the latter had a higher concentration of the caffeic acid derivatives. This showed that these phenolic acids may not play an important role in CYP2E1 inhibition. Further caftaric acid and cichoric acid separated from *Echinacea purpurea* was tested for inhibition of CYP2E1 using the same concentration of the compounds as found in the extract. Both the compounds failed to show any inhibition of the 2E1 activity of cytochrome P450.

3.6.2 Fractionation of Echinacea extracts

The study of the interactions of the hydrophobic compounds like isobutylamides present in Echinacea and Spilanthes was done next. Initially the large scale purification was done with raw Echinacea and Spilanthes extracts based on the solubility of the alkylamides present in the extracts with ethanol. Figure 3.17. shows the chromatogram of the raw *Echinacea purpurea* extract.
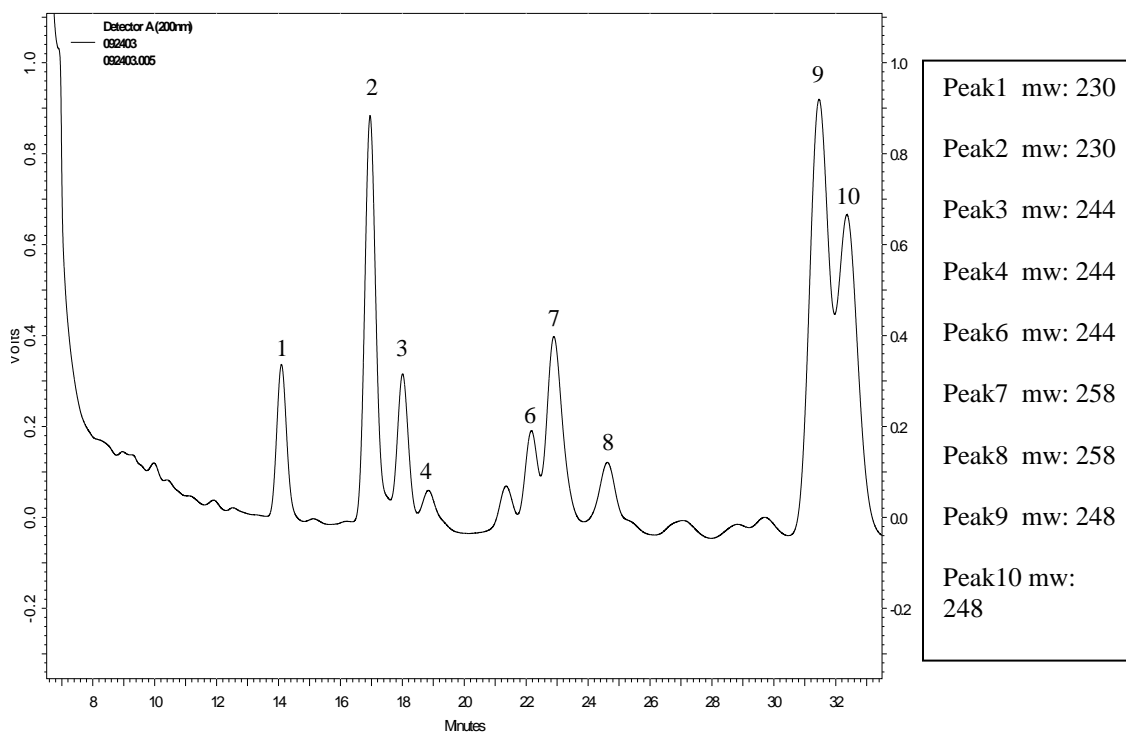
Figure 3.17. Chromatogram of raw *Echinacea purpurea* root extract showing the different alkylamide peaks

The peaks have been identified and their molecular weights determined by comparing the peaks with the standard chromatogram published by Hudaib et al [58].

3.6.2.1 Isolation of peaks 9 and 10

The fractions were tested for inhibition of CYP2E1 using a single substrate concentration. Two of the collected fractions showed about 50% inhibition while the rest did not show any significant inhibition. One of the fractions had 50% ethanol and 50% water while the second one had a 60%-40% combination. The chromatogram showing the first fraction that inhibited CYP2E1 is shown in Figure 3.18.
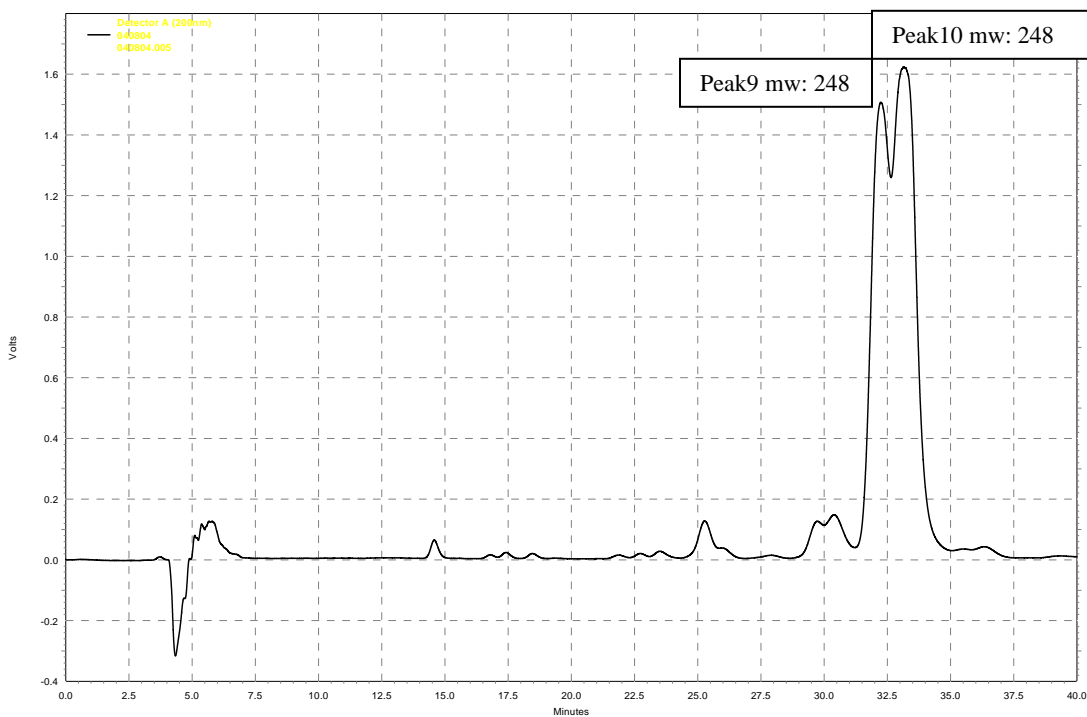
Figure 3.18. Chromatogram showing the fraction collected with 60%-40% ethanol and water solvent combination

The fraction displayed in Figure 3.18. shows mainly the two isomeric isobutylamide

peaks (9, 10) of mass 248 having retention times of 32.228 and 33.147 respectively. The

peaks 9 and 10 have been identified as Dodeca- 2E, 4e, 8Z, 10E- tetraenoic acid

isobutylamide and Dodeca- 2E, 4e, 8Z, 10Z- tetraenoic acid isobutylamide respectively

by comparing with Hudaib et al's work [58]. Further attempt to separate the two isomers

were not successful. The approximate concentration of the pair of isomers present in the

fraction was calculated using an LC- MS analysis based on a one-point calibration. The

concentration was found to be 6.25E-03M. Figure 3.19. and figure 3.20. show the

chromatogram used for the concentration determination and mass spectrometric analysis

of this fraction.
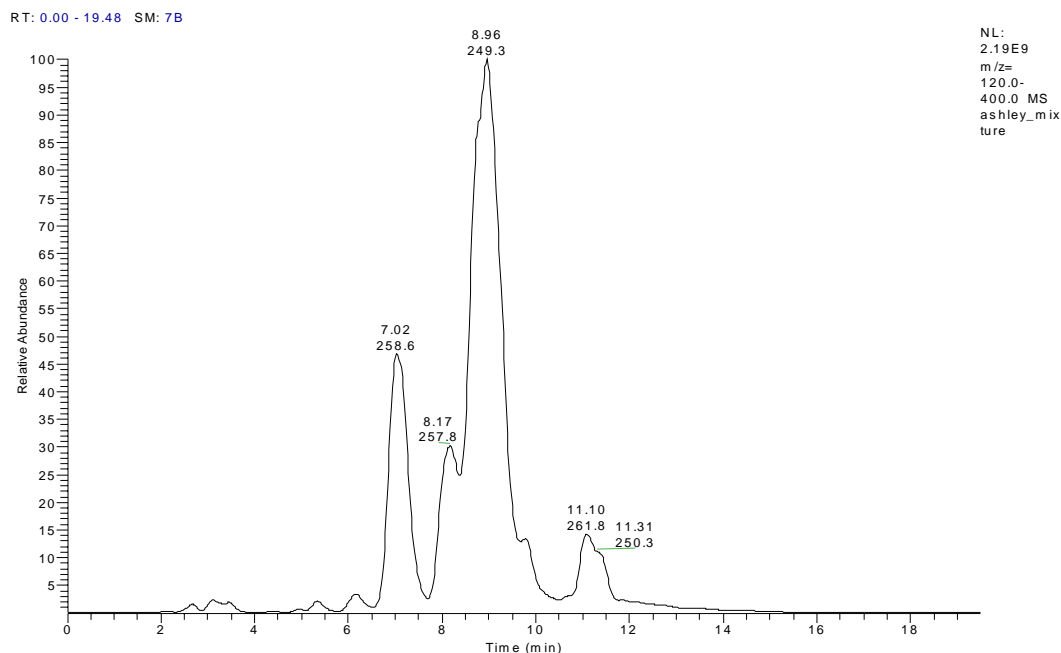
**Figure 3.19. LC chromatogram showing the fraction containing the peaks with a
molecular weight of 248.**
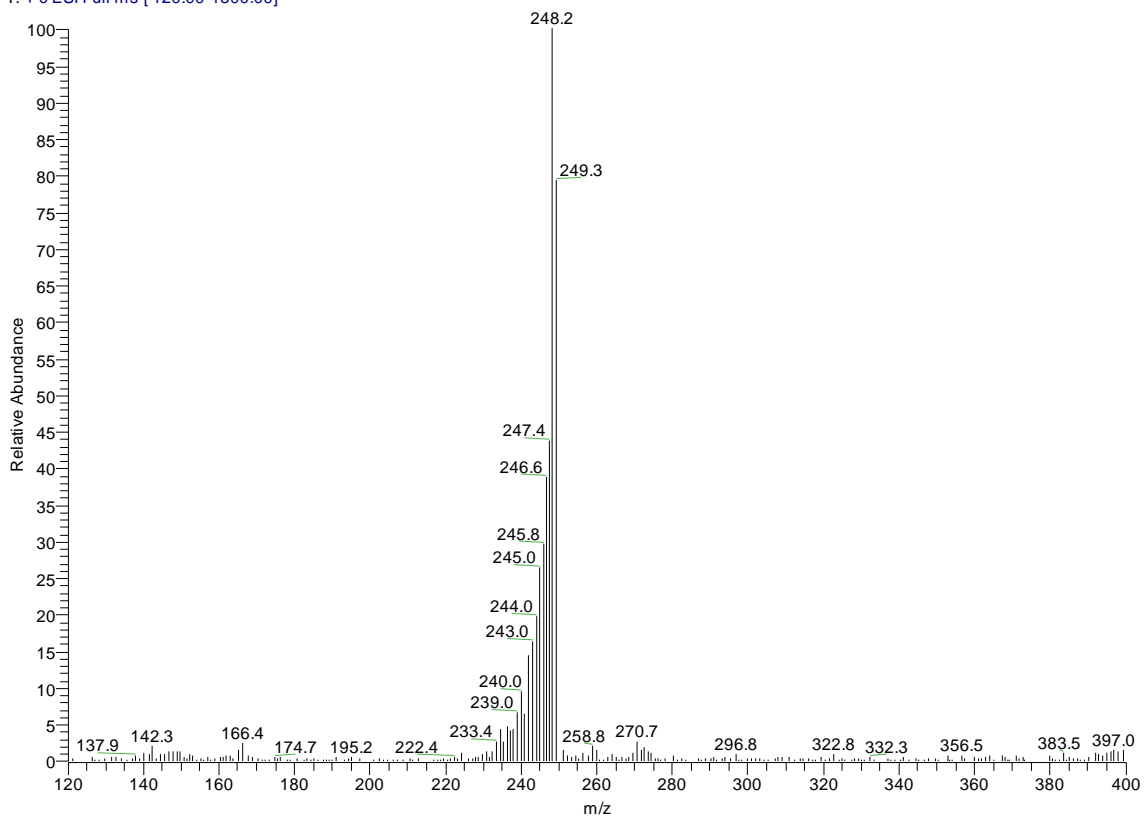
Figure 3.20. Mass spectrum showing the fraction containing the peaks with a molecular weight of 248

