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Cytochrome P4502E1, the ethanol-inducible form, metabolizes and activates a significant number of substrates to more toxic products and the induction of CYP2E1 by ethanol is thought to result in increased oxidative stress in hepatocytes. One proposed mechanism for this increased oxidative stress is the increased production of hydrogen peroxide by CYP2E1 via a so called “uncoupling’’ of its NADPH oxidation activity. A main hypothesis of this research is that the main aldehyde constituent found in Lemongrass, citral, will be able to block the activity of CYP2E1, and consequently demonstrate physiological antioxidant properties.

The induction of the phase II enzyme is an important process involved in cellular oxidative stress response, by which the oxidative toxicants can be eliminated or inactivated before they damage the critical cellular macromolecules. Phase II detoxifying genes provide protection to the cell against the toxicities of ROS and reactive intermediates produced during phase I metabolism.

In this research, cell culture, RT-PCR and electrophoresis gel technology will be used for monitoring the induction of antioxidant genes by a variety of different natural products.

INHIBITION of CYTOCHROME P4502E1 BY LEMONGRASS AND THE PRIMARY ALDEHYDE CONSTITUENT OF LEMONGRASS, CITRAL

by

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in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2009

Approved by

_____

Committee Chair

To almighty God, my family, friends of church in Taiwan, and friends in I-HOUSE

of UNCG Fall2007~Spring2009

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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Date of Acceptance by Committee

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Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

Recent medical studies have shown that oxidative stress is at the root of a number of human diseases such as Parkinson's disease (1), Alzheimer's disease (2) and cancer (3) which directly or indirectly relates to most of human illness and death in the world. Oxidative stress also contributes to aging in the human body (4) and causes many general health problems for humans. The production of reactive oxygen species (ROS) in the human body damages components of the cell including DNA (5), lipids (6) and proteins (7). ROS are ions or molecules including free radicals, oxygen ion or peroxides that can oxidize biological molecules. ROS are formed from a variety of biochemical reactions and cellular functions (8), or through exposure to chemicals (9). The formation and consumption of free radicals can be balanced by antioxidants in the cell. Oxidative stress is caused by an imbalance in rate of formation and consumption of free radicals. In addition, there are some common factors responsible for the generation of free radicals such as air pollution (10), sunlight (11) and smoking (12). In order to reduce oxidative stress, the body produces antioxidants to neutralize

free radicals which otherwise can destroy the human cells. The purpose of this research is to show that specific compounds in the essential oil of lemongrass and the primary aldehyde constituent of lemongrass, citral, have the ability to reduce oxidative stress by scavenging ROS and/or by preventing their formation. The project also addressed the ability of certain essential oils to induce cellular systems that remove ROS in the liver and possibly in other tissues. The hypothesis of this paper is that lemongrass oil stimulates phase II drug metabolizing enzymes in the human liver and reduces the formation of ROS through inhibition of cytochrome $P450_{2E1}$ activity, which is a monooxygenase enzyme that has been implicated in the generation of hydrogen peroxide and superoxide radicals.

1.1 Cytochrome P450 enzymes

1.1.1. General overview

Cytochrome P450 enzymes are most responsible for the metabolism of foreign chemicals. Their primary purpose is the hydroxylation of non-polar molecules, and making the drug more polar to facilitate their elimination in urine. Cytochrome P450 enzymes are commonly found in both prokaryotic and eukaryotic cells in nature. In eukaryotic cells, the cytochrome P450 enzymes are mostly found in the membrane

of the smooth endoplasmic reticulum and some are found in the mitochondria. The cytochrome P450 enzymes work with the reductases which provide the cytochrome P450 enzymes the electrons from NADPH and sequentially use FAD and FMN in the electron transfer process. The two electrons are transferred to the heme cofactor in the active center of cytochrome and the cytochrome transfers two electrons to one of the two oxygen atoms of O_2 to produce water molecules(23). The remaining oxygen atom from O_2 remains coordinated to the iron atom of the heme cofactor in an elevated oxidation state. This so called oxo ferryl species is the activated intermediate in P450 catalysis. The P450 catalytic cycle is shown in figure 1.

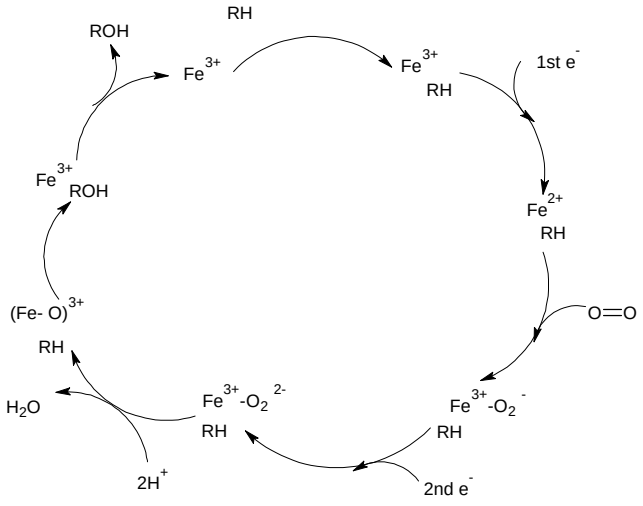
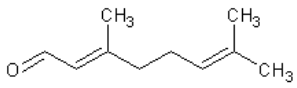
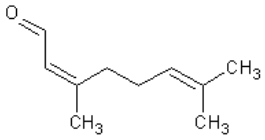


Figure1. The P450 catalytic cycle.

1.1.2. The aldehydes: Citral, Citronellal, and Decanal



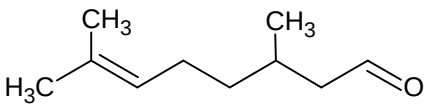
trans(geranial)



cis(neral)

1.1.2.A. Citra

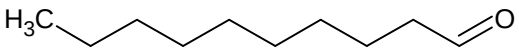
Citral (3,7-dimethyl-2,6-octadienal) is an aldehyde that has two isomers, cis (neral) and trans (geranial). A variety if edible vegetables and fruits, including lemon, lime, orange, grapefruit, apricot, tomatoes, celery, and others, have been reported to contain significant amounts of aldehyde citral(37). It is commonly found in the citrus oils which are common flavor ingredients in foods and beverage products. Citral is susceptible by acid-promoted and oxidative degradation and decomposed during storage by a series of cyclization and oxidation reactions(36). Moreover, citral is the most important constituent of lemongrass oil, containing as much as 75% to 85%(37) and it has been reported to exhibit activity as vitamin A antagonist by inhibiting the oxidation of retinal to retinoic acid(38).



1.1.2.B. Citronellal

Citronellal

Citronellal (3,7-dimethyloct-6-en-1-al), a mono terpenoid is a main component in the mixture of terpenoid chemical compounds that gives the lemon scent associated with citronella oil. It is a major isolate in distilled oils from the plants cymbopogon, lemon-scented gum, and lemon-scented tea tree. Moreover, citronellal has high repellent effect to the insect especially for mosquitoes and also possesses antifungal properties(39).



1.1.2.C. Decanal

Decanal

Decanal is a ten-carbon aldehyde which is used in fragrances and flavor, it is a naturally occurring compound and is an important component in citrus along with octanal, citral, and sinensal(40).

Essential oils containing aldehydes were selected as potential inhibitors of human P450 because prior studies by Raner et al. (41) have shown that the nucleophilic attack of a ferric peroxo species at the electropositive carbonyl carbon of aldehyde

forms a transient peroxohemiacetal. The peroxy-hemiacetal intermediate in aldehyde deformylation resulted heme modification in CYP450.

1.1.3.Aldehyde deformylation

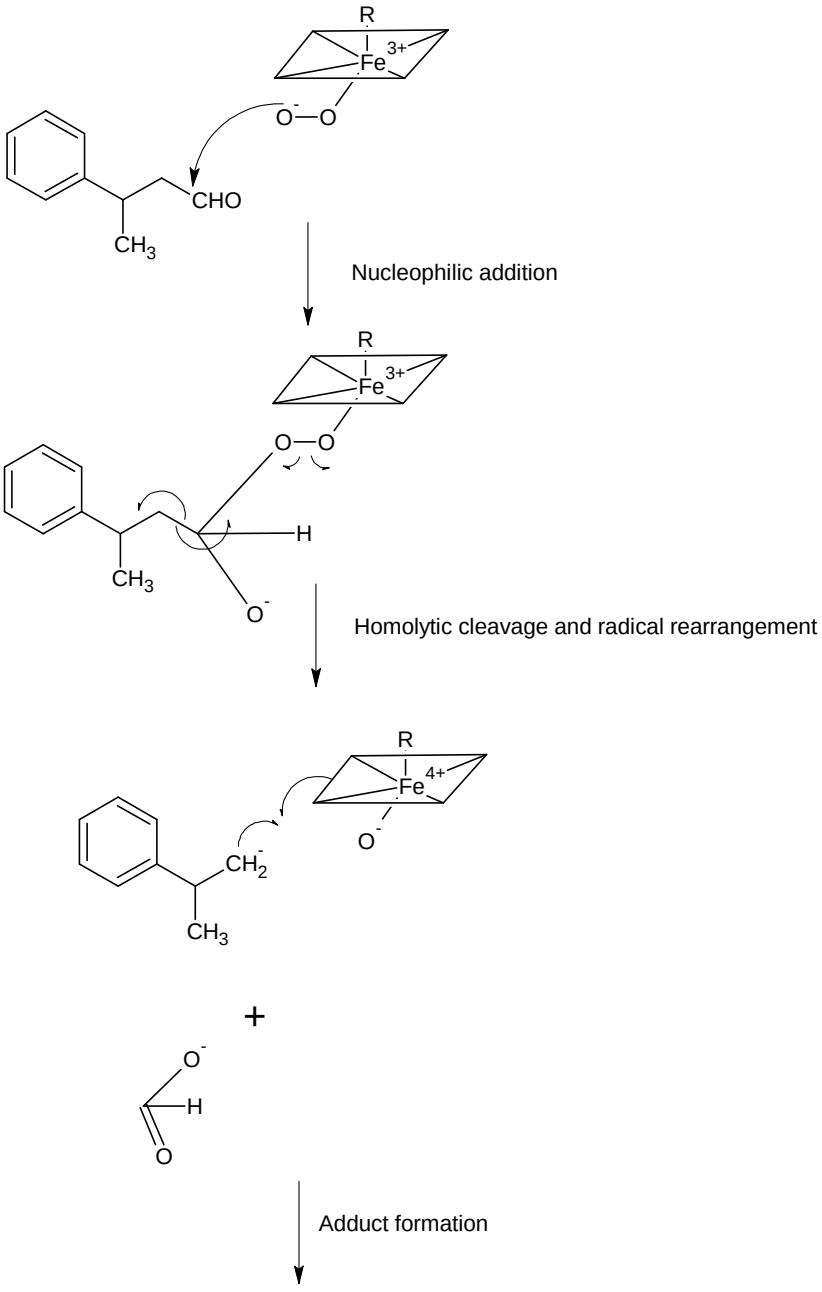


Figure2. Aldehyde deformylation scheme showing heme alkylation mechanism involved in P450 inactivation by aldehydes.

1.2 Cytochrome P450$_{2E1}$

Cytochrome P450$_{2E1}$, the ethanol-inducible form, metabolizes and activates a significant number of substrates to more toxic products and the induction of CYP2E1 by ethanol is thought to result in increased oxidative stress in hepatocytes(15). One proposed mechanism for this increased oxidative stress is the increased production of hydrogen peroxide by 2E1 via a so called “uncoupling’’ of its NADPH oxidation activity. A main hypothesis of this research is that the main aldehyde constituent found in lemongrass, citral is able to block the activity of CYP2E1, and consequently demonstrate physiological antioxidant properties. Many studies have been carried out involving inhibition of CYP2E1and the potential beneficial effects related to a reduction in the activation of carcinogens or hepatotoxins(32). For example Cedarbaun et al. showed NO can effectively inhibite arachidonic acid(AA) toxicity in liver cells which express high levels of CYP2E1. They treated pyrazole-induced rat hepatocytes with AA in the presence of an inhibitor of nitric oxide synthase, L-N^{G}-Nitroarginine Methylester (L-NAME) and added the NO donors S-nitroso-N-acetylpenicillamine (SNAP) to increase NO level to demonstrate that NO can be hepatoprotective against CYP2E1-dependent toxicity and prevent AA-induced oxidative stress(31). In order to demonstrate the inhibition of cytochrome P450$_{2E1}$

activity by these natural products, the Michaelis-Menton model will be used in conjunction with a HPLC-based $P450_{2E1}$ catalytic assay involving the oxidation of p-nitrophenol to nitro catechol.

