Inhibition of Caffeine Metabolism in Humans by Furanocoumarin-Containing Plant Extracts: *In Vivo* and *In Vitro* Studies

by

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Abstract

Caffeine is found at high concentrations in tea, coffee, soft drinks and energy drinks. Daily consumption of caffeinated beverages is considered to be safe but adverse health effects and deaths have been reported in sensitive or overdosed individuals. A variety of furanocoumarin bioactive has been identified in fruits, spices and herbs from plants in the *Apiaceous* and *Rutaceae* families. Since caffeinated beverages are often consumed with food, and both are metabolized by the same CYP1A2 enzyme, we hypothesized metabolic inhibition between caffeine and furanocoumarin-containing food or herbs are common in humans.

The goals of this thesis were: (a) to compare the pharmacokinetics of caffeine in humans before and after pre-treatment with a furanocoumarin-containing herb, (b) to elucidate the mechanism(s) of caffeine-herb interaction using *in vitro* incubations containing pure furanocoumarins and human liver microsomes (HLMs), and (c) to predict *in vivo* herb-caffeine interactions for humans based on *in vitro* caffeine metabolism data and *in vivo* furanocoumarin inhibitor concentrations in the liver.

Chapter 1 of this thesis is a brief introduction of caffeine and furanocoumarin-containing food and herbs. In chapter 2, major furanocoumarin bioactive in 29 food and herbs are identified and quantified using gas chromatography mass spectrometry and high performance liquid chromatography. Chapter 3 describes the pharmacokinetics of caffeine in humans after administering 200 mg of caffeine orally before and after pre-treatment with an herbal extract. Caffeine clearance in the volunteers decreased 33.7-77.3% with concomitant increases in area-under-the-concentration-time curve after oral consumption of *Ammi majus* L., *Angelica archangelica* L., *Angelica pubescens* Maxim, *Cnidium monnieri* (L.) Cusson, or *Ruta graveolens* L. Chapter 4 provides the experimental evidence for irreversible adduct formation between ^{14}C-labeled 8-methoxypsoralen and HLMs. Moreover, the observed caffeine-herb interaction in humans is best explained by mechanism-based inhibition of CYP1A2 enzyme. Chapter 5 demonstrated the use of *in vitro-in vivo* drug-drug interaction models and an integrated furanocoumarin dose/concentration to predict *in vivo* furanocoumarin-caffeine herb interaction in humans. Chapter 6 summarizes the conclusions of this thesis. The experimental and modeling approaches described in this study are also useful in predicting *in vivo* interactions between prescription drugs and dietary supplements or functional food.

Keywords: Caffeine; Cytochrome P-450 1A2; Furanocoumarin; Herb-drug interaction; Human liver microsomes; Irreversible inhibition

I dedicate this work to all who have supported me

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Table of Contents

List of Tables

List of Figures

List of Equations

List of Abbreviations and Acronyms

$[I]_H$	Hepatic Inlet Inhibitor Concentration
°C	Degree Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
^{14}C	Carbon 14
5-MOP	5-Methoxypsoralen, or Bergapten
8-MOP	8-Methoxypsoralen, or Methoxsalen, or Xanthotoxin
ATSDR	Agency for Toxic Substances and Disease Registry
$AUC_{0\text{-}inf}$	Area-Under-Curve From Time Point Zero to Infinity
$AUC_{0\text{-}last}$	Area-Under-Curve From Time Point Zero to Last Measured Point
AUCR	Area-Under-Curve Ratio
BW	Body Weight
CA	Concentration Addition
Ci	Curie
C_I	Inhibitor Concentration
CI	Confidence Interval
CL	Clearance
CM	Chemical Marker
cm	Centimeter
$C_{max,LT}$	Maximum Total (Bound + Unbound) Liver Concentration
$C_{max,LU}$	Maximum Unbound Liver Concentration
$C_{max,PT}$	Maximum Total (Bound + Unbound) Plasma Concentration
$C_{max,PU}$	Maximum Unbound Plasma Concentration
CO	Carbon Monoxide Gas
COPD	Chronic Obstructive Pulmonary Disease
COT	Committee on Toxicity
cpm	Counts Per Minute
CV	Coefficient of Variation
CYP	Cytochrome
DAWN	Drug Abuse Warning Network
DDI	Drug-Drug Interaction
DIN	Deutsches Institut für Normung (German Institute for Standardization)
DIN-HM	Homeopathic Medicine Number
DMSO	Dimethyl Sulfoxide
DWH	Dry Weight of Herb Used to Prepare the Herb Stock Extract
EFSA	European Food Safety Authority
EMEA	European Medicines Agency