3.6.2.2 Isolation of peaks 1and 2

The chromatogram of the second fraction that inhibited CYP2E1 is shown in Figure 3.21.
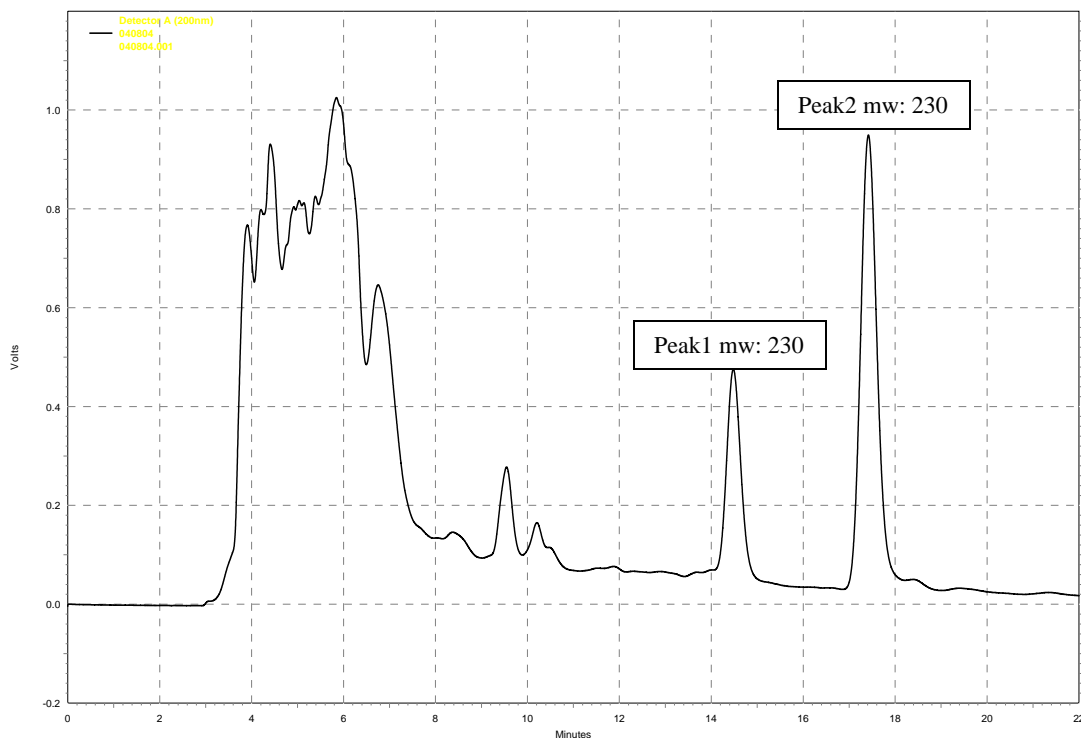
Figure 3.21. Chromatogram showing the fraction collected with 50%-50% ethanol and water solvent combination

The chromatogram in Figure 3.21. shows the presence of the two isomeric peaks with a mass of 230. These peaks had a retention time of 14.4 and 17.4 min respectively. The compounds were identified as Undeca- 2E, 4Z- diene- 8, 10- diynoic acid- isobutylamide and Undeca- 2Z, 4E- diene- 8, 10- diynoic acid- isobutylamide respectively from Hudaib et al's analysis [58]. This fraction was then injected into a C18 column as mentioned in the previous chapter and the two isomers could be separated. Figure 3.22. A and B shows the chromatogram with the isolated 230 peaks.
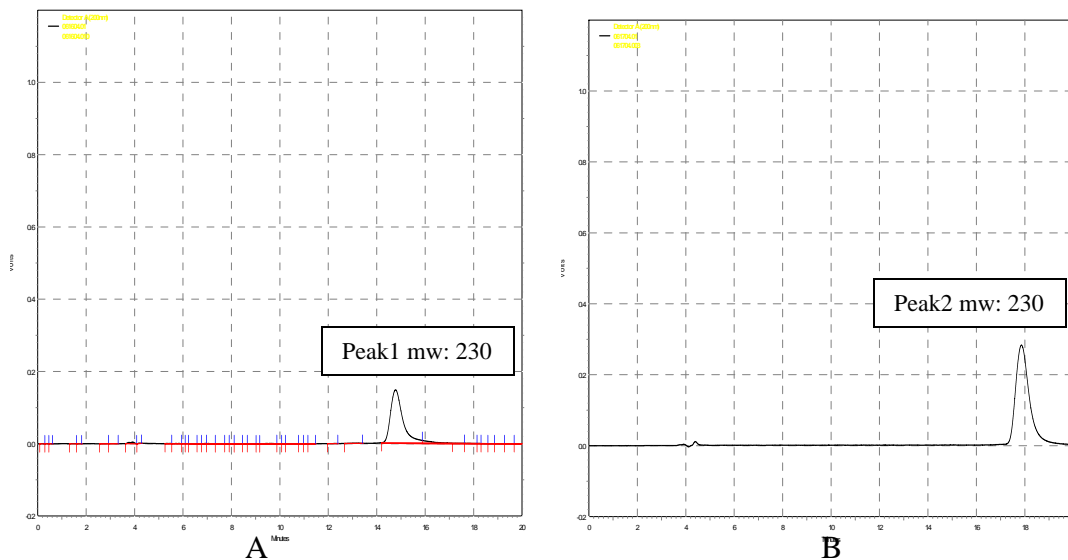
Figure 3.22. A: Chromatogram of the separated peak (1) having a mass of 230,

B: Chromatogram of the separated peak (2) having a mass of 230

The approximate concentrations of the peaks were 6.72E-04M and 5.45E-04M, as calculated using a one-point calibration with LC-MS analysis. Figure 3.23. and 3.24. show the Chromatogram used for determination of concentration and the MS analysis for the 230 peak.
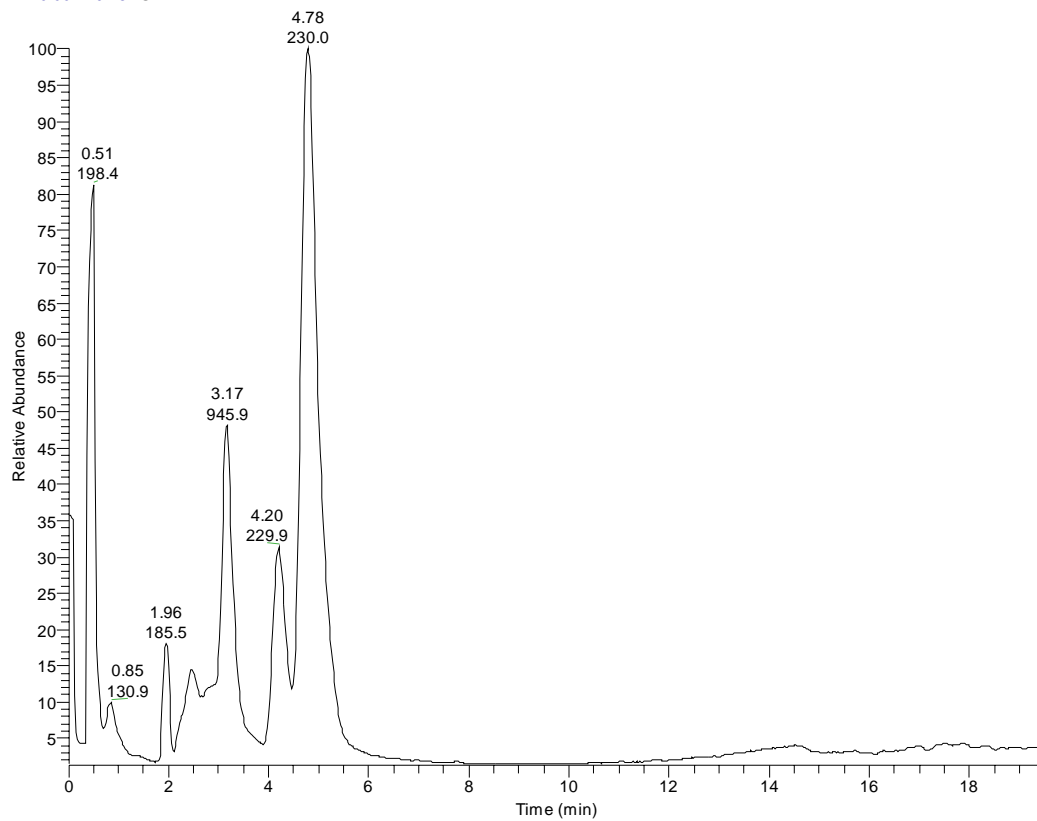
Figure 3.23. LC Chromatogram showing the 1st fraction containing the alkylamide with a molecular weight of 230
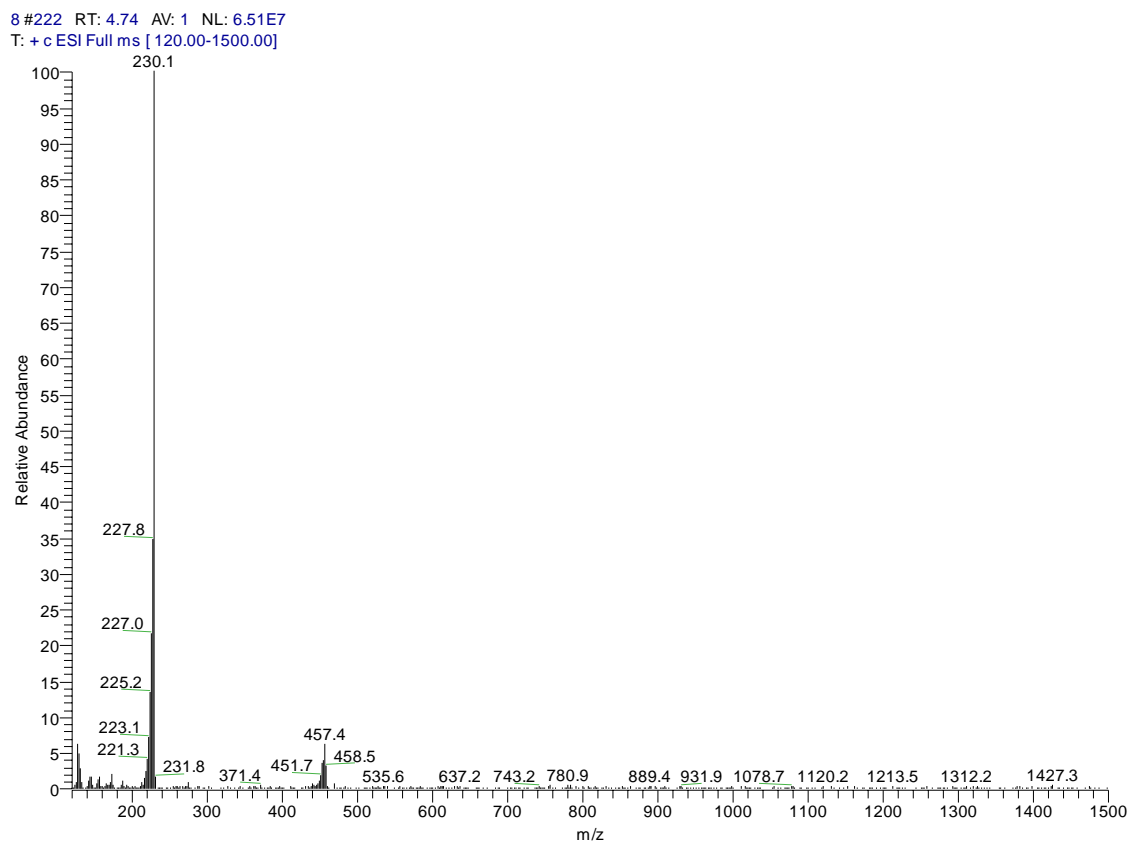
Figure 3.24. Mass spectrum showing the fraction containing the second alkylamide with a molecular weight of 230