1.3 Phase II detoxifying genes

The induction of the phase II enzyme is an important process involved in cellular oxidative stress response, by which the oxidative toxicants can be eliminated or inactivated before they damage the critical cellular macromolecules(13). Phase II detoxifying genes provide protection to the cell against the toxicities of ROS and reactive intermediates produced during phase I metabolism(13). Members of the phase II type enzymes include histone acetyltransferase-1(HAT-1), choline acetyltransferase-1(CHAT-1), histamine N-methyltransferase-1(HNMT-1), Epoxide hydrolase-1(EPHX-1), Heme oxygenase-1(HO-1) and NAD(P)H: quinone oxidoreductase -1(NQO-1). Each of the corresponding gene products play an important role in the cell in quenching ROS and preparing them for elimination. Several recent studies have shown that some novel antioxidant chemicals induce the synthesis of phase II detoxifying enzymes as a mechanism for increasing the ratio of reduced GSH/oxidized GSSG. What appears to be happening is that the transcription factor Nrf2 is activated by these chemicals, allowing it to stimulate the transcription

of a large number of genes in the nucleus. Nrf2 normally binds to the cytoskeleton associated protein Keap1 which is located in the cytoplasm(14). When released from its complex, Nrf2 enters the nucleus and binds to Antioxidant response element(ARE) and regulate the expression and induction of a battery of genes encoding detoxifying/chemopreventive proteins, which are activated in response to oxidants, xenobiotics, UV light, and radiation(14). The increase of the Nrf2-dependent transcription is correlated to the response associated with electrophilic chemicals and oxidative stress. According to a recent study, the activation of Nrf2 involving several kinases such as MAPKs, PKC and PI3K. For example PKC, p38 , JNK, ERK1/2 and Nrf2, all have a role in the induction of HO-1 by oxidized LDL in human muscle cells(13). The study showed that MAPK pathways played an important role in the Nrf2-regulated phase II gene expression(13). The gene product of HO-1 catalyzes the rate-limiting step in heme degradation, which is transformation of heme into biliverdin, carbon monoxide, and free ion(Fe^{2+})(15). The importance of HO-1 expression is that it mediates antioxidant, anti-inflammatory, and antiapoptotic effects within the cell(16-18). The increasing activity of HO-1 correspond to the degradation of the heme moiety which is a potential toxic prooxidant. In addition, increased activity of HO-1 generates

bilirubin which is an antioxidant with the ability to scavenge peroxy radicals and inhibit lipid peroxidation(19-21) (Fig 3). CO is another product generated by the HO-1 induction, it has the vasodilatory effects, anti-apoptotic effect, and anti-inflammatory effect which are mediated by cGMP(15). As for Fe^{2+} ion, Ferritin, an intracellular iron repository, is induced along with HO-1, therefore ferritin binds the unbound iron from heme degradation(15). Cell culture, RT-PCR and electrophoresis gel technology will be used for monitoring the induction of antioxidant genes by a variety of different natural products. The detoxification of foreign substances can be classified into two reaction processes; phase I and phase II. For phase I reactions, foreign chemicals are mainly oxidized by cytochrome P450 (CYP) enzymes to become polarized metabolites. Phase II metabolism involves a variety of enzymes that catalyze group transfer reactions of reductive reactions such as glutathione S-transferase (GST) and NADPH quinone oxidoreductase (NQO1). These enzymes convert the reactive Phase I products to more inert hydrophilic products(22).

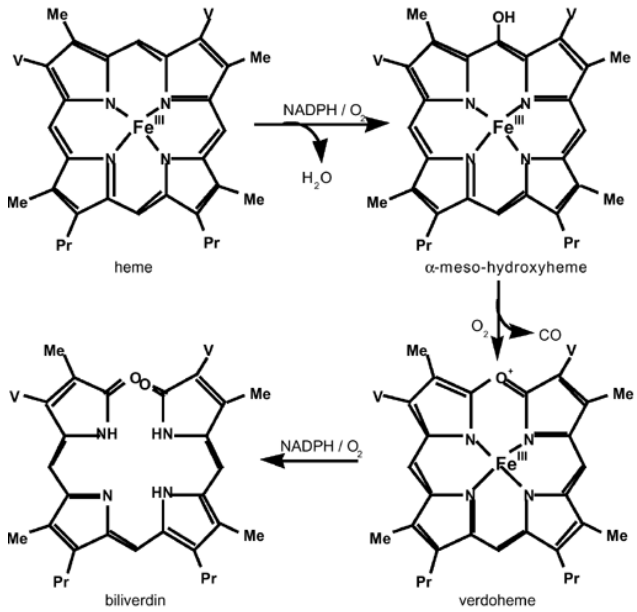
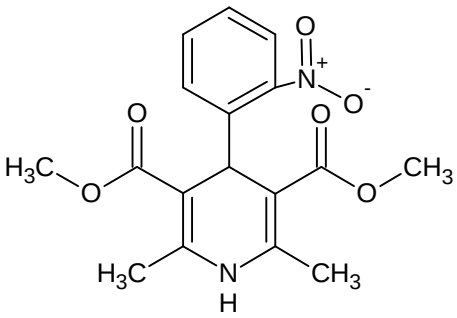


Figure3. Reaction intermediates in the conversion of heme to biliverdin and CO by heme oxygenase.

1.4 Inhibition of Cytochrome P450 3A4

Cytochrome P450 3A4 belongs to the hepatic endoplasmic-reticulum (ER)-anchored P450 family and it is a monotopic hemoprotein with its N-terminus embedded in the ER-membrane with the bulk of its structure exposed to the cytosol. In addition, cytochrome P450 3A4 is induced through transcriptional activation and substrate-mediated stabilization of its degradation(26). Cytochrome P450 3A4 is abundant in the human liver and mainly expressed in the human liver and intestines. The inhibition/induction of CYP3A4 has been reported as a significant reason for herb–drug interaction(35). It is responsible for the metabolism of about 60% of the drugs in current clinical use and regulated by a variety of hormones which include

glucocorticoids, growth hormone, and triiodothyronine. Furthermore, drugs such as Phenobarbital, clotrimazole, mifepristone, and rifamycin have been shown to induce cytochrome P450 3A4 via a transcriptional upregulation(24,25).



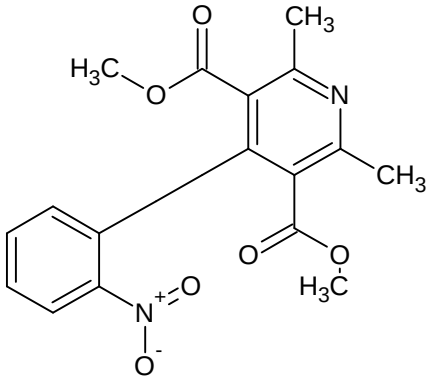
1.5 Nifedipine **Nifedipine**

In an effort to probe the interactions between P450 3A4 and several different essential oils, we developed a method to monitor inhibition of cytochrome P450 3A4 using nifedipine as a test substrate.

Nifedipine(4-(2-nitrophenyl)-2,6-dimethyl-3,5-dicarbomethoxy-1,4-dihydropyridine), one of the most potent calcium-channel blockers belonging to the group of 1,4-dihydropyridines(30), is used in the treatment of a variety of cardiovascular disorders such as Raynaud's phenomenon, hypertension and angina pectoris(27,30). Nifedipine is a photolabile compound which undergoes oxidative biotransformation in the human body into pharmacologically inactive metabolites(30). It is commercially available as yellow crystals with a melting point of 172 to 174ºC(27). Nifedipine, a highly non-polar compound, has very low solubility therefore it was first

dissolved in methanol to facilitate delivery in the current study. In the human body, nifedipine is absorbed completely from the gastrointestinal tract and mostly from jejunum. After absorption, nifedipine is further metabolized in the small intestine and liver to form more polar compounds which enable the kidney to eliminate it via the urine(30). The first analytical instrumental method to detect nifedipine in biological fluids level is gas chromatographic(GC). The disadvantage of GC is that it lacks specificity and selectivity even though the amounts of volumes are in the level of micro liter and the limit of detection could go as low as 2ng/ml. In addition, most GC methods require liquid-liquid and solid-phase extraction which increases the complexity and time required for analysis. Therefore, in order to improve the sensitivity, specificity, and efficiency, a high performance liquid chromatographic(HPLC) method has been developed to detect nifedipine in plasma, however, many of these methods still involved complicated and time-consuming sample extraction. So a major goal has been to determine the plasma level of nifedipine to yield a reliable estimate of its pharmacokinetic parameters for therapeutic drug monitoring and bioavailability/bioequivalence purposes. These estimates rely on the ability to measure the drug level at the lower end of the plasma concentration range found in pharmacokinetic studies following the administration of

therapeutic doses of the drug(30). The method described in this thesis allows rapid accurate measurements of oxidized nifedipine.



Oxidized Nifedipine

1.6 Oxidized Nifedipine

Oxidized Nifedipine is the metabolite of Nifedipine which is formed by CYP3A4 in the oxidation reaction of nifedipine. In my research, nifedipine is used as the substrate for testing the metabolism and inhibition of CYP3A4 activities and the metabolic analysis is performed by Shimadzu HPLC. The structure of oxidized nifedipine is shown above.

1.7 HepG2 human liver cells

In my research, the Hepg2 human liver cancer cell line was used for the cell culture. Many publications indicate that liver cell lines have the activities of drug metabolizing enzymes involved in the activation and detoxification of genotoxic carninogens. In addition, liver cell lines show more pronounced DNA damaging effects caused by test substances than stable mammalian cell lines(29). Among these

cell lines, human HepG2 cell line is the most promising one which has been used in a number of genotoxicity tests. The HepG2 cells possess different phase I and phase II enzymes involved in the activation/detoxification of genotoxic carcinogens(29) and HepG2 cell line is very valuable for the screening purposed in the early phase of pharmaceutical development(28).

1.8 Proposed research

The following two projects were initiated in order to probe the potential antioxidant mechanisms associated with essential oil of lemongrass.

Project 1: **To establish the mechanism and potency of inhibition of human cytochrome P450$_{2E1}$ and P450$_{3A4}$ by essential oil of lemongrass and the primary aldehyde constituent of lemongrass, citral.** According to preliminary studies, lemongrass has promising inhibitory properties toward P450$_{2E1}$ in vitro. The major aldehyde component of lemongrass (citral) appears to be the primary inhibitory constituents of that oil. The experimental method to be used is a HPLC-based assay in which p-nitrophenol(PNP) is oxidized by cytochrome P450$_{2E1}$ by human liver tissue in the presence of the essential oil of Lemongrass or its major constituents. A similar approach using Nifedipine oxidation reaction will be used to show the analogous effects on P450$_{3A4}$**.**

Project 2: To show induction of antioxidant genes by essential oil of lemongrass and primary aldehyde constituent of lemongrass, citral, and to identify those constituents responsible for induction. The hypothesis of this objective is that the Lemongrass aldehyde constituent, citral, can activate the antioxidant response elements found in a variety of antioxidant /phase II drug metabolizing genes. Cultured human liver cells (HepG2) will be used to test this hypothesis where cells will be treated with oil or aldehyde and RT-PCR will be used to monitor antioxidant gene expression.

CHAPTER II

MATERIALS AND METHODS

2.1 CYP2E1 by lemongrass and other essential oils

Several different essential oils along with purified aldehyde constituents were tested for their abilities to inhibit $P450_{2E1}$. In particular Citral, Nonenal, Nonyl aldehyde, Citronellal and Decanal were evaluated with the oils Lemongrass, Eucalyptus Globulous, Cassia, Citronella and Eucalyptus lemon. The following experimental procedure was used for the evaluation of $P450_{2E1}$ inhibition: 5ul of the oil or aldehyde was diluted to 100ml in deionized water and this solution, along with 20ul of microsomes, 50ul of 1M potassium phosphate buffer solution (pH 7.4), 50mM p-nitrophenol(PNP) and deionized water were combined in a final volume of 0.5ml. 25ul of 1mM NADPH was added to initiate the reaction in a 37^{o}C water bath for 1 hr, then the reaction was quenched with the addition of 200ul of 6% perchloric acid. Samples were incubated in an ice bath for 10 minutes then centrifuged at 4000 rpm for 10 minutes and 400ul of supernatant was transferred to HPLC vials and analyzed by HPLC(SHIMADZU) for product formation. The product of p-nitrophenol

oxidation, nitrocatechol, was monitored using a Shimadzu LC 20A Series HPLC system consisting of an SPD-20A UV/Vis detector, LC 20AT solvent delivery, and a Sil 20A autosampler, all controlled using the Shimadzu EZStart version 7.3 SP1 software package. Absorbance detection was set to 340 nm with a mobile phase consisting of 35% acetonitrile, 64.5% H_2O, 0.5% acetic acid. The volume of injection is 40 ul for each sample and the column used was a RP-C18 HPLC column. For the CYP2E1 assay, total oil content in the experiments ranged from 0.5 mg/ml up to 37 mg/ml. Graphical analysis of the Michaelis-Menten data was processed using the non-linear regression analysis function of Slidewrite version 4.1 (advanced Graphics Software Inc), in order to generate K_I values for each of the inhibitors used. All assays were carried out in duplicate and repeated at least twice giving a minimum of four independent experimental values that could be averaged. The Lemongrass oil was purchased from Birch Hill Happenings Aromatherapy, LLC and the aldehyde citral, 95%, mixture of cis-Citral and trans-Citral, was purchased from ACROS ORGANICS.