EN	Exemption Number
FDI	Food-Drug Interaction
FID	Flame Ionization Detector
FLD	Fluorescent Detector
f_m	Fractional Metabolism
f_{up}	Fraction Unbound in Plasma
f_{ut}	Fraction Unbound in Tissue
g	Gram
G	G-force
GC-MS	Gas Chromatography - Mass Spectrometry
GMFE	Geometric Mean-Fold Error
h	Hour
HDI	Herb-Drug Interaction
HLM	Human Liver Microsomes
HPLC	High Performance Liquid Chromatography
ISOP	Isopimpinellin, 5,8-Dimethoxypsoralen
ISTD	Internal Standard
IVIVE	*In Vitro-In Vivo* Extrapolation
k	Elimination Rate Constant
kg	Kilogram
L	Litre
Ln	Natural Logarithm
LOD	Limit of Detection
Log_{10}	Common Logarithm
LOQ	Limit of Quantification
m/z	Mass-to-Charge Ratio
MBI	Mechanism-Based Inhibitor
MFO	Mixed Function Monooxygenase
mg	Milligram
MIC	Metabolite-Inhibitor Complex
min	Minute
mL	Millilitre
mM	Millimolar
MS	Mass Spectrometry
MW	Molecular Weight
MΩ	Mega Ohm
N_2	Nitrogen molecule Gas
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced Form)
NDC	New Drug Candidate
ng	Nanogram
NHP	Natural Health Products
NIST	National Institute of Standards and Technologies

NPN	Natural Product Number
O_2	Oxygen Molecule Gas
OTC	Over The Counter
PFP	Pentafluorophenyl
pg	Picogram
pH	Power of Hydrogen Ions
P-H	Phenyl-Hexyl
pmole	Picomole
$P_{o/w}$	*n*-Octanol/Water Partition
$P_{t:p}$	Tissue:Plasma Partition
PTEF	Phytochemical Toxic Equivalency Factor
PUVA	Psoralen Ultra-Violet A Treatment
R^2	Coefficient of Determination
rCYP1A2	Recombinant Cytochrome 1A2
RPF	Relative Potency Factor
RT	Retention Time
SAMHSA	Substance Abuse and Mental Health Services Administration
SD	Standard Deviation
SEM	Standard Error of Mean
SPE	Solid-Phase Extraction
$t_{1/2}$	Biological Half-life
TCA	Trichloroacetic acid
TDI	Time-Dependent Inhibitor
TLC	Thin-Layer Chromatography
UC	University of California
UK	United Kingdom
US	United States
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
VAM	Volume of the Assay Medium
VET	Volume of Extract Tested
VHE	Volume of Herb Stock Extract
V_{np}	Fraction Weight of Neutral Lipids in Plasma
V_{nt}	Fraction Weight of Neutral Lipids in Tissue
V_{php}	Fraction Weight of Phospholipids in Plasma
V_{pht}	Fraction Weight of Phospholipids in Tissue
V_{wp}	Fraction Weight of Water in Plasma
V_{wt}	Fraction Weight of Water in Tissue
WHO	World Health Organization
WM	Whole Mixture

XDB	Extra Dense Bonding

Glossary

5th Percentile	is a measurement of value in which 5 percent of observed values fall below in a set of observed data.
95th Percentile	is a measurement of value in which 95 percent of observed values fall below in a set of observed data.
Area-Under-Curve	refers to the plot of concentration of substance in the sample against time calculated using the trapezoidal rule. It is also considered the exposure measurment of a biological system by a substance.
Chromatography	is a term to describe various techniques for the separation of complex mixtures based on differential affinities of constituents for a mobile (gas or liquid) medium and for a stationary adsorbing (liquid or solid) medium through which they pass, such as paper, gelatin, or silica.
Clearance	is a pharmacokinetic measurement that describes the volume of plasma that is completely cleared off of a substance per unit time.
C_{max}	is an observed pharmacokinetic measurement that describes the peak drug concentration in tissue after dosage.
Degradation Rate Constant	refers to the first order *in vivo* degradation rate constant (for the specific P-450 enzyme) or the turn-over rate constant.
Elimination Rate Constant	is a pharmacokinetic constant to describe the rate at which a substance is removed from the system.
Geometric Mean	refers to the value reflecting the central tendency of a set of data using the product of values (*i.e.* log) instead of the sum of values.
Geometric Mean-Fold Error	is a common statistical approach for prediction accuracy (or bias) measurement with equal weights for under-prediction and over-prediction.