3.6.3 Fractionation of Spilanthes extracts

A similar procedure was performed with the *Spilanthes acmella* extract. The chromatogram showing the raw extract is given in Figure 3.25.
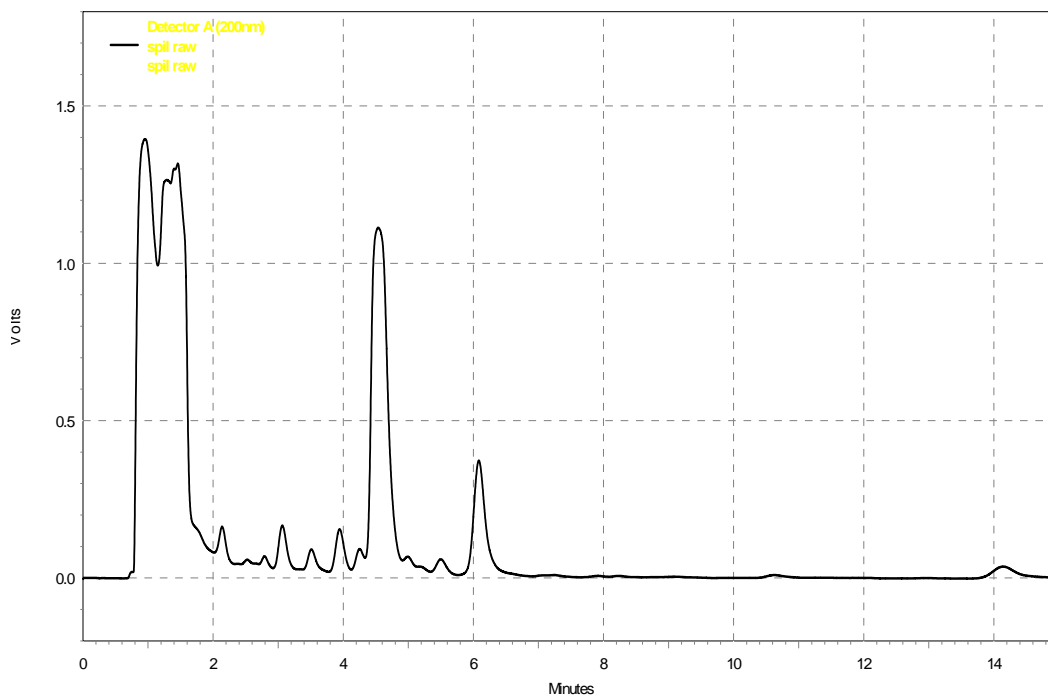
Figure 3.25. Chromatogram of raw *Spilanthes acmella* flowering plant extracts showing the different alkylamide peaks

Fractionation of the whole extract based on the solubility of the isobutylamides in ethanol led to a number of fractions.

3.6.3.1 Isolation of Spilanthol

The fraction with the highest concentration of Spilanthol, the most abundant and bioactive isobutylamide present in Spilanthes was used for further testing. The chromatogram of the fraction having the highest concentration of Spilanthol is given in Figure 3.26.
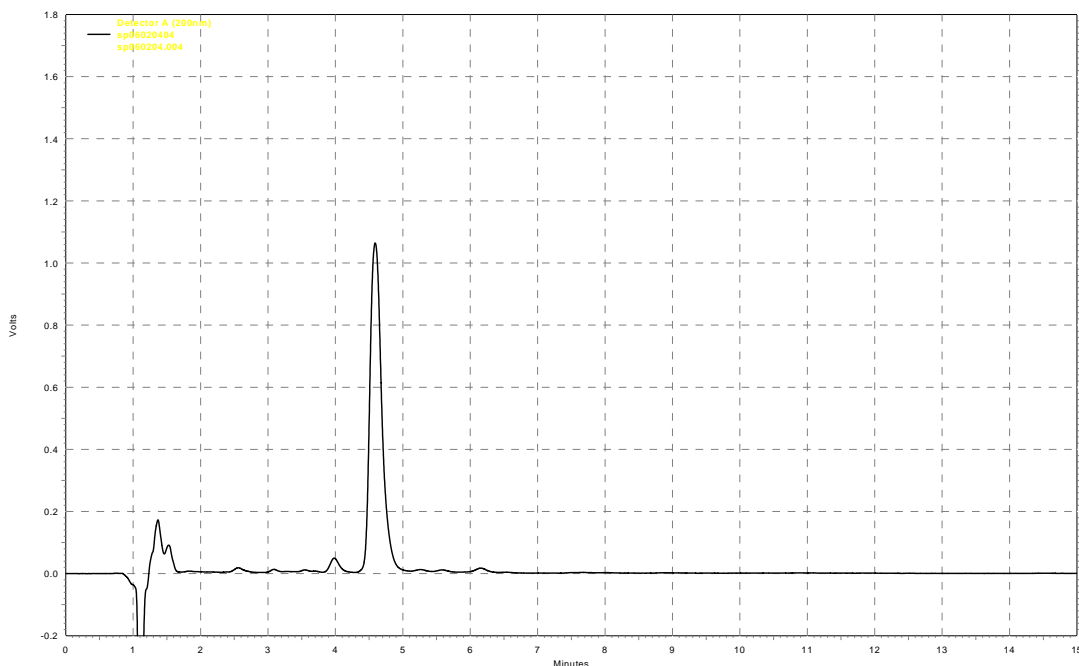
Figure 3.26. Chromatogram showing the fraction of raw *Spilanthes acmella* flowering plant extract with highest concentration of Spilanthol

Spilanthol had a retention time of 4.588. The fraction did not have a comparable amount of any other isobutylamide as is evident from the chromatogram shown in Figure 3.26. and was further confirmed in the mass spectral analysis. Thus further separations were not attempted. This fraction was analyzed using an LC-MS for the concentration of Spilanthol in the fraction. Figures 3.27. and 3.28. show the chromatogram used for the determination of concentration and the mass spectrometric analysis, respectively. The concentration of Spilanthol was calculated in a similar fashion as Echinacea using a one point calibration and the approximate concentration of Spilanthol in the fraction was found to be 1.66E-03M.
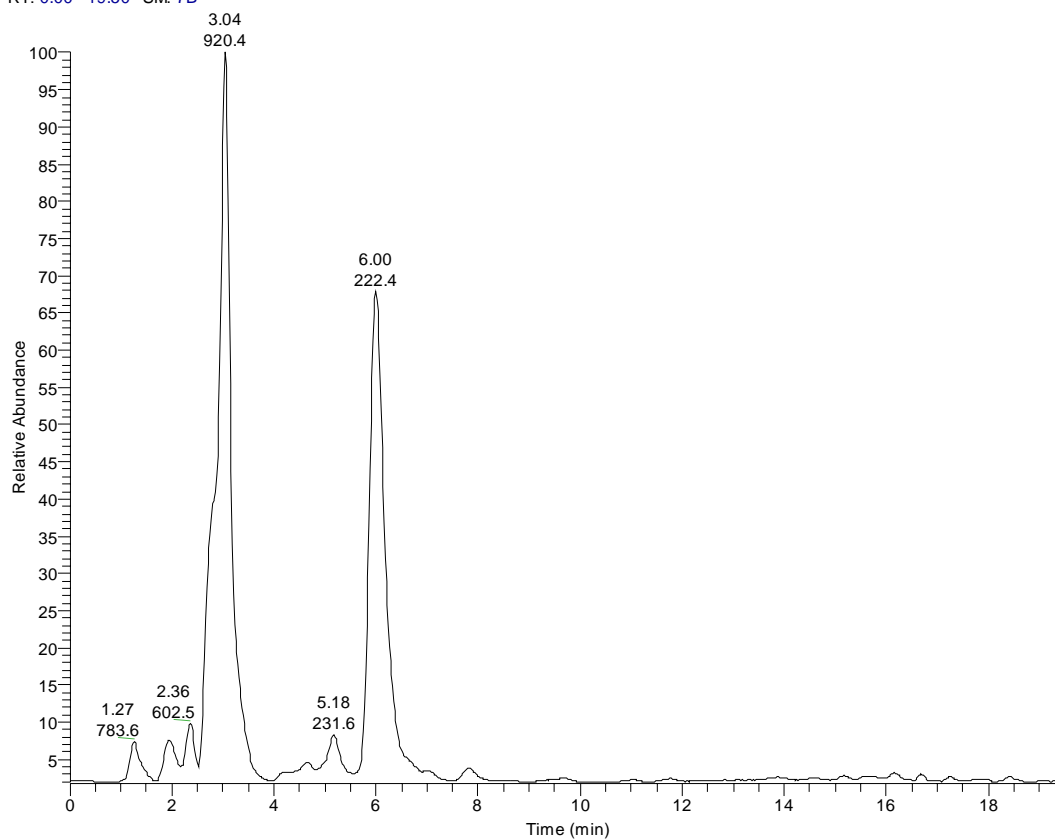
Figure 3.27. LC Chromatogram showing the fraction of *Spilanthes acmella* flowering plant extract containing Spilanthol
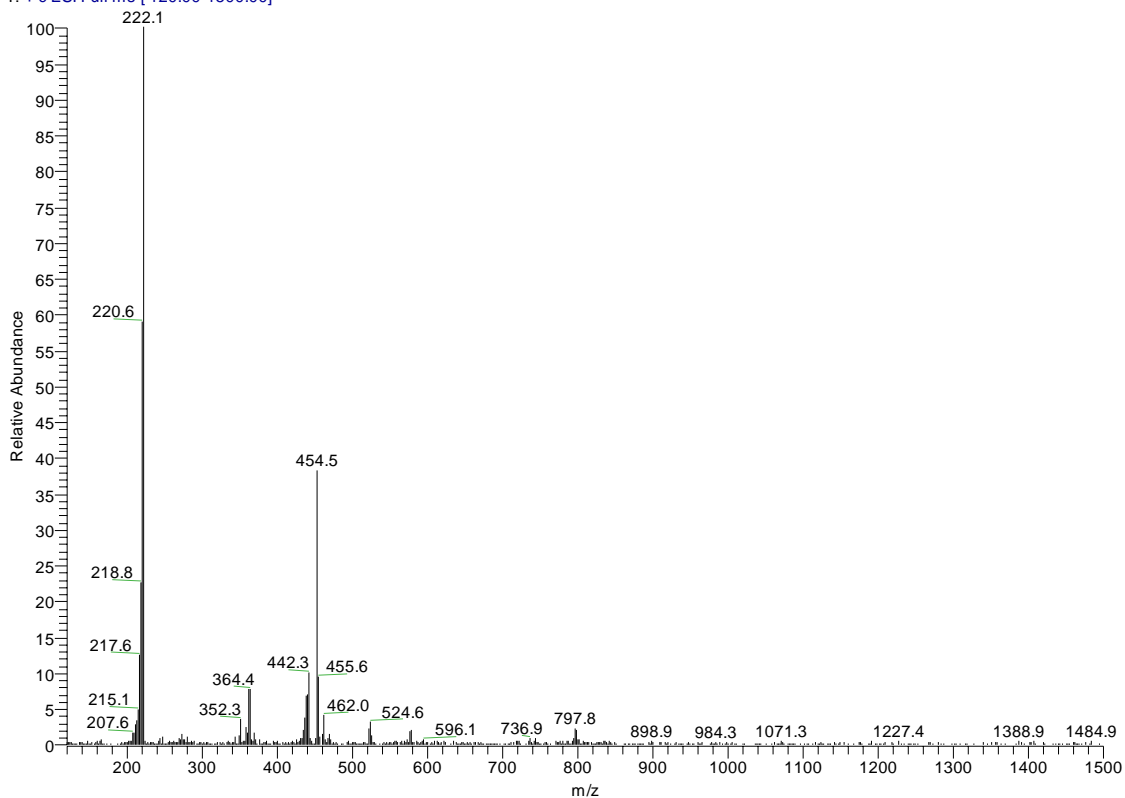
Figure 3.28. Mass spectrum showing the fraction of *Spilanthes acmella* flowering plant extract containing Spilanthol

3.6.4 Inhibition of 2E1 activity by individual components of Echinacea and Spilanthes

Next, inhibition by these isolated compounds was studied with respect to CYP2E1 and the K_I values for each compound or pair of compounds were calculated. Reactions were carried out with both human liver microsomes and CYP2E1 supersomes for each of the samples.