2.2 Growth and treatment of HepG2 cells

HepG2 cells were grown in 75 cm^2 tissue culture flasks at 37^oC 5% CO_2 under

standard conditions: (Dulbecco's Modified Eagle Medium(DMEM) 1X with 1g/L glucose, 584 mg/L L-glutamine & 110 mg/L sodium pyruvate+ 10% Fetal bovine serum). When the cells were about 80% confluency, they were treated with diluted essential oils at concentrations between 20ug/ml and 200ug/ml for 5h. The cells were then treated with DMEM containing trypsin and incubated in the 37^{o}C environment for 5 minutes until they separated from the flask. Following this incubation, cells were harvested by scraping from the plate and were collected in a 15ml Falcon tube. The samples were then subjected total RNA isolation which is described in the following section.

2.2.1 Total RNA isolation and RT-PCR analysis

The RNA of the cells was isolated by using the SV Total RNA Isolation System protocol which includes disrupting the cells, denaturing the nucleoprotein complexes, inactivation of the RNase activity and removal of the proteins and DNA. The protocol used was as follows: In the RNA isolation section, ***1***. 175ul cell lysate was placed into the centrifuge tube then 350ul of SV RNA Dilution Buffer was added. The sample was mixed by inverting the tube 3~4 times and centrifuged at 14000g for 10 minutes at room temperature. ***2***. The cleared lysate solution was transferred to a fresh microcentrifuge tube by pipeting, while avoiding disturbing the

pelleted debris. A 200ul 95% ethanol aliquot was added to the cleard lysate and mixed by pipeting 3-4 times. This mixture was transferred to a spin column assembly and centrifuged for one minute at 14000g. ***3***. The spin basket was removed from the spin column assembly and the liquid in the collection tube was discarded. The spin basket was placed back into the collection tube, and 600ul of SV RNA Wash Solution was added to the spin column assembly then centrifuged at 14000g for one minute. ***4***. The collection tube was emptied as before and set in a rack. The DNase incubation mix was prepared by combining 40ul yellow core buffer, 5ul 0.09M $MnCl_2$ and 5ul of DNase I enzyme, per sample, in a sterile tube (in this order). The DNase incubation mix was disbursed by gentle pipeting. A 50ul aliquot of freshly prepared DNase incubation mix was applied directly to the membrane inside the spin basket for 15minutes incubation at the room temperature. ***5***. After the incubation, 200ul of SV DNase Stop Solution was added to the spin baset and centrifuged at 14000g for one minute. Then 600ul SV RNA Wash Solution (with ethanol added) was added and centrifuged at 14000g for one minute. ***6***. The collection tube was emptied and 250ul SV RNA Wash Solution (with ethanol added) was added and the assembly was then centrifuged at 14000g for two minutes. Finally, 100ul Nuclease-Free Water was added to the membrane and centrifuged for

one minute to elute the RNA and stored at -70 °C. A 10 ul sample of the RNA was used for reverse transcription to the corresponding cDNA, as described in the protocol provided with the kit.

2.2.2 RT-PCR

The cDNA obtained from the previous step was used as a template for PCR reactions, which were performed in a final volume of 20 µl according to the specifications given with the DNA polymerase. The cycling conditions were as follows: mixtures were heated to 95 °C for 5 min and then cycled 35 times through a 30 sec denaturation step at 95 °C, a 1 min annealing step at a specified annealing temperature 54°C-62°C, and a 45 sec extension step at 72 °C in a Perkin Elmer 9600 DNA cycler (Wellesley, MA). A 4.0 min extension time at 72 °C was included at the end of 35 cycles, and this was followed by incubation at 4 °C until the samples were analyzed. All PCR products were separated by gel electrophoresis on a 1.5% agarose gel, prepared by dissolving 0.6g agarose into 35ml 1xTAE buffer. A 10ul aliquot of each RT-PCR was mixed with 5ul 5X loading buffer and the samples were electrophoresed at 120V until the bromophenol blue dye reached the bottom of the gel. After running the gel, 50ul of a 0.625mg/ml Ethiudium Bromide solution was

added to DI water (100ml) and the gel was placed in this solution for one to two hours, and the DNA was visualized on a transilluminator with UV excitation.

2.2.3 Induction of phase II genes

2.2.3.1 Determination of effective dose of lemongrass for induction of phase II genes

Primer sets were used to amplify the cDNA of certain phase II mRNA produced in the HepG2 cells: the genes of interest consisted of histone acetyltransferase-1(HAT-1), choline acetyltransferase-1(CHAT-1), histamine N-methyltransferase-1(HNMT-1), Epoxide hydrolase-1(EPHX-1), Heme oxygenase-1(HO-1) and NAD(P)H dehydrogenase, quinone-1(NQO-1). These genes were selected on the basis of their observed induction upon exposure to Green tea extract in a prior study(34). Cells were treated with the essential oils at concentrations of 20ug/ml, 50ug/ml, and 200ug/ml. HepG2 cells were grown in 25 cm^2 tissue culture flasks at 37^oC. When the cells reached about 80% confluence, they were treated with the essential oils for 5h. The cells were then treated with trypsin and incubated in the 37^oC environment for 5 minutes as described previously. Following this incubation, cells were harvested by scraping from the flask. The cells were collected in a 15ml

Falcon tube and subjected to total RNA isolation which is described in the previous section. Control experiments were carried out using the same conditions, only DI water was added in place of the essential oil mixture.

Name	Primer sequence	Product
β- actin 1	5’-AGCGAGCATCCCCCAAAGTT-3’	285bp
β- actin 2	5’-GGGCACGAAGGCTCATCATT-3	285bp
CHAT-1	5’-GTCTACGCCTGTGGAGCCGATAC-3’	255bp
CHAT-2	5’-GGAACCAAGCTTAGTGGCTGGCAGC-3’	255bp
HNMT-1	5’-GGACAAGAAGCTGCCAGGC-3’	219bp
HNMT-2	5’-CTCGAGCTTCGATGTCTTGGC-3’	219bp
HAT1-1	5’-CAGTTCTCAGTCCAACAGGAGGAG-3’	215bp
HAT1-2	5’-CGGTCGCAAAGAGCGTAGCTCCA-3’	215bp
EPHX1-1	5’-GGCTTCTCAGAGGCATCCTCC-3’	273bp
EPHX1-2	5’-CCACATCCCTCTCAGTGAGGCC-3’	273bp
GAPDH1	5’-AGAAGGCTGGGGCTCATTTG-3’	258bp
GAPDH2	5’-AGGGGCCATCCACAGTCTTC-3’	258bp
HO-1	5’-GCTTCACATAGCGCTGCA-3’	270bp
HO-2	5’-CAGGCAGAGAATGCTGAGTTC-3’	270bp

Table1. The primer sequences and base pairs.

2.2.4 Time dependent induction of phase II genes

Experiments were also carried out in an attempt to establish the kinetics of induction of these genes. To do this, HepG2 cells were treated with lemongrass oil at the dose of 20ug/ml, as indicated in the previous section. After treatment, cells were grown in 25 cm^2 tissue culture flasks at 37^oC for additional 0hr, 2hrs, 6hrs and 24hrs. The cells were then trypsinized and incubated in the 37^oC environment for 5 minutes. Following this incubation, cells were harvested by scraping from the flask. The cells were collected in a 15ml Falcon tube and subjected to total RNA isolation as described in the previous sections. Samples were analyzed by RT-PCR in an attempt to determine kinetics of induction. Control experiments were carried out using the same conditions, only DI water was added in place of the essential oil mixture.

2.2.5 Effect of essential oil of Lemongrass on CYP3A4 activity

As with the 2E1 study, the effect of Lemongrass on CYP3A4 activity was determined. A specific assay for monitoring CYP3A4 has been developed utilizing the specific ability of this enzyme to oxidize nifedipine. Initially an assay for the oxidation of the nifedipine by CYP3A4 had to be developed. Using five different types of microsomes; human liver microsomes, rabbit liver microsomes 1, rabbit liver microsomes 2, rat liver microsomes, and supersomes enriched in CYP3A4,

experiments were carried out in the absence and presence of NADPH. The experimental procedure used for the evaluation of CYP3A4 inhibition was as follows: Along with 20ul of the five different kinds of microsomes, 50ul of 0.1M potassium phosphate buffer solution (pH 7.4), 40ul of 1mM Nifedipine and deionized water were initially combined in a final volume of 0.5ml. 25ul of 20mM NADPH was added to initiate the reaction in a 37^{o}C water bath for 10 minutes, then the reaction was quenched with the addition of 1ml of dichloromethane and 100ul of 1M Na_2CO_3 buffer (pH 10.5) containing 2M NaCl. Samples were extracted by liquid-liquid extraction then centrifuged at 4000 rpm for 10 minutes and 1ml of organic layer was transferred to centrifuge tube, reduced to dryness at 23^{o}C under vacuum and 500ul of 45% methanol was added to the centrifuge tube, transferred to HPLC vials, and analyzed by HPLC(SHIMADZU) for product formation. The product of Nifedipine oxidation, oxidized Nifedipine, was monitored using a Shimadzu LC 20A Series HPLC system consisting of an SPD-20A UV/Vis detector, LC 20AT solvent delivery, and a Sil 20A autosampler, all controlled using the Shimadzu EZStart version 7.3 SP1 software. Absorbance detection was set to 254 nm with a mobile phase consisting of 60% methanol, 40% H_2O, 0.5% acetic acid at a flow rate of 0.6ml/min. The volume of injection was 40 ul for each sample and the

column was a RP-C18 HPLC column 100cm x 3.0mm. All assays were carried out in duplicate. After the trials with five different microsomes, rat liver microsomes were chosen to perform the oxidation of the nifedipine by CYP3A4 assay due to the greater activity of these microsomes in the oxidation of nifedipine. First, the screen experiments of both Lemongrass oil and the aldehyde citral were carried out to determine the potency of inhibition of CYP3A4 with 20ul of rat microsomes, 50ul of 1.0M potassium phosphate buffer solution (pH 7.4), 40ul of 1mM Nifedipine , and deionized water were initially combined in a final volume of 0.5ml. 25ul of 20mM NADPH was added to initiate the reaction in a 37^{o}C. After establishing the potency of inhibition of CYP3A4, Michaelis-Menten plots were used for the evaluation of CYP3A4 inhibition by Lemongrass oil and the aldehyde citral. 5ul of the Lemongrass oil or the aldehyde citral was diluted to 100ml in deionized water and 5ul of this solution, along with 20ul of rat microsomes, 50ul of 1.0M potassium phosphate buffer solution (pH 7.4), 0.2mM nifedipine and deionized water were combined in a final volume of 0.5ml. 25ul of 20mM NADPH was added to initiate the reaction in a 37^{o}C water bath for 10 minutes, then the reaction was quenched with the addition of 1ml of dichloromethane and 100ul of 1M Na_2CO_3 buffer (pH 10.5) containing 2M NaCl. Samples were extracted again by liquid-liquid extraction

then centrifuged at 3000 rpm for 10 minutes and 1ml of organic layer was transferred to centrifuge tube, reduced to dryness at 23^{o}C under vacuum. 500ul of 45% methanol was added to the centrifuge tube, transferred to HPLC vials, and analyzed by HPLC(SHIMADZU) for product formation. The product of Nifedipine oxidation, oxidized Nifedipine, was monitored using the Shimadzu HPLC as described previously.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Inhibition of CYP2E1 by Lemongrass oil and other essential oils

3.1.1 Introduction

The inhibition of CYP2E1 by natural compounds may have significant implications in the field of pharmacology or toxicology, given the role of this isoform in production of ROS or reactive drug metabolites. CYP2E1 metabolizes and activates many toxicological substrates to more toxic products and the induction of CYP2E1 by ethanol is thought to result in increased oxidative stress in hepatocytes. One proposed mechanism for this increased oxidative stress is the increased production of hydrogen peroxide by CYP2E1 via uncoupling of its NADPH oxidation activity. The main hypothesis of this thesis is that the main aldehyde constituent found in Lemongrass, Citral, will be able to block the activity of CYP2E1, and demonstrate physiological antioxidant properties. For this reason we initiated studies to probe the interaction between a variety of oils and pure aldehydes with CYP2E1, specifically to determine whether these oils or aldehydes possessed inhibitory properties toward this enzyme.

Furthermore the mechanism of inhibition was also addressed.

3.1.2 Michaelis-Menten kinetic analysis

3.1.2.A. Inhibition of CYP2E1 by Lemongrass oil and the aldehyde citral

Figure 4 and figure 5 show the results of inhibition studies carried out by using a single dose of Lemongrass oil of 5ug/ml. The final concentration of p-nitrophenol in the reaction was (10-100uM) and that of NADPH was 1.0mM. The Michaelis-Menten plot for Lemongrass (shown in Fig.4) shows significant enzyme inhibition across the entire range of substrate concentrations used. Because of the selectivity of CYP2E1 in the human liver microsomes for p-nitrophenol, the inhibition effect observed must be due to reduction in activity of this isoform. The observed value for Vmax of 59 and K_M of 87 in the presence of inhibitor compared to the control Vmax of 76 and K_M of 16 suggest a competitive type inhibition. The K_I value was evaluated using the equation $K_{M'}=K_M(1+[I]/K_I)$ and was found to be 1.1ug/ml. The lineweaver-Burk plot (Fig.5) although the curves do not cross precisely at the Y axis is consistent with the competitive model of inhibition.

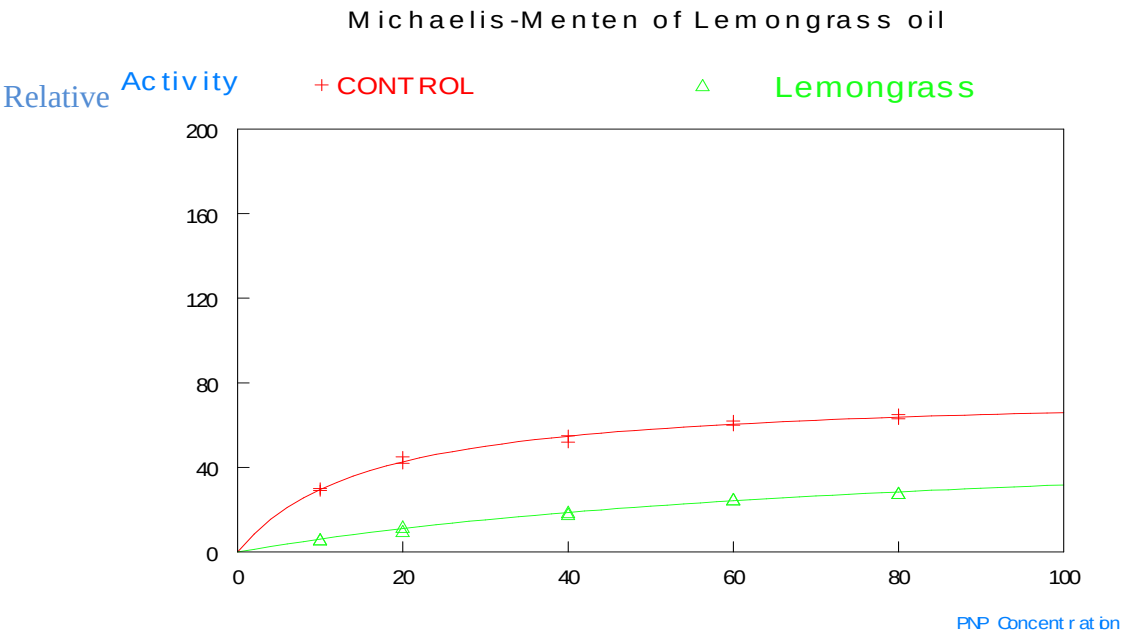


Figure 4. Michaelis-Menten plot of 50mM p-nitrophenol oxidation by CYP2E1 and inhibition by 5ug/ml Lemongrass oil in 20ul human liver microsomes. Each data point is the average of two samples.

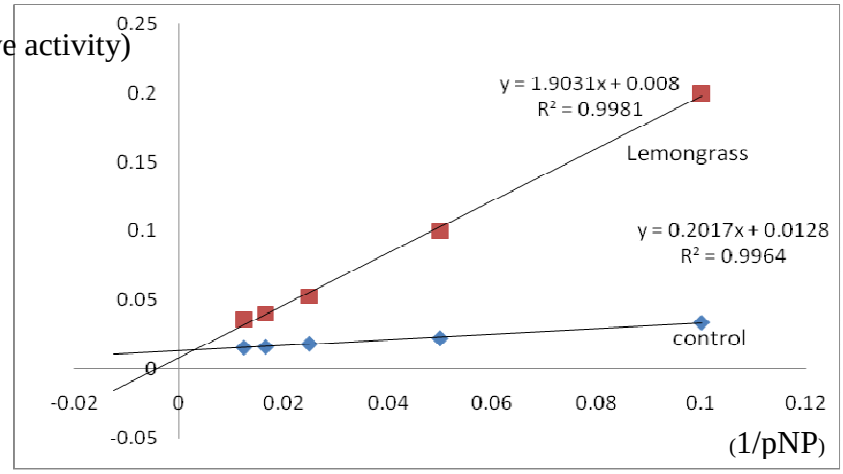


Figure 5. The Lineweaver-Burk plot of p-nitrophenol oxidation by CYP2E1 and inhibition by 5ug/ml Lemongrass oil in 20ul human liver microsomes, the x-axis= 1/(S) and the y-axis=1/(V), each data point is the average of two samples.

Figure 6 and figure 7 show the results of inhibition studies carried out using a single dose of the aldehyde citral of 0.033mM. The final concentration of paranitrophenol in the reaction was (10-100uM) and that of NADPH was 1.0mM. The Michaelis-Menten plot for the aldehyde citral (shown in Fig.6) shows significant enzyme inhibition across the entire range of substrate concentrations used. The observed value for Vmax of 58 and K_M of 98 in the presence of inhibitor compared to the control Vmax of 87 and K_M of 21 suggest a competitive type inhibition. The K_I value was evaluated using the equation $K_{M'}=K_M(1+[I]/K_I)$ and was found to be 1.4ug/ml which correspond to K_I value 9.0uM. The lineweaver-Burk plot(Fig.7) although the curves do not cross precisely at the Y axis is consistent with the competitive model of inhibition.

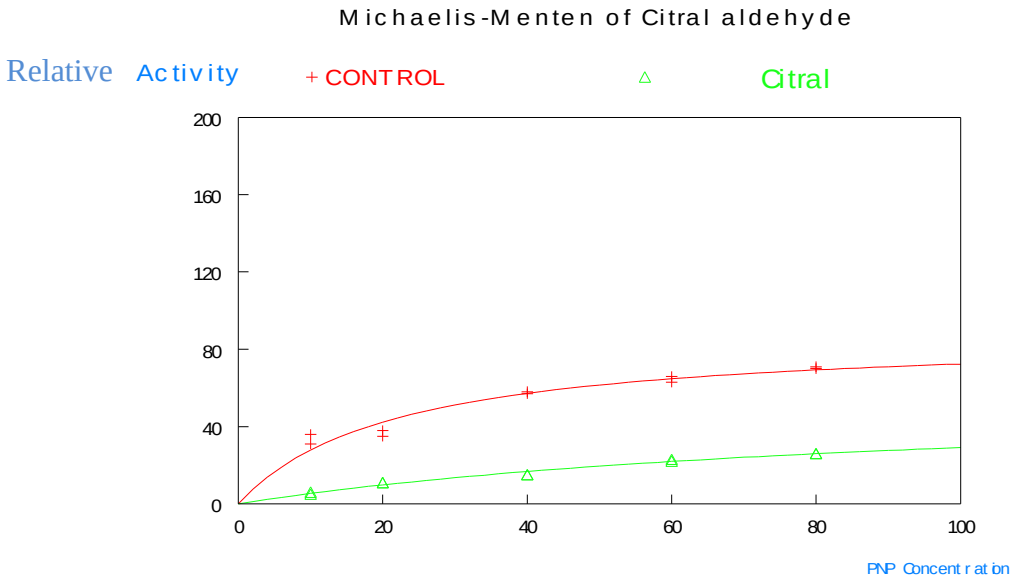


Figure 6. Michaelis-Menten plot of 50mM p-nitrophenol oxidation by CYP2E1 and inhibition by 0.033mM the aldehyde citral in 20ul human liver microsomes. Each data point is the average of two samples.

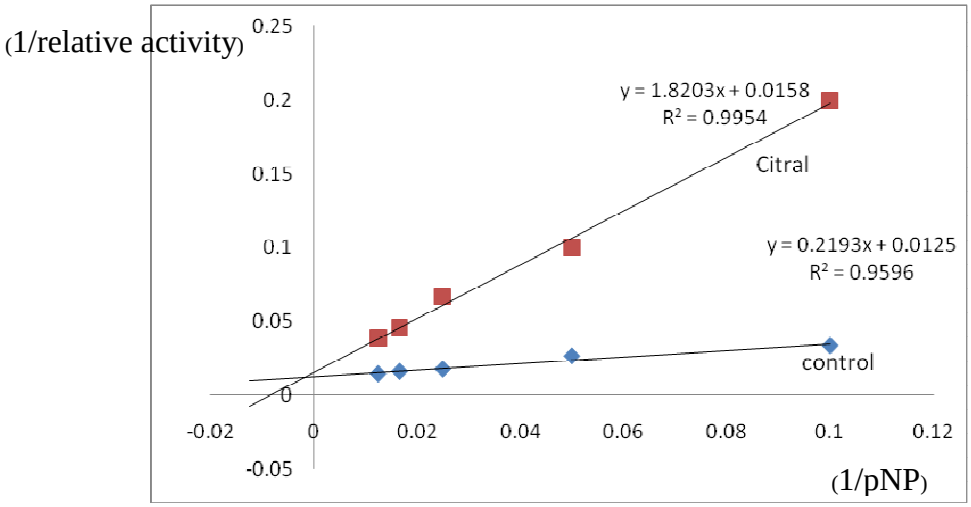


Figure 7. The Lineweaver-Burk plot of p-nitrophenol oxidation by CYP2E1 and inhibition by the 0.033mM aldehyde citral in 20ul human liver microsomes, the x-axis= 1/(S) and the y-axis=1/(V), each data point is the average of two samples.

3.1.2.B. Inhibition of CYP2E1 by Eucalyptus Lemon oil, Citronella oil, and the aldehyde citronellal

Figure 8 shows the results of inhibition studies carried out using a single dose of the Eucalyptus Lemon oil of 5ug/ml. The final concentration of p-nitrophenol in the reaction was (10-100uM) and that of NADPH was 1.0mM. The Michaelis-Menten curve shows a little enzyme inhibition to CYP2E1. The observed value for Vmax of 134 and K_M of 47 in the presence of inhibitor compared to the control Vmax of 130 and K_M of 32 suggest a competitive type inhibition. The K_I value was evaluated using the equation $K_{M'}=K_M(1+[I]/K_I)$ and was found to be 3.4ug/ml.

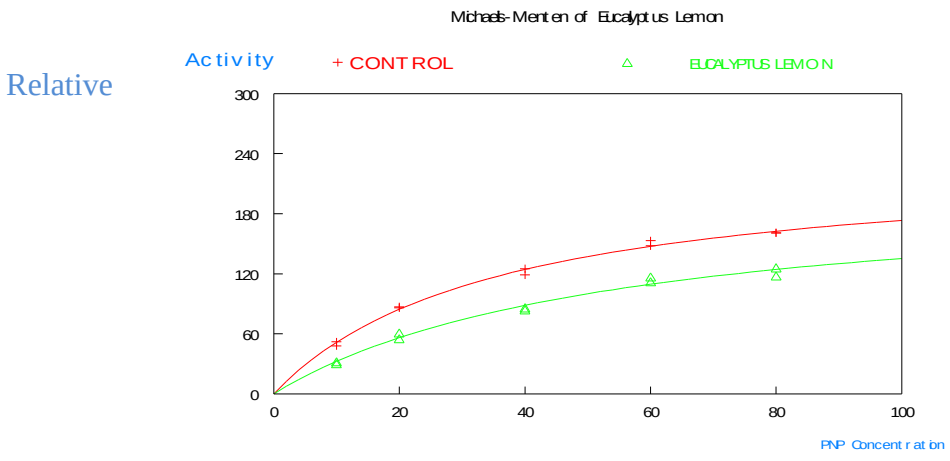


Figure 8. Michaelis-Menten plot of 50mM p-nitrophenol oxidation by CYP2E1 and inhibition by 5ug/ml Eucalyptus Lemon oil in 20ul human liver microsomes. Each data point is the average of two samples.

Figure 9 shows the results of inhibition studies carried out using a single dose of the Citronella oil of 5ug/ml. The final concentration of p-nitrophenol in the reaction was (10-100uM) and that of NADPH was 1.0mM. The Michaelis-Menten curve shows slight enzyme inhibition for the citronella oil. The observed value for Vmax of 149 and K_M of 55 in the presence of inhibitor compared to the control Vmax of 149 and K_M of 29 suggest a competitive type inhibition. The K_I value was evaluated using the equation $K_{M'}=K_M(1+[I]/K_I)$ and was found to be 5.6ug/L.