3.6.4.1 Inhibition of 2E1 activity by peaks 9 and 10 using human liver microsome

The fraction corresponding to the isomeric compounds with mass of 248 was shown to inhibit CYP2E1 with the addition of 10μL of the fraction. The Michaelis-Menten plot showing inhibition by using human liver microsomes is shown in Figure 3.29. and the corresponding Lineweaver-Burk plot is shown in Figure 3.30.

Figure 3.29. Michaelis-Menten plot showing inhibition of CYP2E1 using human liver microsomes with the addition of 10μL of fraction containing peaks 9 and 10 isolated from *Echinacea purpurea*

Figure 3.30. Lineweaver-Burk plot showing inhibition of CYP2E1 using human liver microsomes with the addition of 10μL of fraction containing peaks 9 and 10 isolated from *Echinacea purpurea*

The nature of inhibition is reversible and appears to be mixed here as can be determined from Figure 3.30. V_{max} and K_M values have been calculated from the plot. The V_{max} values both in absence and in presence of inhibitor are similar and are given as 72μM/min and 81μM/min respectively. The K_M value in absence of inhibitor is 36μM and that in presence of inhibitor is 87.7μM. The K_I value was calculated using this data and has been found to be 43.3μM .

3.6.4.2 Inhibition of 2E1 activity by peaks 9 and 10 using CYP2E1 supersomes

The same procedure was followed using CYP2E1 supersomes. The Michaelis-Menten plot and the corresponding Lineweaver-Burk plot are given in Figures 3.31. and 3.32. respectively.
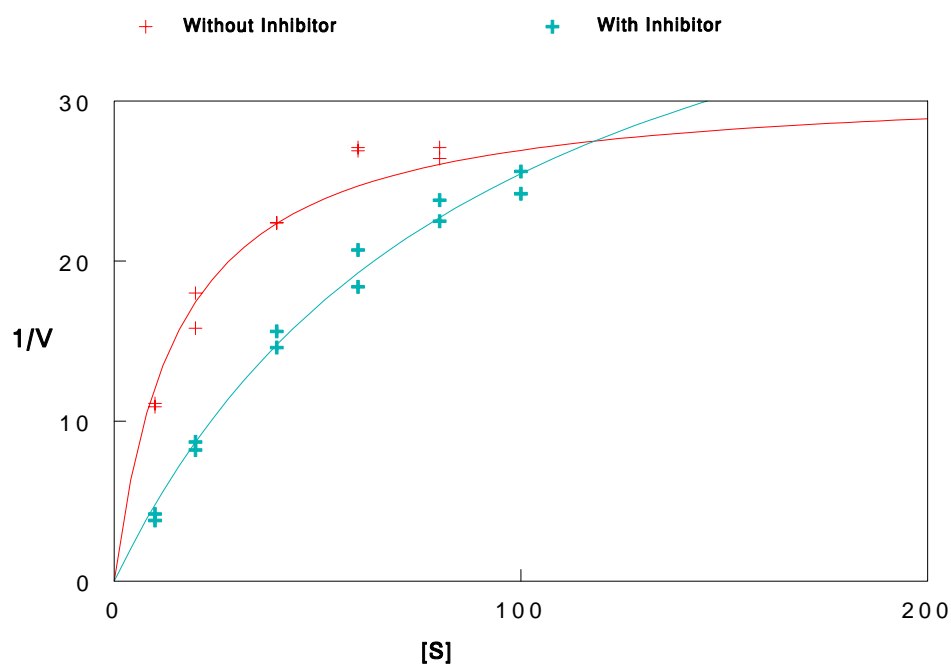
Figure 3.31. Michaelis-Menten plot showing inhibition of CYP2E1 using CYP2E1 supersomes with the addition of 10μL of fraction containing peaks 9 and 10 isolated from *Echinacea purpurea*

Figure 3.32. Michaelis-Menten plot showing inhibition of CYP2E1 using CYP2E1 supersomes with the addition of 10μL of fraction containing peaks 9 and 10 isolated from *Echinacea purpurea*

The nature of inhibition appears to be mixed. The V_{max} value is found to be similar both in presence and in absence of inhibitor, the value being 48μM/min and 35μM/min respectively. The K_M values are 93μM with the extract and 21μM without it. Using the V_{max} and K_M, the K_I value was determined to be 18.2μM .

3.6.4.3 Inhibition of 2E1 activity by peak 1 using human liver microsome

The addition of 20μL of the isolated fractions both in case of peak 1 and peak 2 caused significant inhibition of CYP2E1. Figure 3.33. gives the Michaelis-Menten plot

generated using 20μL of the fraction containing the isolated peak 1. The corresponding

Lineweaver-Burk plot is given in Figure 3.34. which shows the inhibition to be

competitive.

Figure 3.33. Michaelis-Menten plot showing inhibition of CYP2E1 using human liver microsomes with the addition of 20μL of fraction containing peaks 1 isolated from *Echinacea purpurea*

Figure 3.34. Lineweaver-Burk plot showing inhibition of CYP2E1 using human liver microsomes with the addition of 20μL of fraction containing peak 1 isolated from *Echinacea purpurea*

The V_{max} value was similar both in presence and in absence of inhibitor and it is calculated to be 53μM/min in presence and 71μM/min in absence of the extract. The K_M value is observed to be 36μM and 51μM for the plots without and with the inhibitor respectively. The K_I value is calculated with this data and has been found to be 31.1μM.

3.6.4.4 Inhibition of 2E1 activity by peak 1 using CYP2E1 supersomes

The same experiment was carried out using supersomes instead of human liver microsomes and the resulting Michaelis-Menten and Lineweaver-Burk plots are given in Figures 3.35. and 3.36. respectively.
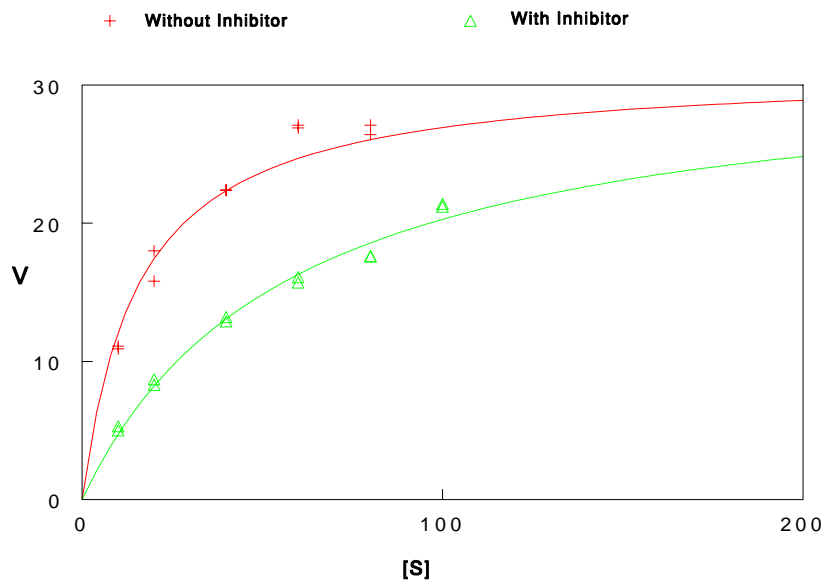
Figure 3.35. Michaelis-Menten plot showing inhibition of CYP2E1 using CYP2E1 supersomes with the addition of 20μL of fraction containing peak 1 isolated from *Echinacea purpurea*
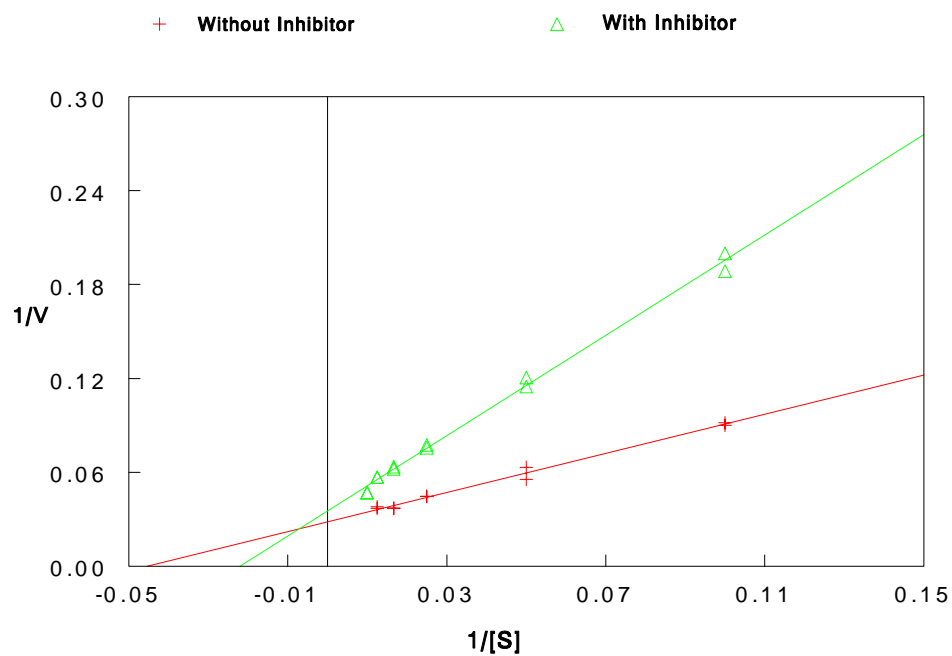
Figure 3.36. Lineweaver-Burk plot showing inhibition of CYP2E1 using CYP2E1 supersomes with the addition of 20μL of fraction containing peak 1 isolated from *Echinacea purpurea*

The nature of inhibition appears to be mixed here, the V_{max} value being very similar. The V_{max} value was 35μM/min in absence of inhibitor and 32μM/min in presence of it. The K_M values without and with the inhibitor are observed to be 21μM and 58μM respectively. The calculated K_I value is found to be 7.4μM.

3.6.4.5 Inhibition of 2E1 activity by peak 2 using human liver microsome

Next the same procedure was followed with the fraction containing peak 2. The

Michaelis-Menten and the Lineweaver-Burk plot generated using human liver

microsomes are shown in Figures 3.37. and 3.38. respectively.
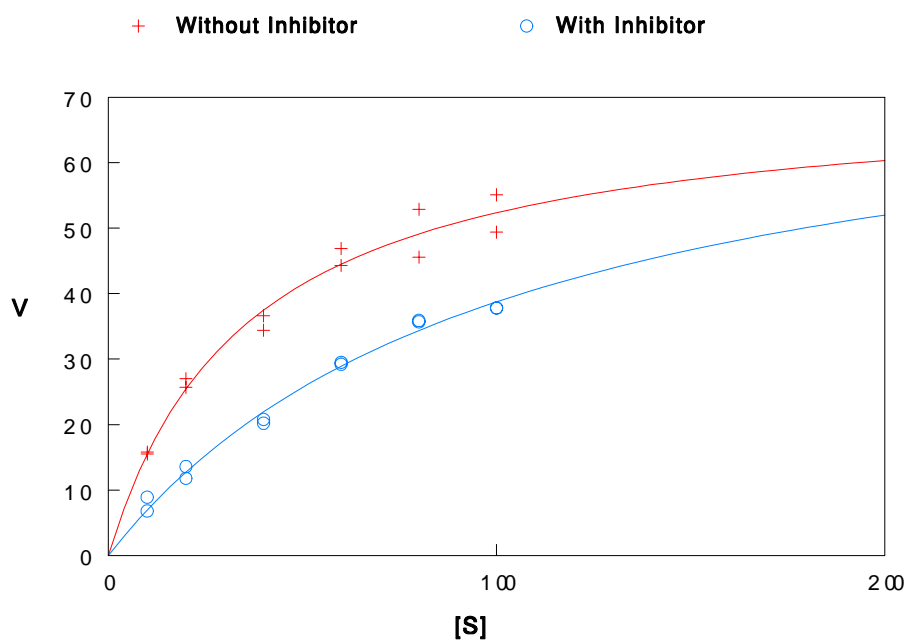
Figure 3.37. Michaelis-Menten plot showing inhibition of CYP2E1 using human liver microsomes with the addition of 20μL of fraction containing peak 2 isolated from *Echinacea purpurea*

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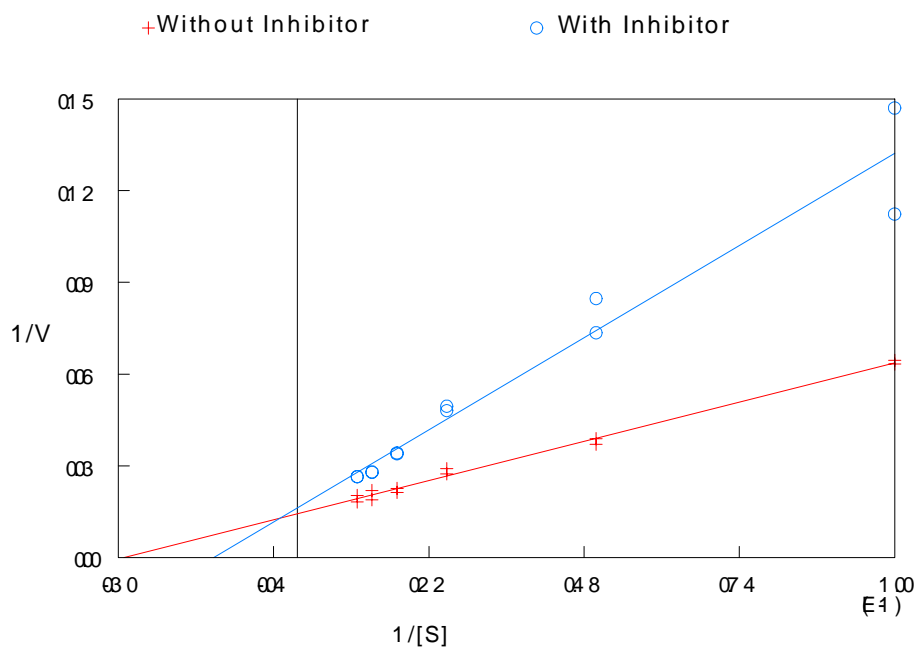
Figure 3.38. Lineweaver-Burk plot showing inhibition of CYP2E1 using human liver microsomes with the addition of 20μL of fraction containing peak 2 isolated from *Echinacea purpurea*

This inhibition as observed from the Lineweaver-Burk plot is mixed in nature the V_{max} value for both the plots in Figure 3.37. are 71μM/min without inhibitor and 79μM/min in its presence. The K_M values are calculated in absence and in presence of the inhibitor and are found to be 36μM in absence and 104μM in presence of inhibitor. The K_I value as calculated from this data is found to be 5.8μM.

3.6.4.6 Inhibition of 2E1 activity by peak 2 using CYP2E1 supersomes

The same experiment was done using CYP2E1supersomes. The Michaelis-Menten and Lineweaver-Burk plot generated are given in Figures 3.39. and 3.40. respectively.
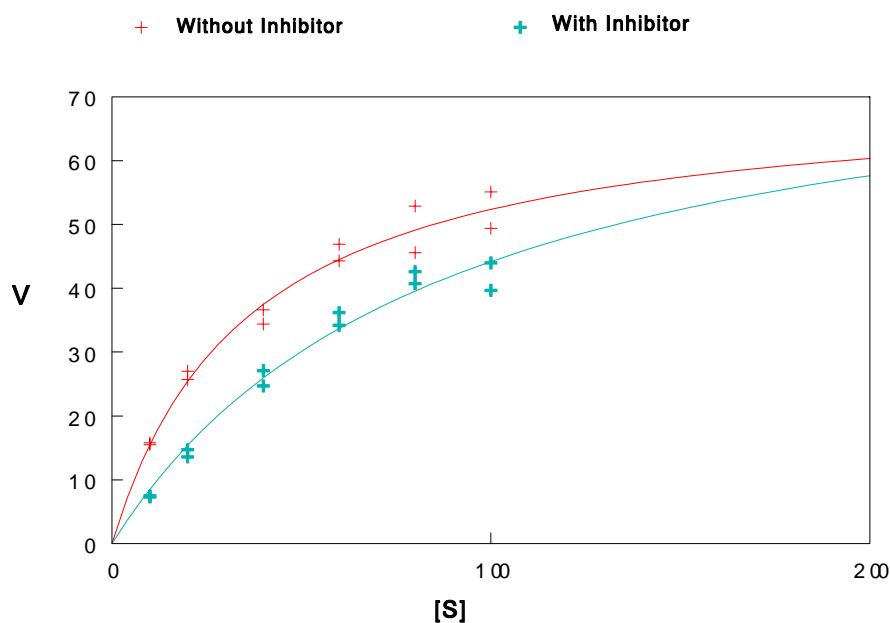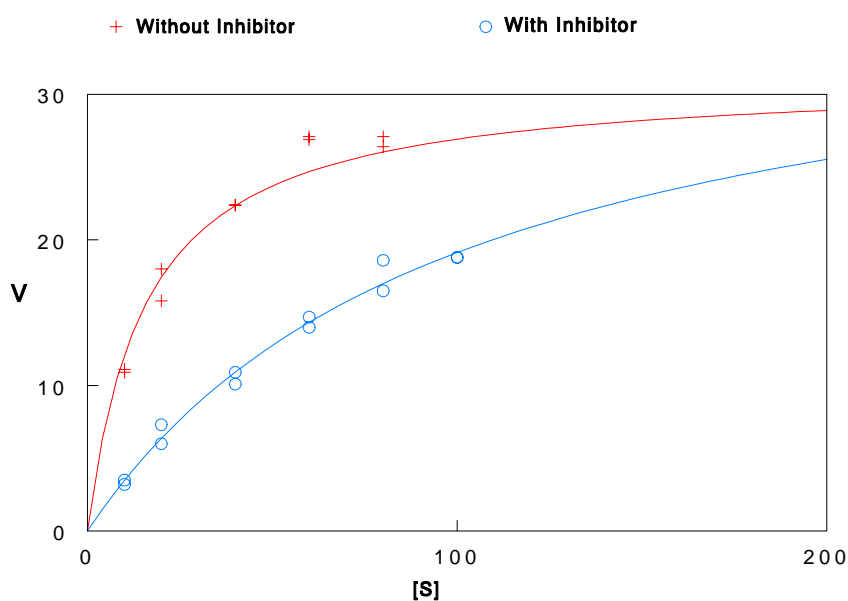

Figure 3.39. Michaelis-Menten plot showing inhibition of CYP2E1 using CYP2E1 supersomes with the addition of 20μL of fraction containing peak 2 isolated from *Echinacea purpurea*
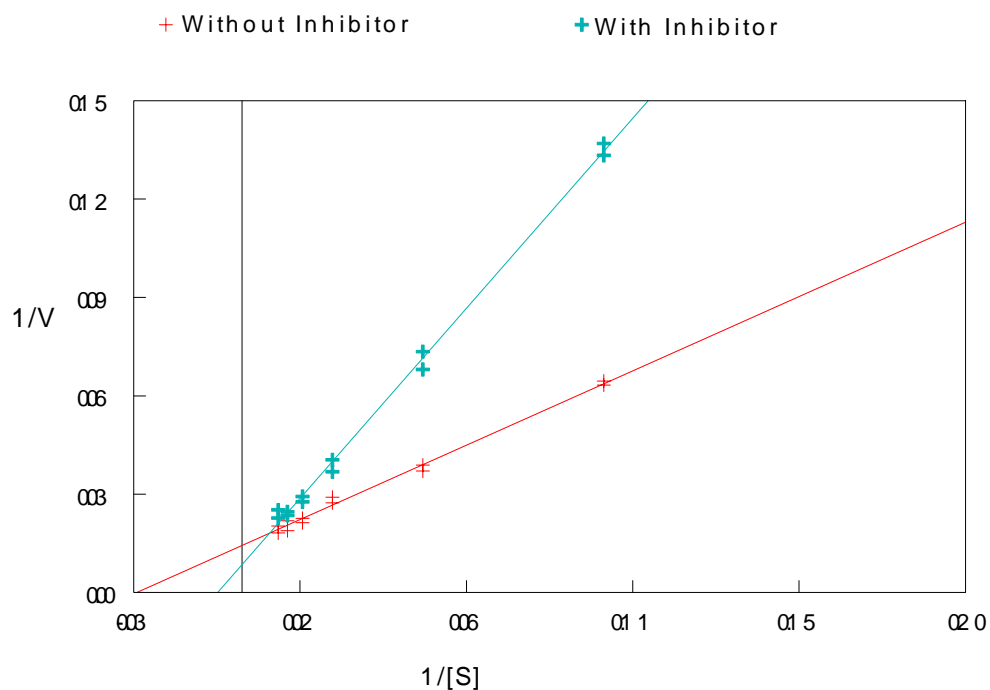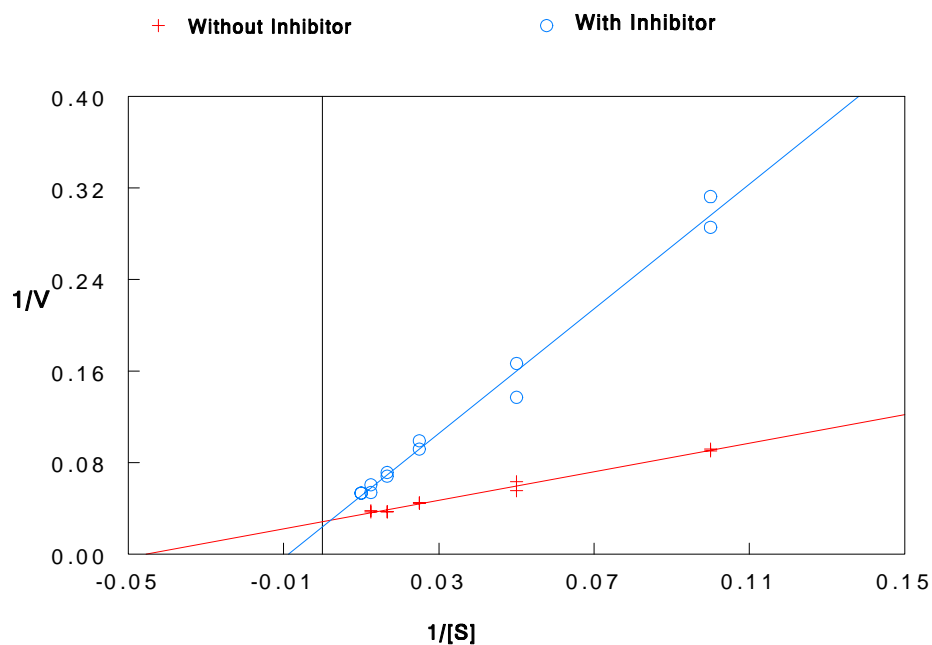
Figure 3.40. Lineweaver-Burk plot showing inhibition of CYP2E1 using CYP2E1

supersomes with the addition of 20μL of fraction containing peak 2 isolated from

Echinacea purpurea

Here the V_{max} values are very similar for plots with and without inhibitor and are observed to be is 38μM/min and 35μM/min respectively. The K_M value without inhibitor is 21μM and that with inhibitor is 101μM. The K_I value has been calculated to be 2.9μM.