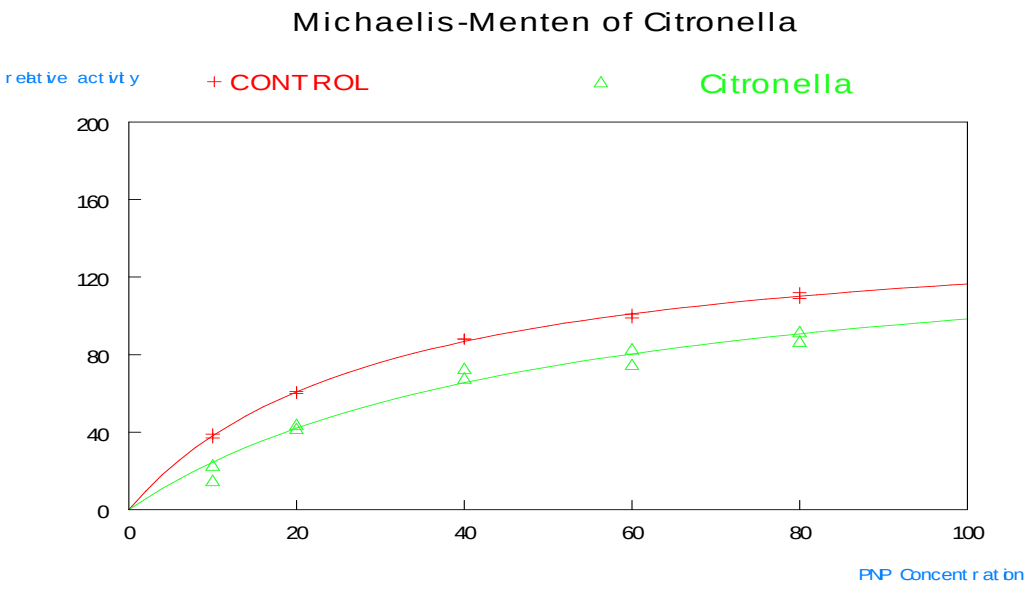


Figure 9. Michaelis-Menten plot of 50mM p-nitrophenol oxidation by CYP2E1 and inhibition by 5ug/ml Citronella oil in the 20ul human liver microsomes. Each data point is the average of two samples.

Figure 10 shows the results of inhibition studies carried out using a single dose of the Citronellal of 5ug/ml. The final concentration of p-nitrophenol in the reaction was (10-100uM) and that of NADPH was 1.0mM. The Michaelis-Menten plot for Citronellal (shown in Fig.10) shows slightly enzyme inhibition across the entire range of substrate concentrations used. The observed value for Vmax of 136 and K_M of 86 in the presence of inhibitor compared to the control Vmax of 121 and K_M of 35 suggest a competitive type inhibition. The K_I value was evaluated using the equation $K_{M'}=K_M(1+[I]/K_I)$ and was found to be 3.4ug/ml.

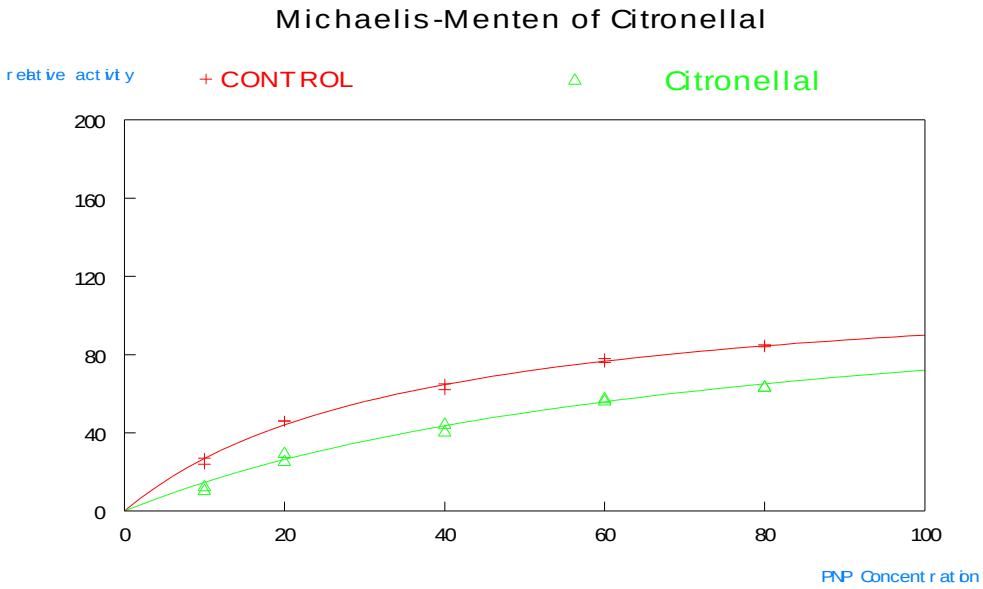


Figure 10. Michaelis-Menten plot of 50mM p-nitrophenol oxidation by CYP2E1 and inhibition by 5ug/ml Citronellal in 20ul human liver microsomes. Each data point is the average of two samples.

3.1.2.C. Inhibition of CYP2E1 by Eucalyptus Globulous oil and it's main constituent 1,8 Cineole

Figure 11 shows the results of inhibition studies carried out using a single dose of the Eucalyptus Globulous oil of 5ug/ml. The final concentration of p-nitrophenol in the reaction was (10-100uM) and that of NADPH was 1.0mM. The Michaelis-Menten plot for Eucalyptus Globulous oil (shown in Fig.11) shows very little enzyme inhibition across the entire range of substrate concentrations used. In fact the observed value for Vmax of 150 and K_M of 40 in the presence of inhibitor compared to the control Vmax of 162 and K_M of 39 suggest Eucalyptus globulous, at this concentration does not significantly affect the activity of CYP2E1. This is in contrast to earlier studies performed in this lab during a random screening of oils. It is possible that the Eucalyptus globulous oil contains unstable compounds that inhibit CYP2E1. Upon storage these compounds degrade.

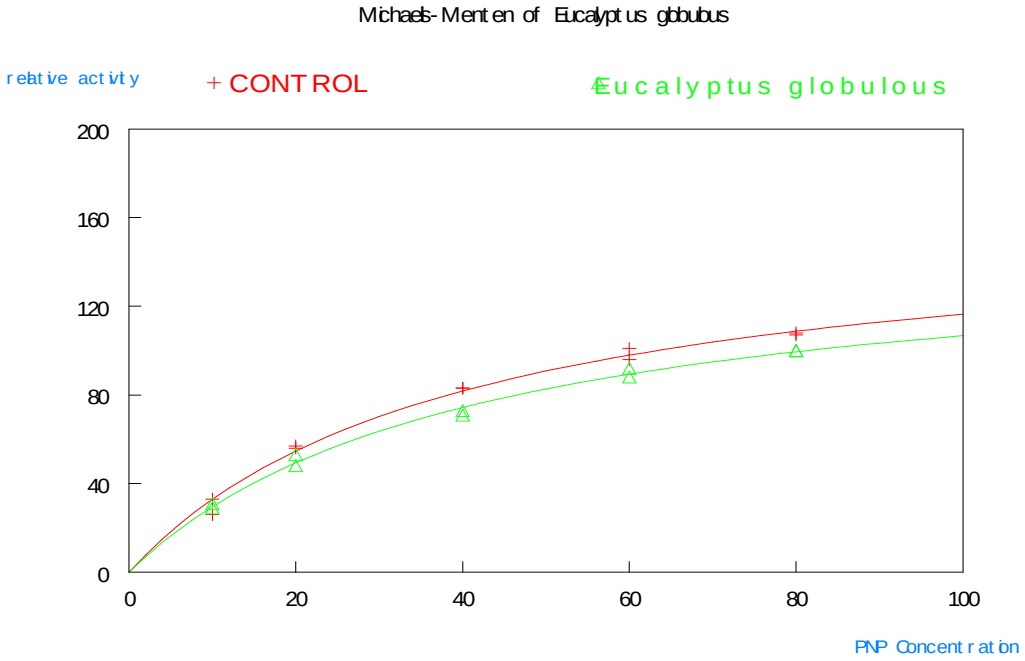


Figure 11. Michaelis-Menten plot of 50mM p-nitrophenol oxidation by CYP2E1 and inhibition by 5ug/ml Eucalyptus Globulous oil in 20ul human liver microsomes. Each data point is the average of two samples.

Figure 12 shows the results of inhibition studies carried out using a single dose of the 1,8 Cineole of 5ug/ml. The final concentration of p-nitrophenol in the reaction was (10-100uM) and that of NADPH was 1.0mM. The Michaelis-Menten plot for 1,8 Cineole (shown in Fig.12) shows no enzyme inhibition across the entire range of substrate concentrations used. The observed value for Vmax of 99 and K_M of 30 in the presence of inhibitor compared to the control Vmax of 104 and K_M of 31.

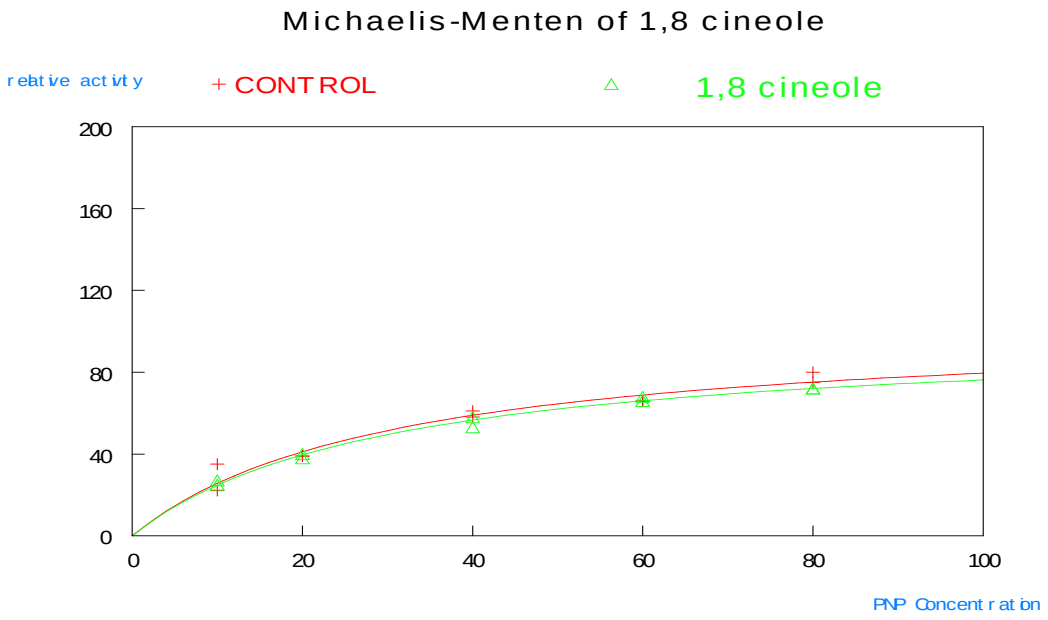


Figure 12. Michaelis-Menten plot of 50mM p-nitrophenol oxidation by CYP2E1 and inhibition by 5ug/ml 1,8 Cineole in 20ul human liver microsomes. Each data point is the average of two samples.

3.1.2.D. **Inhibition of CYP2E1 by Cassia oil**

Figure 13 shows the results of inhibition studies carried out using a single dose of the Cassia oil of 5ug/ml. The final concentration of p-nitrophenol in the reaction was (10-100uM) and that of NADPH was 1.0mM. The Michaelis-Menten plot for Cassia oil (shown in Fig.13) shows a little enzyme inhibition across the entire range of substrate concentrations used. The observed value for Vmax of 146 and K_M of 46 in the presence of inhibitor compared to the control Vmax of 142 and K_M of 25 suggest a competitive type inhibition. The K_I value was evaluated using the equation $K_{M'}=K_M(1+[I]/K_I)$ and was found to be 6.0ug/ml.

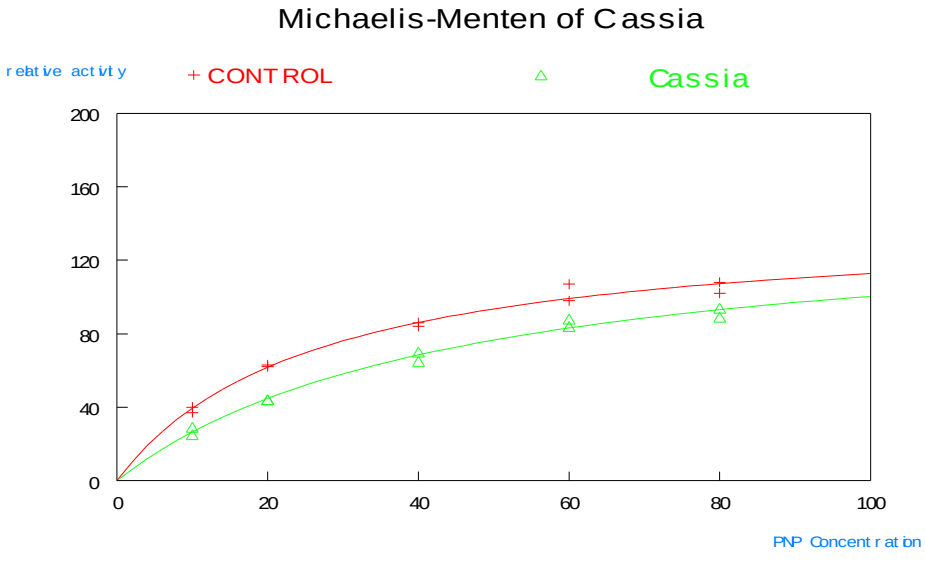


Figure 13. Michaelis-Menten plot of 50mM p-nitrophenol oxidation by CYP2E1 and inhibition by 5ug/ml Cassia oil in 20ul human liver microsomes. Each data point is the average of two samples.