3.6.4.7 Inhibition of 2E1 activity by Spilanthol using human liver microsome

The fraction with the highest concentration of Spilanthol separated from raw Spilanthes extract was also tested for inhibition of CYP2E1 activity. The addition of 20μL of the sample showed considerable inhibition. The Michaelis-Menten plot using

human liver microsomes is shown in Figure 3.41. The corresponding Lineweaver-Burk

plot is given in Figure 3.42.
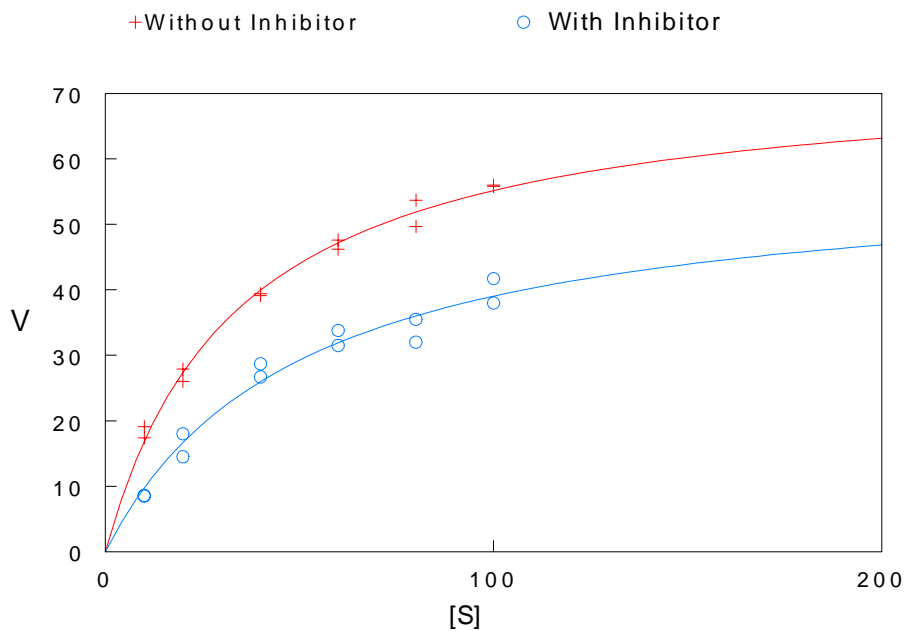
Figure 3.41. Michaelis-Menten plot showing inhibition of CYP2E1 using human liver microsomes with the addition of 20μL of fraction containing Spilanthol isolated from *Spilanthes acmella*
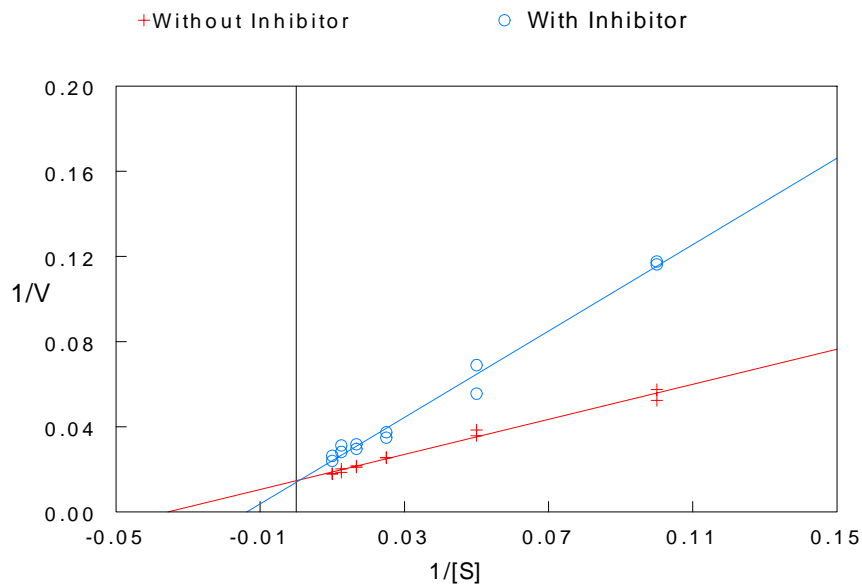
Figure 3.42. Lineweaver-Burk plot showing inhibition of CYP2E1 using human liver microsomes with the addition of 20μL of fraction containing Spilanthol isolated from *Spilanthes acmella*

The inhibition appears to be competitive with some mixed characteristics from the nature of the graph. The V_{max} value is calculated to be 74μM/min in absence and 66μM/min in presence of the inhibitor. The K_M value in absence of the inhibitor is 34μM while that in presence of inhibitor is 40μM. The K_I value is calculated to be 70.1μM.

3.6.4.8 Inhibition of 2E1 activity by Spilanthol using CYP2E1 supersomes

The same analysis was done using CYP2E1supersomes. The Michaelis-Menten plot and the corresponding Lineweaver-Burk plot are shown in Figures 3.43. and 3.44. respectively.
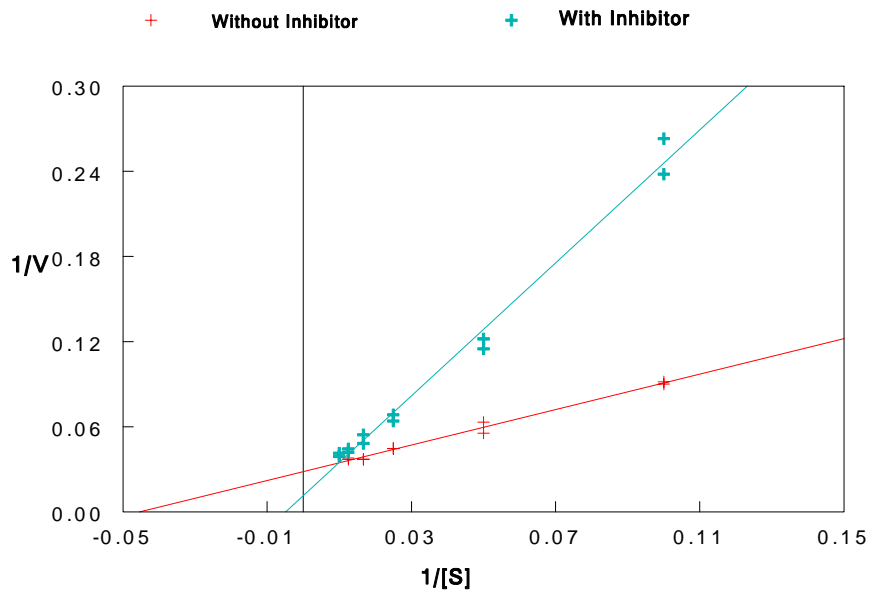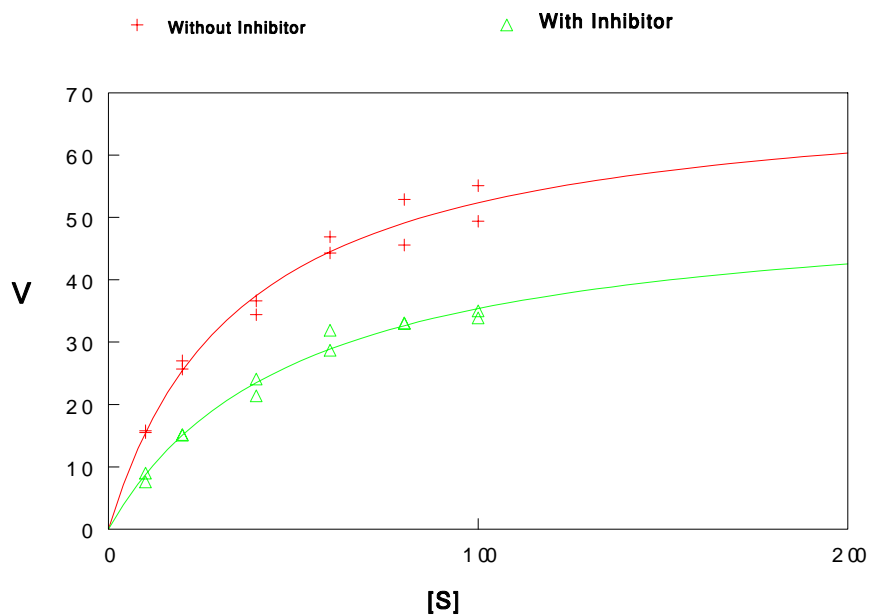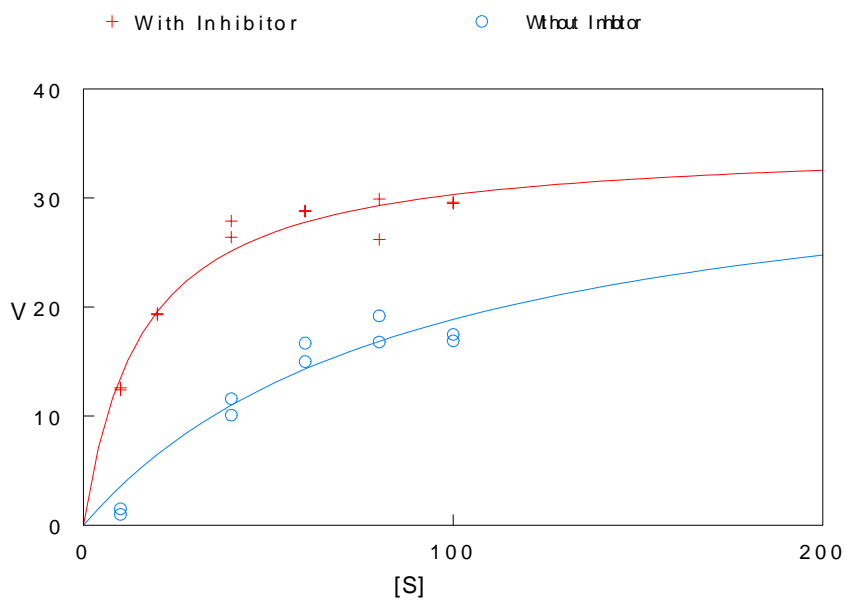
Figure 3.43. Michaelis-Menten plot showing inhibition of CYP2E1 using CYP2E1 supersomes with the addition of 20μL of fraction containing Spilanthol isolated from *Spilanthes acmella*
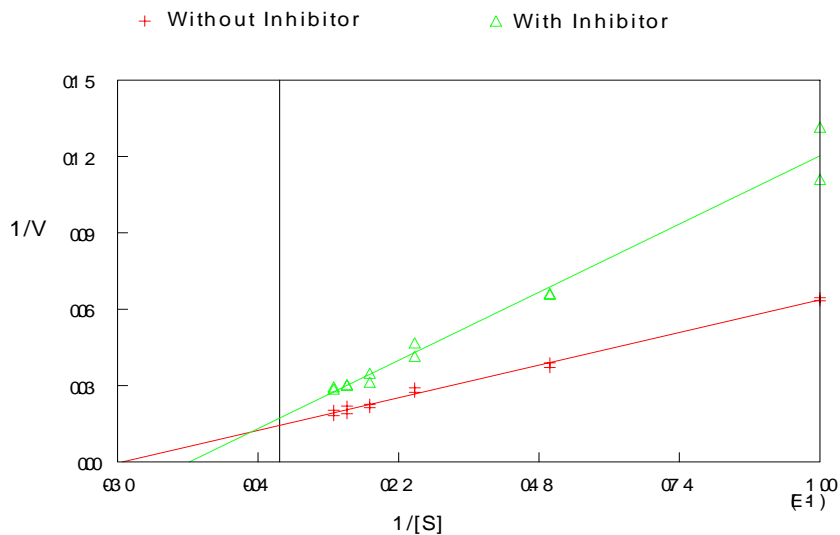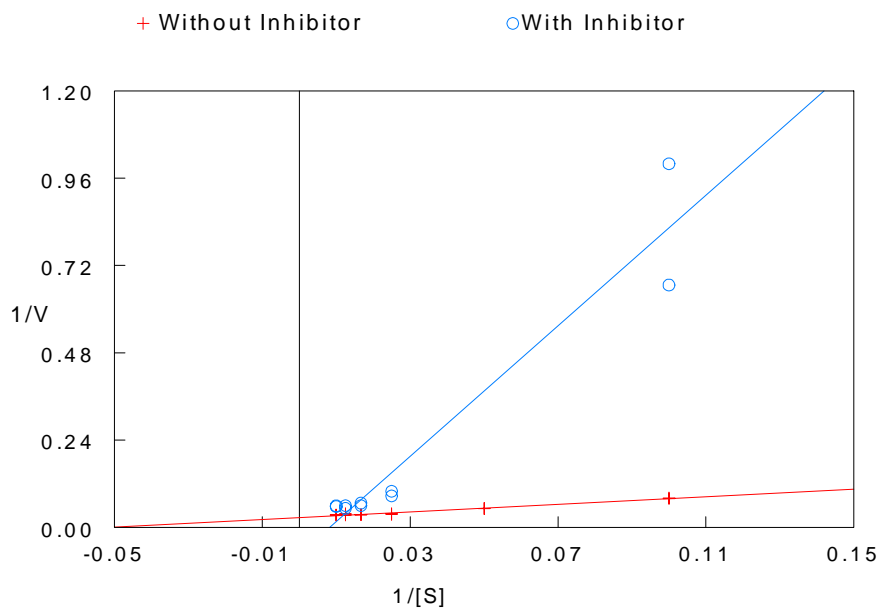
Figure 3.44. Lineweaver-Burk plot showing inhibition of CYP2E1 using CYP2E1 supersomes with the addition of 20μL of fraction containing Spilanthol isolated from *Spilanthes acmella*

The nature of inhibition appears to be mixed. Here the V_{max} value in presence and absence of inhibitor is very similar and is observed to be 36μM/min and 35μM/min respectively. The K_M value in absence of inhibitor is 15μM and that in presence of Spilanthol is 91μM. The K_I value is calculated to be 6.5μM.

3.6.4.9 Summary of inhibition of 2E1 activity by individual components of Echinacea and Spilanthes extract using human liver microsomes

Table 3.3. shows the K_M, V_{max} and K_I values of the inhibition by the compounds separated from *Echinacea purpurea* and *Spilanthes acmella* with human liver microsomes.

Table 3.3. Concentration, K_M, V_{max} and K_I values of the inhibition of 2E1 activity by the compounds separated from *Echinacea purpurea* and *Spilanthes acmella* with human liver microsomes

Name of the compound	Amount Added μL	Concentration	K_M (μM)	V_{max} (μM/min)	K_I μM
Raw Echinacea extract	2	500mg/mL*	58	75	-
	5	500mg/mL*	60	69	-
Caftaric acid	-	4.3E-04M(Aerial)	-	-	-
	-	9.4E-05M(Root)	-	-	-
Cichoric acid	-	3.74E-04M(Aerial)	-	-	-
	-	8.95E-05M(Root)	-	-	-

Name of the compound	Amount Added μL	Concentration	K_M (μM)	V_{max} (μM/min)	K_I nM
Dodeca- 2E, 4e, 8Z, 10E- tetraenoic acid isobutylamide and Dodeca- 2E, 4e, 8Z, 10Z- tetraenoic acid isobutylamide	10	6.25E-03M	88	82	43.3
Undeca- 2E, 4Z- diene-8, 10- diynoic acid-isobutylamide	20	6.72E-04M	51	53	31.1
Undeca- 2Z, 4E- diene-8, 10- diynoic acid-isobutylamide	20	5.45E-04M	104	79	5.8
Raw Spilanthes extract	40	500mg/mL*	34	24	-
Spilanthol	20	1.66E-03M	34	74	70.1

* Note: Calculation based on the fact that 1g of plant material was dissolved in 2mL ethanol

3.6.4.10 Summary of inhibition of 2E1 activity by individual components of Echinacea and Spilanthes extract using CYP2E1 supersomes

Table 3.4. shows the K_M, V_{max} and K_I values of the inhibition by the compounds separated from *Echinacea purpurea* and *Spilanthes acmella* with CYP2E1 supersomes.

Table 3.4. Concentration, K_M, V_{max} and K_I values of the inhibition by the compounds separated from *Echinacea purpurea* and *Spilanthes acmella* with CYP2E1 supersomes

Name of the compound	Amount Added µL	Concentration in the reaction mixture (M)	K_M (µM)	V_{max} (µM/ min)	K_I µM
Raw Echinacea extract	2	500mg/mL*	50	38	-
	5	500mg/mL*	51	38	-
Caftaric acid	-	4.3E-04M(Aerial)	-	-	-
	-	9.4E-05M(Root)	-	-	-
Cichoric acid	-	3.74E-04M(Aerial)	-	-	-
	-	8.95E-05M(Root)	-	-	-

Name of the compound	Amount Added μL	Concentration in the reaction mixture (M)	K_M (μM)	V_{max} (μM/ min)	K_I nM
Dodeca- 2E, 4e, 8Z, 10E- tetraenoic acid isobutylamide and Dodeca- 2E, 4e, 8Z, 10Z- tetraenoic acid isobutylamide	10	6.25E-03M	93	49	18.2
Undeca- 2E, 4Z- diene- 8, 10- diynoic acid- isobutylamide	20	6.72E-04M	58	32	7.4
Undeca- 2Z, 4E- diene- 8, 10- diynoic acid- isobutylamide	20	5.45E-04M	101	38	2.9
Raw Spilanthes extract	40	500mg/mL*	91	60	-
Spilanthol	20	1.66E-03M	91	36	6.5

* Note: Calculation based on the fact that 1g of plant material was dissolved in 2mL ethanol

CHAPTER IV

DISCUSSION

Plants are a complex mixture of different compounds which when administered in human body may individually and in combination affect human physiology in a variety of ways. Compounds discovered from herbs have always been a source of many valuable medicines and even today herbs still continue to be a great source of compounds having therapeutic importance. Various studies have been conducted so far on *Echinacea purpurea* often with conflicting results(48, 56). One of the factors contributing towards this could be the fact that herbal medicines are not regulated and much variation occurs between two preparations of the same herbal product including amount of active ingredients or inclusion of additional substances(57). Several *Echinacea purpurea* products have been found to lack adequate levels of one or more clinically effective compounds (57). Moreover, often an herb has been found to possess multiple ways by which it modulates human physiology. Thus studies are required to fully understand the efficacy of this herb. Many studies have reported Echinacea to be effective for immune system stimulation (58). The proposed mechanism of action being increase in the number of granulocytes, enhanced phagocytic activity by macrophages and T lymphocytes, release of immunomodulators such as tumor necrosis factors and interferon, inhibition of virus proliferation and cytokine activation (58, 59). In most of the studies, efficacy of Echinacea has been demonstrated at a dose equivalent to 900mg of dried herb (59). In a

study conducted by Randolph et al, *in vitro* exposure of THP-1 cells to 250µg/mL of Echinacea species extract induced 10 fold expression of tumor necrosis factor-α and interleukin-1α, 1β, 8, 10 genes (59). The overall gene expression pattern after consumption of a commercial blended Echinacea product 1518mg/day for 2 days and one additional dose of 506mg on day 3 was consistent with an anti inflammatory response (59). In the current study the *Echinacea purpurea* root preparations have been made using 1g of plant material in 2mL of solvent.

Spilanthes acmella has been shown to possess strong diuretic potential in rats (17). A cold-water extract of 1500mg/kg oral administration in hydrated rats exhibited strong diuretic action (17). It has also been shown to possess larvicidal effect against *Culex quinquefasciatus* (60). Not much is known about this plant native to South America and there is scope for considerable research to achieve a comprehensive idea about the true potential of this herb in therapeutics.

The bioavailability of the components of Echinacea is not fully known. In fact out of the many compounds identified from Echinacea species so far, only alkylamides have been shown to be bioavailable in both *in vitro* and *in vivo* experiments (61). In a study conducted using roots of *Echinacea angustifolia* on humans, plasma samples of 11 healthy individuals were analyzed following oral administration of 60% ethanolic extract from the roots of *Echinacea angustafolia* (62). The maximum concentration of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides, the main alkamides in the roots of *E. angustifolia*, appeared already after 30 minutes and was 10.88ng/mL for the 2.5mL dose (62). In another study using ethanolic extract of fresh blooming aerial parts of *Echinacea*

purpurea, oral intake of 65mL extract containing 4.3mg of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides resulted in identification of the compound in blood, the amount being 44ng/mL of blood (63). Matthias et al conducted a study where a combination of 2700mg of *Echinacea purpurea* root and 600mg of *Echinacea angustifolia* was orally administered to volunteers, the total alkylamine content being about 54.6 mg where the total amount of Undeca- 2E, 4Z/E- diene- 8, 10- diynoic acid-isobutylamide was 1mg and the total amount of Dodeca- 2E, 4e, 8Z, 10E- tetraenoic acid isobutylamide and Dodeca- 2E, 4e, 8Z, 10Z- tetraenoic acid isobutylamide combined was 4.2 mg (64). The average of the sum of alkyamides in human plasma was 336 ± 131 ng eq/ml plasma (64).

A matter of concern with these herbal products is related to their safety. Drug interactions are responsible for more than 100,000 deaths per year in the United States and some of these may be linked to the use of herbs (58). When an herb is taken in combination with drug, many aspects including absorption, distribution, metabolism and excretion may be affected. Herbal products may interact with drugs in several ways changing the rate of elimination or amount of drug absorbed. Most known drug interactions are due to altered expression or functionality of cytochrome P450 enzymes. Elevated CYP activity may result in a rapid metabolic rate and thus cause decrease in plasma concentrations and in loss of therapeutic effect whereas inhibition of CYP activity may cause a rise in plasma concentrations and lead to toxicity related to overdose.

Finally, herbal medicines are not regulated and much variation can occur in the composition of the preparation. The safety and efficacy of these substances have not been

proved beyond doubt. Thus bioactive components need to be isolated and studied. Recently presence of similar bioactive alkylamides in both Echinacea and Spilanthes has raised interest in minds of researchers regarding their ability to modulate human physiology. .

4.1 Inhibition of CYP2C9 by Echinacea and Spilanthes

In the current study, 5μL of *Echinacea purpurea* root extract, equivalent to 2.5mg of the herb, showed about 33.2% inhibition of CYP2C9. The nature of inhibition was observed to be mixed thus suggesting a complex mechanism. In an *in vivo* study conducted by Gorski et al on the effect of *Echinacea purpurea* root on CYP2C9, it significantly reduced the oral clearence and increased the systemic exposure of tolbutamide, the probe for CYP2C9, indicating inhibition of hepatic CYP2C9 activity (48). He used 400mg of *Echinacea purpurea* root from Nature's Bounty, 4 times a day for 8 days (48). This result, though statistically significant was not considered clinically important based on the guidelines established by Food and Drug Administration (48). It was mentioned in the study however that the degree of inhibition varied between individuals, some of them showing moderate inhibition (48).

It is known that commercially available brands of Echinacea differ widely in their phytochemical content. Thus coadministration of an Echinacea product other than the one used by Gorski et al with CYP2C9 substrates having a narrow therapeutic index such as phenytoin may need careful monitoring (48). In this context, it may also be stated that in case of drug known to undergo biotransformation by multiple cytochrome P450 pathways, this result may acquire clinical relevance. Several studies have established the mixed

effect of Echinacea on CYP3A activity. Echinacea has been shown to inhibit intestinal CYP3A and induce hepatic CYP3A activity. Thus in case of drugs like warfarin which is a substrate for both CYP3A and CYP2C9, offsetting changes may be observed due to reduction in efficacy due to CYP3A induction and toxicity due to inhibition of CYP2C9 (48).

Spilanthes acmella seems to be an even more potent inhibitor of CYP2C9. Here 5μL of the extract, equivalent to 2.5mg of the herb showed an inhibition of 51.7%. The nature of inhibition was observed to be mixed. Contribution from both competitive and uncompetitive inhibition may thus be possible resulting in a complex mechanism. There have not been any studies on *in vivo* administration of this herb on humans. Care needs to be taken not to take this herb concurrently with drugs metabolized by CYP2C9.

4.2 Inhibition of CYP1A2 by Echinacea and Spilanthes

The *in vivo* study conducted by Gorsky et al showed that short-term administration of Echinacea reduced the oral clearance of caffeine, a probe for CYP1A2, to a significant extent (48). Gurley et al however did not see an effective inhibition (56). None of the studies however used 7-ethoxycoumarin as substrates. In the current studies neither raw *Echinacea purpurea* root extract, nor raw Spilanthes extract showed any inhibition of CYP1A2 using 7-ethoxycoumarin as a substrate. CYP1A2 is involved in the metabolism of a number of prescribed drugs, a few of them being theophyllin, cyclobenzaprine, tacrine and clozapine (48). According to the current study, coadministration of Echinacea or Spilanthes with drugs metabolized by CYP1A2 may be considered safe.