3.1.2.E. Inhibition of CYP2E1 by the aldehyde decanal

Figure 14 and figure 15 shows the results of inhibition studies carried out using a single dose of Decanal of 5ug/ml. The final concentration of p-nitrophenol in the reaction was (10-100uM) and that of NADPH was 1.0mM. The Michaelis-Menten plot for Decanal (shown in Fig.14) shows that decanal is a very efficient inhibitor of CYP2E1 at this dose. The observed value for Vmax of 81 and K_M of 177 in the presence of inhibitor compared to the control Vmax of 113 and K_M of 40 suggest a competitive type inhibition. The K_I value was evaluated using the equation $K_{M'}=K_M(1+[I]/K_I)$ and was found to be 2.6ug/ml. The lineweaver-Burk plot (Fig.15) although the curves do not cross precisely at the Y axis is consistent with the competitive model of inhibition.

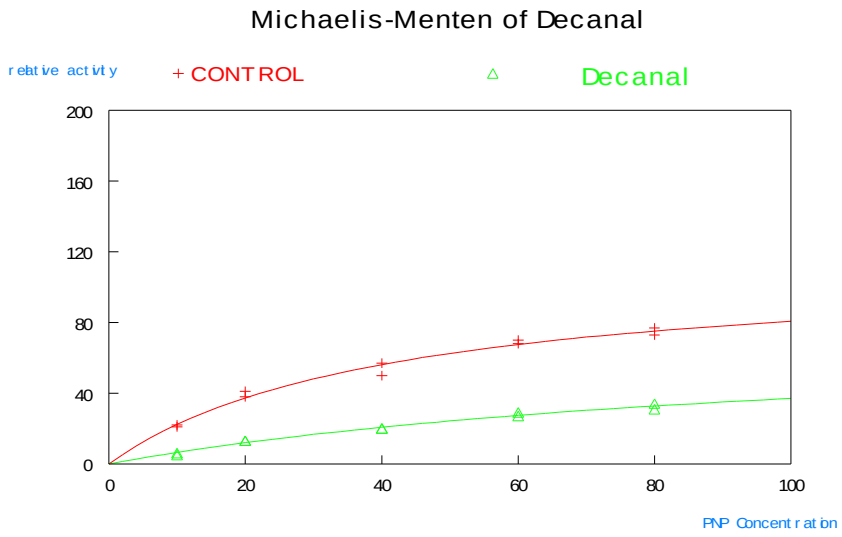


Figure 14. Michaelis-Menten plot of 50mM p-nitrophenol oxidation by CYP2E1 and inhibition by 5ug/ml Decanal in 20ul human liver microsomes. Each data point is the average of two samples.

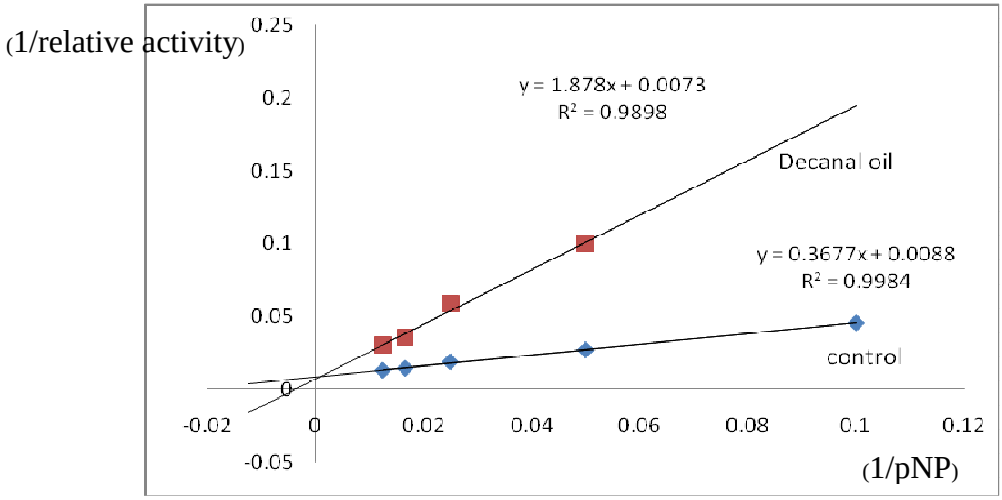


Figure 15. The Lineweaver-Burk plot of p-nitrophenol oxidation by CYP2E1 and inhibition by 5ug/ml Decanal in 20ul human liver microsomes, the x-axis= 1/(S) and the y-axis=1/(V), each data point is the average of two samples.

Essential oils and aldehydes	KI values ug/ml
The aldehyde Citral	1.4
Lemongrass oil	1.1
The aldehyde Citronellal	3.4
Citronella oil	5.6
Eucalyptus lemon oil	3.5
The aldehyde Decanal	2.6
1,8 Cineole	N/A
Eucalyptus globulous oil	N/A
Cassia oil	6.0

Table 2. KI values of essential oils and the aldehydes

3.2 Growth and treatment of HepG2 cells

This experiment was to show Lemongrass oil,which contains the aldehyde constituent Citral, can activate the antioxidant response elements found in a variety of antioxidant/phase II drug metabolizing genes. In order to demonstrate the induction of phase II genes, cultured human liver cells, HepG2 cells, were used to test the induction of genes of interest and these cells were treated with Lemongrass oil and the aldehyde citral. The RT-PCR and gel electrophoresis were used to monitor antioxidant genes expression.

3.2.1. The gel electrophoresis result

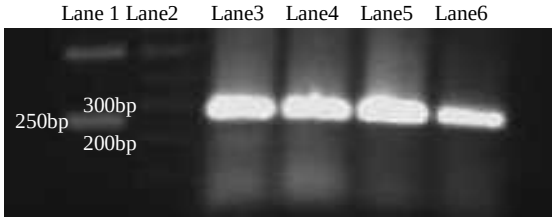


Figure 16. 1.5% agarose gel showing the resulting band from RT-PCR amplification of β-actin RNA produced in HepG2 cells with increasing doses of Lemongrass oil. Lanes 3-6 correspond to doses of 0ug/ml, 20ug/ml, 50ug/ml, and 200ug/ml Lemongrass, respectively.

Figure 16 shows the transcriptional level of β-actin-1 with increasing doses of the Lemongrass oil applied to the HepG2 cells. Based on visual inspection of the bands, the β-actin levels appear to be similar, although lane6 may be slightly less intense than the others, which may suggest a small change in expression of β-actin at the very highest concentration of oil. Lane1 is the DNA ladder, lane 2 is also DNA ladder, lane3 is the control without Lemongrass, lane4 is the sample of HepG2 cell treated with 0.02 ug/ml of Lemongrass oil, lane5 is the sample of HepG2 cell treated with 0.05 ug/ml of Lemongrass oil, lane 6 is the sample of HepG2 cell treated with 0.2 ug/ml of Lemongrass oil. β-actin in each sample was confirmed by the presence of a PCR product at just under 300bp in length. Several experimental conclusions had to be tested before consistent amplification of the β-actin gene was observed, more importantly the most effective annealing temperature for the PCR was determined to be 56 ^{o}C.

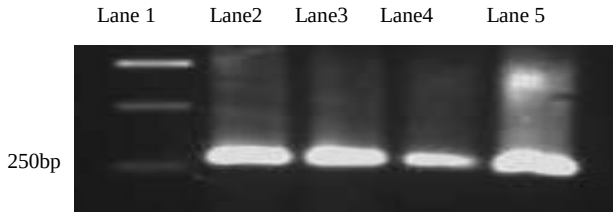


Figure 17. 1.5% agarose gel showing the resulting band from RT-PCR amplification of β-actin RNA produced in HepG2 cells at 20ug/ml Lemongrass oil time-dependent experiment. Lanes 2-5 correspond to the time of 0hr, 2hrs, 6hrs, and 24hrs respectively.

Figure 17 shows the expression of β-actin-1 for time dependent when HepG2 cells were treated with 20ug/ml Lemongrass oil. In this experiment lane1 is the DNA ladder, lane2 is the HepG2 cells treated with Lemongrass oil at 20ug/ml for 0hr incubation, lane3 is the HepG2 cells treated with Lemongrass oil at 20ug/ml for 1hr incubation, lane4 is the HepG2 cells treated with Lemongrass oil at 20ug/ml for 6hr incubation, lane5 is the induction of HepG2 cells treated with Lemongrass oil at 20ug/ml for 24hr incubation post treatment. It appeared that the intensity of 4 bands were uneven, however, lane 4 had lower intensity compared to the other lanes, which may be the result of artifact, perhaps loading error. And lane 5 had stronger intensity which could be the number of the cells were more than the other samples.

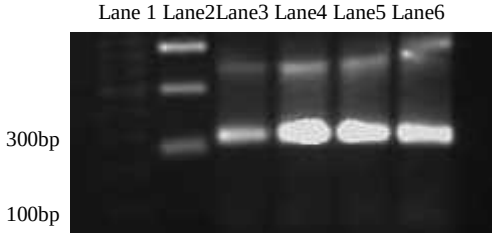


Figure 18. 1.5% agarose gel showing the resulting band from RT-PCR amplification of β-actin RNA produced in HepG2 cells with increasing doses of the aldehyde citral. Lanes 3-6 correspond to doses of 0ug/ml, 20ug/ml, 50ug/ml, and 200ug/ml citral, respectively.

Figure 18 showed the expression of β-actin-1 for dose dependent when treated the aldehyde citral to the HepG2 cells. Lane1 and lane2 are the DNA ladders, lane3 is the control without the aldehyde citral, lane4 is the HepG2 cells treated with 20 ug/ml of the aldehyde citral, lane5 is the HepG2 cells treated with 50 ug/ml of the aldehyde citral**,** lane6 is the HepG2 cells treated with 200 ug/ml of the aldehyde citral. There is a slight stronger intensity in both lane3 and lane 4 with increasing concentration. All the expression of β-actin-1 are much stronger than the control, it could be the artifact of gel loading or the cell numbers were lower in the control cells than the other cells, which make β-actin-1 expression slightly less intense.

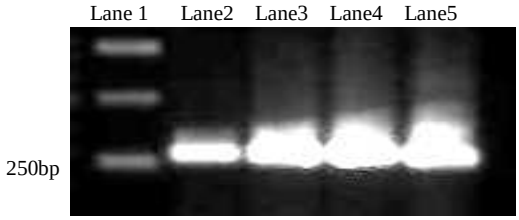


Figure 19. 1.5% agarose gel showing the resulting band from RT-PCR amplification of β-actin RNA produced in HepG2 cells at 20ug/ml Citral time-dependent experiment. Lanes 2-5 correspond to the time of 0hr, 2hrs, 6hrs, and 24hrs respectively.

Figure 19 shows the expression of β-actin-1 for time dependent when HepG2 cells were treated with 20ug/ml Citral. In this experiment lane1 is the DNA ladder, lane2 is the HepG2 cells treated with Citral at 20ug/ml for 0hr incubation, lane3 is the HepG2 cells treated with Citral at 20ug/ml for 2hrs incubation, lane4 is the HepG2 cells treated with Citral at 20ug/ml for 6hrs incubation, lane5 is the induction of HepG2 cells treated with Citral at 20ug/ml for 24hrs incubation post treatment. There is a increasing trend of β-actin expression with increasing time of citral treated which may be the numbers of cells were different and made the expression of β-actin uneven.

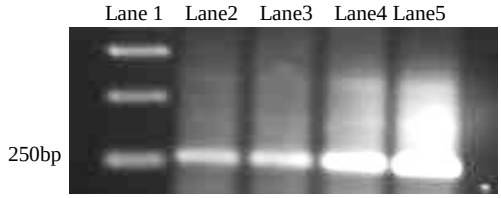


Figure 20. 1.5% agarose gel showing the resulting band from RT-PCR amplification of HO-1 RNA produced in HepG2 cells with increasing doses of Lemongrass oil. Lanes 2-5 correspond to doses of 0ug/ml, 20ug/ml, 50ug/ml, and 200ug/ml Lemongrass, respectively.

Figure 20 shows the transcriptional level of HO-1 with doses increasing of the Lemongrass oil applied to the HepG2 cells. Based on visual inspection of the bands, the HO-1 expression levels appear to be increased with increasing concentration of lemongrass. Lane1 is the DNA ladder, lane2 is the control without Lemongrass, lane3 is the sample of HepG2 cell treated with 20 ug/ml of Lemongrass oil, lane4 is the sample of HepG2 cell treated with 50 ug/ml of Lemongrass oil, lane 5 is the sample of HepG2 cell treated with 200 ug/ml of Lemongrass oil. HO-1 in each sample was confirmed by the presence of a PCR product at just 270bp in length. The most effective annealing temperature for PCR was determined to be 55 ^{o}C. When compared with β-actin expression in the dose dependent experiment, HO-1 expression appeared

to increase with increasing dose of lemongrass. This may also correspond to increasing antioxidant properties induced by lemongrass oil.

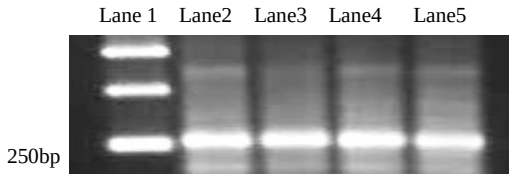


Figure 21. 1.5% agarose gel showing the resulting band from RT-PCR amplification of HO-1 RNA produced in HepG2 cells at 20ug/ml Lemongrass oil time-dependent experiment. Lanes 2-5 correspond to the time of 0hr, 2hrs, 6hrs, and 24hrs respectively.

Figure 21 shows the expression of HO-1 for time dependent when HepG2 cells were treated with 20ug/ml Lemongrass oil. In this experiment lane1 is the DNA ladder, lane2 is the HepG2 cells treated with Lemongrass oil at 20ug/ml for 0hr incubation, lane3 is the HepG2 cells treated with Lemongrass oil at 20ug/ml for 2hr incubation, lane4 is the HepG2 cells treated with Lemongrass oil at 20ug/ml for 6hr incubation, lane5 is the induction of HepG2 cells treated with Lemongrass oil at 20ug/ml for 24hr incubation post treatment. The bands here appeared to be constant brightness with increasing time of HepG2 cells treated with Lemongrass oil. The result indicates that lemongrass did not have an inductive effect at this dose over a 24 hr period. Because the HO-1 expression in this figure remain constant along with increasing times of lemongrass treating. In looking at the previous figure, it is not totally unexpected, as the dose of 20ug/ml appears to be below the effective dose.

Therefore, to examine the time dependence of this induction, future studies should be carried out at 100ug/ml or higher.

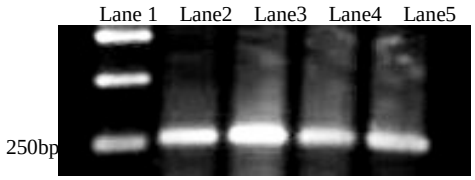


Figure 22. 1.5% agarose gel showing the resulting band from RT-PCR amplification of HO-1 RNA produced in HepG2 cells with increasing doses of the aldehyde citral. Lanes 2-5 correspond to doses of 0ug/ml, 20ug/ml, 50ug/ml, and 200ug/ml citral, respectively.

Figure 22 shows the expression of HO-1 with doses increasing of the aldehyde citral applied to the HepG2 cells. Lane1 is the DNA ladder, lane2 is the control without citral, lane3 is the sample of HepG2 cell treated with 20 ug/ml of citral, lane4 is the sample of HepG2 cell treated with 50 ug/ml of citral, lane 5 is the sample of HepG2 cell treated with 200 ug/ml of citral. HO-1 in each sample was confirmed by the presence of a PCR product at just 270bp in length. In lane 3, it shows a strongest HO-1 expression in 20ug/ml of citral compared to the higher dose in the lane 4 and lane 5. It may suggest that 20ug/ml is the most effective dose for the induction of antioxidant property. Citral is, in fact, known to kill cancer cells at elevated doses.

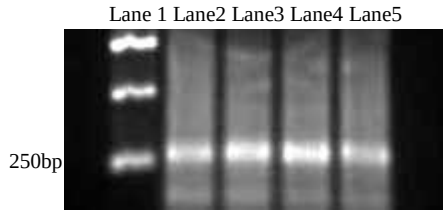


Figure 23. 1.5% agarose gel showing the resulting band from RT-PCR amplification of HO-1 RNA produced in HepG2 cells at 20ug/ml the aldehyde citral time-dependent experiment. Lanes 2-5 correspond to the time of 0hr, 2hrs, 6hrs, and 24hrs respectively.

Figure 23 shows the expression of HO-1 for time dependent when HepG2 cells were treated with 20ug/ml the aldehyde citral. In this experiment lane1 is the DNA ladder, lane2 is the HepG2 cells treated with the aldehyde citral at 20ug/ml for 0hr incubation, lane3 is the HepG2 cells treated with the aldehyde citral at 20ug/ml for 2hr incubation, lane4 is the HepG2 cells treated with the aldehyde citral at 20ug/ml for 6hr incubation, lane5 is the induction of HepG2 cells treated with the aldehyde citral at 20ug/ml for 24hr incubation post treatment. Both lane 3 and lane 4 have the same intensity of HO-1 expression compare to lane 1 and lane 4, it shows that with 2 and 6 hours citral treating, there is a stronger antioxidant property. Moreover the effect of antioxidant may be lesser when the cell treated with citral for 24 hours, probably due to metabolism of the citral. Liver enzymes, including cytochrome

P450's, are known to degrade this compound in a matter of hours.

3.3 Inhibition of CYP3A4 by nifedipine oxidation

The assay for monitoring CYP3A4 were developed utilizing the specific ability of this enzyme to oxidize nifedipine and rat microsomes were used in the assay. Experiments were carried out in the absence and presence of NADPH for identifying the peak of oxidized nifedipine on the chromatogram. The experimental procedure used for the evaluation of CYP3A4 inhibition was as follows: Along with 20ul of rat microsomes, 50ul of 1M potassium phosphate buffer solution (pH 7.4), 40ul of 1mM Nifedipine and deionized water were initially combined in a final volume of 0.5ml. 25ul of 1mM NADPH was added to initiate the reaction in a 37^{o}C water bath for 10 minutes, then the reaction was quenched with the addition of 1ml of dichloromethane and 100ul of 1M Na_2CO_3 buffer (pH 10.5) containing 2M NaCl. Samples were extracted by liquid-liquid extraction then centrifuged at 3000 rpm for 10 minutes and 1ml of organic layer was transferred to centrifuge tube, reduced to dryness at 23^{o}C under vacuum, added 500ul of 45% methanol to centrifuge tube, transferred to HPLC vials, and analyzed by HPLC(SHIMADZU) for product formation. The product of Nifedipine oxidation, oxidized Nifedipine, was monitored using a Shimadzu LC 20A Series HPLC system consisting of an SPD-20A UV/Vis detector, LC 20AT solvent

delivery, and a Sil 20A autosampler, all controlled using the Shimadzu EZStart version 7.3 SP1 software. Absorbance detection was set to 254 nm with a mobile phase consisting of 60% methanol, 40% H_2O, 0.5% acetic acid at a flow rate of 0.6ml/min. The volume of injection was 40 ul for each sample and the column was a RP-C18 HPLC column. All assays were carried out in duplicate.

3.3.1. The retention time of Nifedipine and Oxidized nifedipine was identified

Figure 24 shows the peaks of standard oxidized nifedipine and standard nifedipine on the HPLC with UV detection. The peak at 4.0 minute was identified as the retention time of standard oxidized nifedipine using an authentic standard purchased for Oxford Biomedical and the peak at 6.2 minute was identified as the retention time of nifedipine.

Relative activity

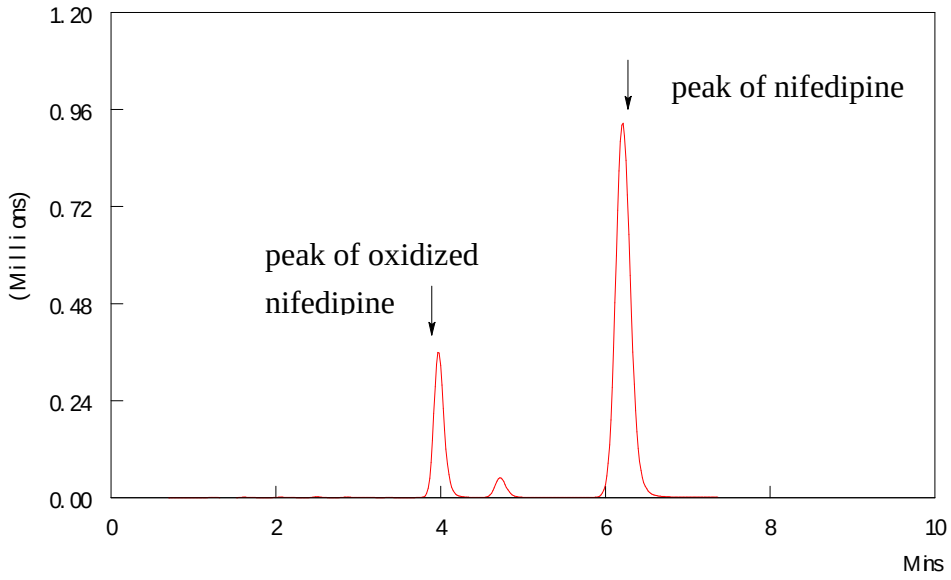


Figure24. HPLC-UV chromatogram of standard oxidized nifedipine(4.0minute) and nifedipine(6.2minute). Absorbance detection was set to 254 nm with a mobile phase consisting of 60% methanol, 40% H_2O, 0.5% acetic acid at a flow rate of 0.6ml/min.

Figure 25 shows the HPLC chromatogram of 40ul of 1mM of nifedipine combined with 20ul of rat microsomes, 50ul of 1.0M potassium phosphate buffer solution (pH 7.4), and deionized water in a final volume of 0.5ml. The reaction was incubated at 37^{o}C water bath for 10 minutes, then the reaction was quenched with the addition of 1ml of dichloromethane and 100ul of 1M Na_2CO_3 buffer (pH 10.5) containing 2M NaCl. Samples were extracted by liquid-liquid extraction then centrifuged at 3000 rpm for 10 minutes and 1ml of organic layer was transferred to centrifuge tube, reduced to dryness at 23^{o}C under vacuum, 500ul of 45% methanol was added to centrifuge tube, the contents were transferred to HPLC vials, and analyzed by HPLC(SHIMADZU) for product formation. In figure 25, the peak of nifedipine matched with figure 24 in the same retention time and the peak of oxidized nifedipine was undetectable because of no NADPH in the reaction.

Relative activity

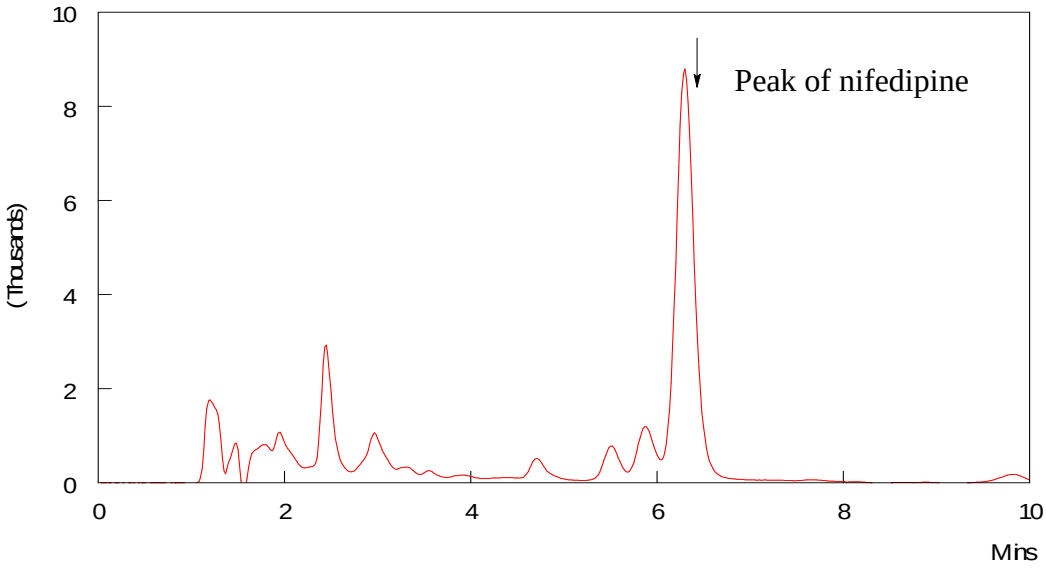


Figure25. HPLC-UV chromatogram of nifedipine(6.2minute) when the reaction incubated without the addition of NADPH. Absorbance detection was set to 254 nm with a mobile phase consisting of 60% methanol, 40% H_2O, 0.5% acetic acid at a flow rate of 0.6ml/min.