4.3 Inhibition of CYP2A6 by Echinacea and Spilanthes

CYP2A6 is the main enzyme that metabolizes nicotine into an inactive metabolite, cotinine (65). It is well established that tobacco derived nitrosamines are potent carcinogens in animal models. The 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of the most abundant pulmonary carcinogen present in tobacco and it requires metabolic activation by CYP2A family to activate its tumorigenic potential (65). CYP2A6 is partially responsible for its bioactivaton (64). The ability of CYP2A6 to bioactivate NNK by its methylene hydroxylation coupled with the fact that it catalyzes nitotine to cotinine makes it an anti cancer therapeutic target (65). In fact, pretreatment of female mice with 8-methoxypsoralen has been shown to strongly inhibit lung tumorigenesis induced by NNK (65). The ability of CYP2A6 to catalyze metabolic activation of nitrosamine derivatives have also been linked to gastric cancer (66). Studies suggest that CYP2A6 deletion is associated with gastric adenocarcinoma in Japanese population (66). No studies have been conducted so far to observe the effect of Echinacea or Spilanthes on CYP2A6. In the current study addition of 10μL or 10μg/mL of Echinacea extract inhibited CYP2A6 activity by 80% whereas 10μg/mL of Spilanthes extract inhibited liver microsome to about 75%. The nature of inhibition in case of Echinacea was found to be uncompetitive while that in case of Spilanthes was found to be mixed. More research needs to be carried out in this direction to better understand the reason for this kind of inhibition. According to the results by Matthias et al, the amount of bioactive alkylamides in Echinacea is about 1.7% of the total extract (64). Calculating the possible amount of bioavailable alkylamides in the raw Echinacea extract used in the

current study based on the results of Matthias et al, it seems10μg of extract should have about 170ng/mL of bioavailable alkylamides. According to this result Echinacea might be considered as a potent inhibitor of CYP2A6. More experiments need to be carried out in this direction considering the fact that potency of Echinacea as inhibitors of CYP2A makes it a probable candidate in the field of anticancer therapeutics. In case of Spilanthes, considerable inhibition was observed. Thus it may be said that *Spilanthes acmella* is also an effective inhibitor of CYP2A6.

4.4 Inhibition of CYP2E1 by Echinacea and Spilanthes

Cytochrome P450 2E1 is one of the enzymes that metabolizes ethanol in the liver, the other one being alcohol dehydrogenase (67). Ethanol is a systemic toxin responsible for severe form of hepatic apoptosis. CYP2E1 is induced by alcohol and this phenomena has been directly linked with alcoholic liver injury (67). When induced, CYP2E1 produces a highly toxic derivative of the reactive oxygen species known as alpha-hydroxy ethyl radical (67). The increase of this species seems to play an important role in ethanol-induced hepatocellular damage (67). Thus inhibition of CYP2E1 can be considered as a protective measure against ethanol-induced hepatotoxicity.

In the current study, both Echinacea and Spilanthes whole plant extracts were initially tested for their ability to inhibit CYP2E1 activity. Addition of 5μL or 5μg/mL of Echinacea showed about 42.3% inhibition. Spilanthes failed to show any significant inhibition. Addition of 40μL of Spilanthes extract caused inhibition of CYP2E1 of about 29.1%. The nature of inhibition in both the cases was determined to be reversible and they appeared competitive in nature with the possibility of some mixed elements. There

have not been many studies on the effect of Echinacea or Spilanthes on CYP2E1. The only study on the inhibition of CYP2E1 by Echinacea *in vivo* was done by Gurley et al where only cichoric acid was present out of the phenolic compounds and the content of alkylamides have not been mentioned (56). He did not observe any significant inhibition of CYP2E1 by Echinacea. According to the results by Matthias et al and considerations as mentioned in the previous section of this chapter, 85ng/mL Echinacea extract managed to cause 42.3% inhibition of CYP2E1 (64).

4.5 Inhibition of CYP2E1 by Alkylamides in Echinacea and Spilanthes

In the current study, it was observed that aerial portions of *Echinacea purpurea* failed to show any significant inhibition of CYP2E1. The ethanolic extract of roots of *Echinacea purpurea* however showed considerable inhibition. Next owing to the therapeutic importance linked with CYP2E1 major components of Echinacea and Spilanthes are isolated and tested for their ability to inhibit CYP2E1. None of the phenolic extracts showed any inhibition of CYP2E1. In a recent *in vitro* study conducted to examine the enzyme-mediated metabolism of alkylamides present in Echinacea no degradation of alkylamides was noticed in cystolic fractions while NADPH-dependent degradation of alkylamides was observed in microsomal fractions suggesting cytochrome p450 mediated metabolism in liver (61). A couple of interesting observations were made in this study. Firstly, the extent of metabolism of 2-ene that is, the (2E)-N-isobutylundeca-2-ene-8, 10-diynamide was one tenth of the 2,4-diene, the (2E,4E,8Z,10Z)-N-isobutyldodeca-2,4,8,10-tetraenamide (61). Also remarkably less degradation of the 2,4-diene was seen in the mixture of alkylamides in ethanolic extract

of Echinacea than when used alone suggesting contribution of both chemistry and combinaton of alkylamides in their metabolism by cytochrome P450 in human liver (61).

In the current study, all of the alkylamides tested both for Echinacea and Spilanthes proved to be moderate inhibitors of CYP2E1, particularly Undeca- 2Z, 4E- diene- 8, 10- diynoic acid- isobutylamide. The inhibition seen while using the CYP2E1 was greater than in liver microsomes. The combination of Dodeca- 2E, 4E, 8Z, 10E- tetraenoic acid isobutylamide and Dodeca- 2E, 4e, 8Z, 10Z- tetraenoic acid isobutylamide inhibited CYP2E1 at a concentration of 31ng/mL. The K_I was determined to be 6.5µM when CYP2E1 supersomes were used, while the K_I for inhibition of CYP2E1 using human liver microsomes by *Echinacea purpurea* root extract was 31.1µM. In case of Undeca- 2E, 4Z- diene- 8, 10- diynoic acid- isobutylamide 6.2 ng/mL was needed to show considerable inhibition. Here K_I with CYP2E1 supersome was 7.4 µM while that using human liver microsome was 31.1µM. Undeca- 2Z, 4E- diene- 8, 10- diynoic acid- isobutylamide inhibited CYP2E1 at a concentration of 5ng/mL. The K_I determined for CYP2E1 supersomes was 2.9µM and that for inhibition of CYP2E1 in human liver microsome was 5.8µM. These results look very promising considering the bioavailability of these alkylamides as shown in different studies. In the study carried out by Gorski et al, 1600mg was used for 8days and considerable inhibition was noticed (48). In a study using *Echinacea purpurea* root, administration of 4.3mg alkylamides resulted in plasma concentration of 44ng/mL (63). Matthias et al used 3300mg Echinacea with an alkylamide content of 54.6mg, the contribution from our alkylamides being about 6mg, and observed 44ng/mL concentration (64). Spilanthol, a bioavailable alkylamide

component of Spilanthes was also very effective in inhibiting CYP2E1. In this case 14.6ng/mL showed considerable inihibition of CYP2E1 in human liver microsomes. The K_I value observed in this case was 6.5µM when CYCP2E1 supersomes were used and was 70.1µM when inhibition of CYP2E1 was tested using human liver microsomes. This was a bit surprising considering the fact that raw Spilanthes was not a potent inhibitor of CYP2E1. This could be owing to the concentration of Spilanthol in Spilanthes or interactions by other components of Spilanthes which suppresses the activity of Spilanthol in raw Spilanthes extract.

Thus it may be said that the current study revealed two more possible ways by which we can be benefited by *Echinacea purpurea* root. Its ability to inhibit CYP2A6 makes it a possible therapeutic target as anticancer agent and the effective way by which Echinacea and the isolated alkylamides inhibit CYP2E1 make them potential agents to combat liver injury. Spilanthol isolated from *Spilanthes acmella* whole flowering plant also has a promising future as a therapeutic agent against alcohol induced liver injury.

4.6 Future Direction

In the current research *in vitro* analysis showed two possible benefits of *Echinacea purpurea* root extract due to its interaction with CYP2A6 and CYP2E1. To the best of my knowledge, until now no studies have investigated the interaction between Echinacea and CYP2A6. Thus *in vivo* studies need to be carried out to confirm the potency of this inhibition. Also, different components of Echinacea needs to be tested for their ability to inhibit CYP2A6 to understand which component of Echinacea is contributing towards this activity. The bioavailabilty of the components need to be tested

as well. In case of CYP2E1, *in vivo* studies are required with the individual components of Echinacea to confirm the potency of these compounds against CYP2E1. The root extract of *Echinacea purpurea* was shown to inhibit CYP2C9 in the current study. Further, the bioavailable components of Echinacea needs to be tested individually and also *in vivo* to get a clearer picture.

Very few *in vivo* studies could be found which have tested the potency of *Spilanthes acmella* and none of them with humans. The interactions of this herb with cytochrome P450 have not been studied before. Studies are thus require to investigate the interaction of this herb with the CYP isoforms which have not been under the scope of current research, like CYP3A or CYP2D to get a comprehensive idea about how this herb interacts with different CYP isoforms. Next, the bioavailable components need to be investigated for their contribution towards these interactions if any. *In vivo* analysis of the interaction of Spilanthol with CYP2E1 would be another future direction for this research.

4.7 Conclusion

Echinacea is one of the most commonly used alternative medicines in the world mainly used for the treatment of cold and flu. Recently the potential of this herb to as an immunostimulant is being widely studied. At the same time, the safety of this herb related to drug-herb interactions is also being investigated. In the current study *in vitro* analysis of the interaction of *Echinacea purpurea* root with different isoforms of cytochrome P450 (CYP1A2, CYP2C9, CYP2A6 and CYP2E1) have been investigated to throw more light on the efficacy and safety of this herb. This study has led to some interesting observations as regards to the possible benefits from this herb. Echinacea was found to

inhibit CYP2A6 and CYP 2E1 to considerable extent, thus making it a potential

therapeutic target in the field of anticancer therapy and alcohol induced liver injury. At

the same time analysis of the interaction of Echinacea with 2C9 has revealed that this

herb poses risk of adverse drug-herb interactions when taken concurrently with drugs

metabolized by CYP2C9. On the other hand, coadministration of this herb with drugs

metabolized by CYP1A2 seems safe. Further, it was observed that neither the

components of the aerial portions of *Echinacea purpurea* nor the phenolic components of

this herb are responsible for the inhibition of CYP2E1. The four isobutyl amides

(Dodeca- 2E, 4E, 8Z, 10E- tetraenoic acid isobutylamide, Dodeca- 2E, 4e, 8Z, 10Z-

tetraenoic acid isobutylamide, Undeca- 2E, 4Z- diene- 8, 10- diynoic acid- isobutylamide

and Undeca- 2Z, 4E- diene- 8, 10- diynoic acid- isobutylamide) present in major

proportions in the ethanolic preparation of *Echinacea purpurea* root are potent inhibitors

of CYP2E1. In fact, Undeca- 2Z, 4E- diene- 8, 10- diynoic acid- isobutylamide inhibited

CYP2E1 at a concentration of 5ng/mL, the K_I being 5.8nM with human liver microsomes.

 Spilanthes acmella is known for its antidiuretic and larvicidal activities[16, 17].

Recently Spilanthol, an isobutylamide in Spilanthes has been isolated and shown to be

bioavailable which has similarity in structure with the isobutylamides found in Echinacea.

In the current research, *Spilanthes acmella* raw flowering plant extract was not found to

be a significant inhibitor of CYP2A6 or CYP2E1. However, Spilanthol, the major

alkylamide component of Spilanthes was found to be a potent inhibitor of CYP2E1. This

observation further suggests functional similarity between the isobutylamides in

Echinacea and Spilanthes. When tested with CYP2C9 and CYP1A2, Spilanthes failed to

show inhibition against CYP1A2 but showed considerable inhibition against CYP2C9, thus making it unsafe with drugs metabolized by CYP2C9.

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