Figure 26 shows the HPLC chromatogram of 40ul of 1mM of nifedipine combined with 20ul of rat microsomes, 50ul of 1M potassium phosphate buffer solution (pH 7.4), 0.2mM nifedipine, and deionized water in a final volume of 0.5ml. The reaction was incubated at 37^{o}C water bath for 10 minutes, then the reaction was quenched with the addition of 1ml of dichloromethane and 100ul of 1M Na_2CO_3 buffer (pH 10.5) containing 2M NaCl. Samples were extracted by liquid-liquid extraction then centrifuged at 3000 rpm for 10 minutes and 1ml of organic layer was transferred to centrifuge tube, reduced to dryness at 23^{o}C under vacuum, added 500ul of 45% methanol to centrifuge tube, transferred to HPLC vials, and analyzed by HPLC(SHIMADZU) for product formation. In the figure 26, the peak of oxidized nifedipine and nifedipine were match with the figure 24 at the same retention time. In the reaction, nifedipine was oxidized by CYP3A4 and formed the product of oxidized nifedipine when the reaction incubated with the addition of NADPH.

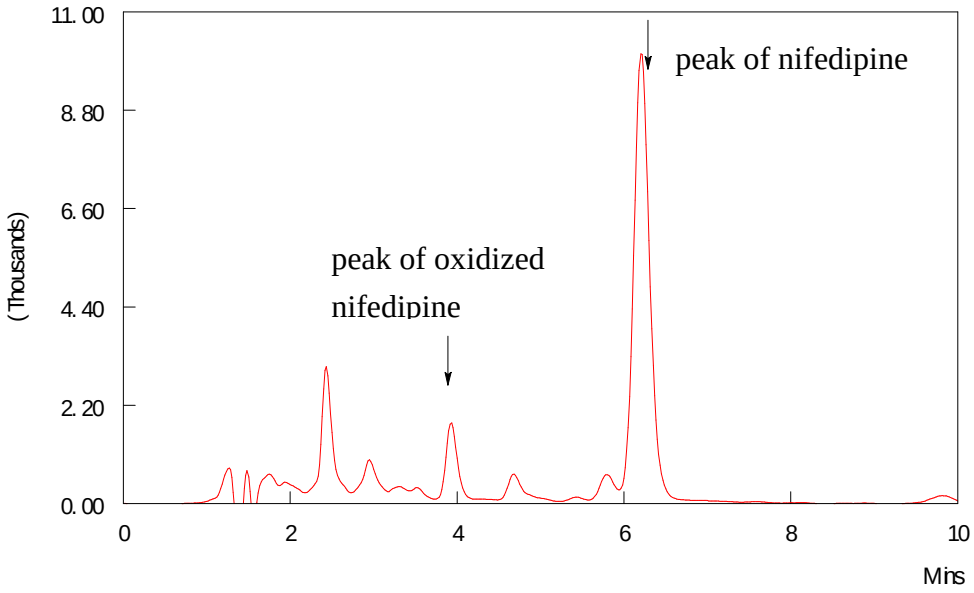


Figure26. HPLC-UV chromatogram of oxidized nifedipine when the reaction incubated with the addition of NADPH(4.0minute) and nifedipine(6.2minute). Absorbance detection was set to 254 nm with a mobile phase consisting of 60% methanol, 40% H_2O, 0.5% acetic acid at a flow rate of 0.6ml/min.

3.3.2. The screen experiments of nifedipine oxidation by CYP3A4 with and without Lemongrass oil and the aldehyde citral

Figure 27 shows the percentage of inhibition with Lemongrass added in the reaction of oxidation of nifedipine. Over 50% of inhibition of activity was observed by adding only 5ul of the diluted Lemongrass oil. 5ul of Lemongrass oil was diluted into 100ml deionized water. The final concentration of Lemongrass oil was 0.5ug/ml, 5ug/ml, and 35ug/ml. From the figure it is apparent that at relatively low doses, the lemongrass oil had a significant inhibitory effect on the activity of CYP3A4. For example, a 5ug/ml dose reduced the activity by more than 50%. This corresponds to a solution that is the equivalent of 5mg(~5ul) diluted to 10L with water.

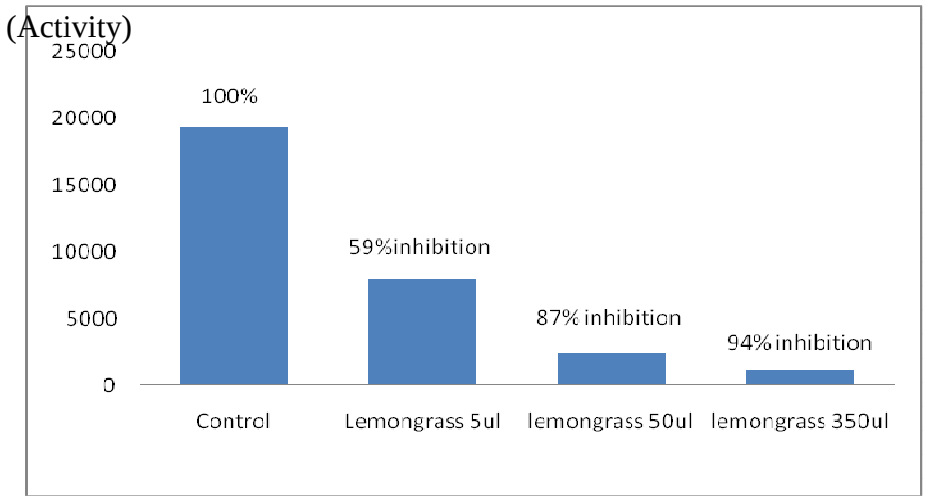


Figure 27. The screen experiment of Lemongrass oil with 0ul, 5ul, 50ul, and 350ul correspond to the oil concentration of 0ug/ml, 0.5ug/ml, 5ug/ml, and 35ug/ml respectively.

The potent inhibition of CYP3A4 by lemongrass oil, combined with the high content of citral in lemongrass led us to look at the ability of this naturally occurring aldehyde with regard to its inhibition of CYP3A4 using similar doses of citral, ranging from 0ug/ml, 0.5ug/ml, 5ug/ml, and 35ug/ml, the activity of CYP3A4 was monitored as in the previous section. Figure 28 shows the percentage of inhibition with the aldehyde citral added in the reaction of oxidation of nifedipine. Over 50% of inhibition of activity was observed by adding only 5ul of the diluted citral. 5ul of citral was diluted into 100ml deionized water. The final concentration of citral was 0.5ug/ml, 5ug/ml, and 35ug/ml. As with lemongrass, the citral was a very potent inhibitor of CYP3A4, even more so than lemongrass. At the lowest dose used, 5ug/ml, only 30% of the activity of the enzyme remained. This dose corresponds to a concentration of 3.3uM. Based on this potent inhibition, the inhibition of CYP3A4 by this common food additive may have significant pharmacological consequences.

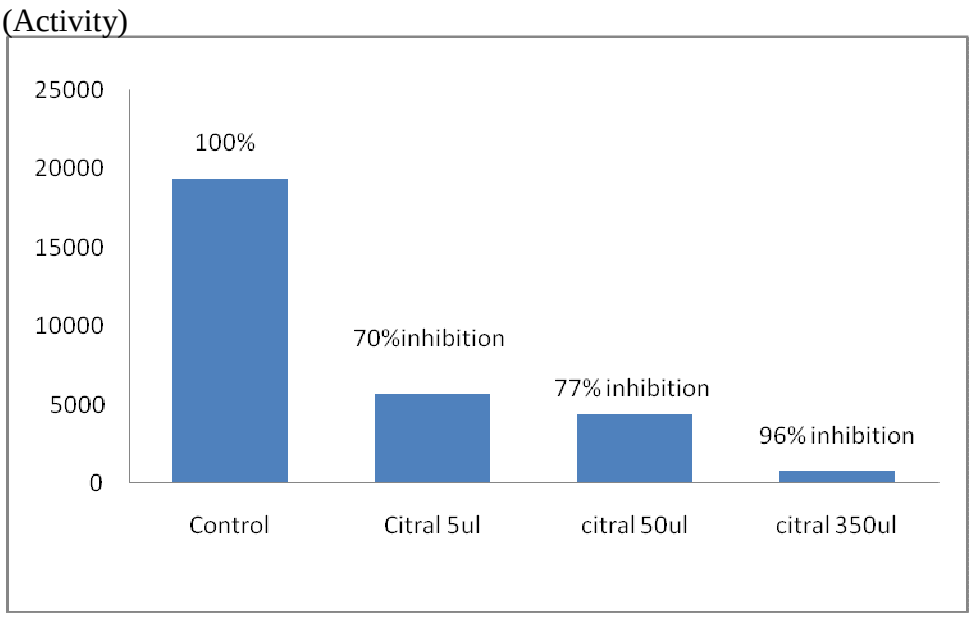


Figure28. The screen experiment of citral with 0ul, 5ul, 50ul, and 350ul correspond to the oil concentration of 0ug/ml, 0.5ug/ml, 5ug/ml, and 35ug/ml respectively.

CHAPTER IV

DISCUSSION

From the Michaelis-Menten plots for inhibition of CYP2E1, there was a correlation seen between the aldehyde content and the potency with regard to cytochrome $P450_{2E1}$ inhibition. Lemongrass, which contains the unsaturated aldehyde citral, showed a significant level of cytochrome CYP2E1 inhibition. The essential oil of lemongrass contains very high amounts of the aldehyde citral, which is the name for the pair of cis, trans isomers neral and geranial. Citral is an α,β-unsaturated aldehyde and has been shown to possess anti-inflammitory properties that may relate to its ability to control redox balance within the cell. The anti-inflammitory response results from suppression of iNOS expression and NF-kB activation. The relationship of both of these aldehydes to a reduction in oxidative stress and their ability to inhibit the human cytochrome $P450_{2E1}$ isoform suggests that the two effects may be related, given the involvement of this isoform in initiating lipid peroxidation.

According to my experimental data and gel electrophoresis experimental results, the HO-1 showed significant increased expression in human liver cells in response to

increasing concentrations of both Lemongrass oil and the aldehyde citral. Furthermore, the time-dependence experiment for both Lemongrass oil and the aldehyde citral also showed the levels of mRNA for these genes increased in a time dependent manner up to 8 hrs incubation. For the gene expression of HO-1, in the dose-dependence experiment, it showed an increasing trend with respective to the increasing concentration of Lemongrass oil and the aldehyde citral in the agarose gel electrophoresis from the control sample to the highest concentration one, which directly validated that the presence of Lemongrass oil induced phase II detoxyfying genes in the HepG2 cells with increasing doses. It also assertively demonstrated the hypothesis that Citral which is a component of Lemongrass oil plays the major role of the induction of phase II detoxyfying genes. In the dose-dependence experiment, Citral caused significant increases in the expression of HO-1 genes. This experimental result suggests that the Citral is the main component of Lemongrass which induces the expression of HO-1. From prior studies we know the activation of HO-1 is a ubiquitous cellular response to oxidative stress, which produces ROS, such as hydrogen peroxide and ultimately leads to lipid peroxidation. The expression of HO-1 gene is related to the production of biliverdin and its subsequent metabolite bilirubin,

both products have antioxidant properties against oxidative stress caused by xenobiotics, oxidants, UV light, and radiation (33). In addition to the dose-dependent experiment, I also performed time-dependent experiment to show the time manner of the induction of HO-1. With the different period of time, the bands showed stronger band with increasing time of oil treatment in both Lemongrass oil and Citral in the gel electrophoresis up to 8hrs. The 24hr time resulted in a return to basal expression levels, which may result from the metabolism of control over that time period. This experiment results lead to the conclusion that with the same concentration of oil but different time treating periods, the induction of HO-1 is time dependent.

In the method development of CYP3A4 inhibition assay, CYP3A4 is the enzyme to metabolize Nifedipine to oxidized nifedipine. In order to identify the retention time of Nifedipine and oxidized nifedipine, standard compounds of both Nifedipine and oxidized nifedipine were acquired and injected onto HPLC and the retention time of both compounds were determined using a variety of different mobile phases. After identification of the peaks of Nifedipine and oxidized nifedipine and determination of the exact retention time on the chromatogram, incubations were performed to demonstrate the Nifedipine was oxidized to oxidized nifedipine by using 5 different microsomes which included human liver microsomes, rabbit liver microsomes 1,

rabbit liver micorsomes 2, rat liver microsomes and supersomes which is enriched of CYP3A4. The chromatograms showed that there was a significant absorbance for oxidized nifedipine in the retention time of 3.8minutes with the presence of supersomes and rat liver microsomes when the NADPH was added in the reaction. In the human liver microsomes and rabbit liver microsomes 1, there was very little or no significant difference for both with NADPH and without NADPH reaction. Finally, it was shown that both lemongrass and citral inhibit CYP3A4 significantly at very low micromolar concentrations. This may have very important implications in drug metabolism as CYP3A4 is extremely important pharmacologically, in that it is responsible for metabolism of nifedipine.

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