IC_{50}	is the concentration of substance needed to inhibit a biological process (*e.g.* biological reaction, pathway, receptor, cell growth) by fifty percent or half.
K_I	is a measurement of inhibitor concentration at 50% the value of k_{inact} at optimal reaction conditions.
k_{inact}	is a measurement of maximum enzyme inactivation rate at optimal reaction conditions.
k_{obs}	is defined as the observed *in vitro* first-order enzyme decay rate constant.
Limit of Detection	is defined as the lowest quantity of a substance that can be distinguished from the absence of that substance within a stated confidence limit.
Limit of Quantification	is defined as the minimum concentration of substance that can be quantified at a specified level of precision or accuracy (or both).
Mass Spectrometry	refers to an analytical chemistry technique that identifies the amount and type of chemicals present in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions.
Mean	is defined as the central value of a discrete set of numbers or the sum of the values divided by the number of values. The terms of arithmetic mean and average are used synonymously.
Mechanism-based Inhibition	is defined as time-dependent inhibition which involves enzyme inactivation by a chemically reactive metabolite.
Median	refers to the value situated at the midpoint of a frequency distribution of observed data or the 50th percentile value.

Metabolic Pathway Fraction Percentage	refers to the fraction percentage of total elimination for the victim drug mediated by the specific CYP isoform.
Retention Time	is defined as the time it takes a solute to travel through the column. It is calculated as the time from injection to detection.
Standard Deviation	refers to a value that quantifies the extent of deviation (or scatter) from the mean for a whole set of data.
Standard Error of Mean	refers to the a value that determines how well the mean of samples estimates the mean of population using the standard deviation and sample size.
Time-dependent inhibition	is defined as an interaction where there is an enhanced inhibition if the inhibitor is pre-incubated with the enzyme prior to addition of the substrate.
T_{max}	is an observed pharmacokinetic measurement that describes the time to reach C_{max} after dosage.
Ultra-Violet Detector	refers to chemical measurement based on absorption spectroscopy in the ultraviolet spectral region. It is one of the most common detectors in liquid chromatography.

Chapter 1.

Introduction

1.1. Traditional Medicine and Natural Health Products

1.1.1. What is traditional medicine?

The World Health Organization (WHO) defines traditional medicine as the knowledge, skills, approaches, and practices that are used to prevent, diagnose, improve, and treat illness based on theories, beliefs, and experiences indigenous to different cultures (WHO, 2001). Non-conventional medicine, complementary medicine, alternative medicine, holistic medicine, and integrative medicine are different terms used to describe traditional medicine depending on the country and jurisdiction (WHO, 2002). At present, traditional and complementary medicine include medicinal herbs, naturopathy, acupuncture, osteopathy, chiropractic, cupping, acupressure (shiatsu), thermal treatments (cauterization), and mental/spiritual treatments such as qi-gong, tai-chi, and yoga (WHO, 2005).

1.1.2. What are natural health products?

Natural health products (NHP) are defined by Health Canada as naturally-occurring substances from botanical, microbial, animal, and marine sources that are used to restore or maintain good health (Health Canada, 2012). The majority of NHP are plant products which include the whole plant, parts of the plants, or a combination of plants as active ingredients. NHP are also available in different forms such as tablets, capsules, tinctures, solutions, creams, shampoos, soaps, ointments, powder, and drops. In some countries herbal medicines may contain, by tradition, natural organic or inorganic active ingredients that are not of plant origin such as animal parts or minerals (WHO, 2002).

1.1.3. How much natural health products do we consume?

The numbers of Canadians seeking alternative and complementary medicines are rapidly increasing as a result of personal beliefs that NHP are safer than conventional medicines. Based on a Canadian survey, 71% of the participants indicate that NHP are better for them than chemicals and pharmaceutical drugs (Ipsos Reid, 2011). It is estimated that four billion people around the world choose NHP for healing purposes prior to seeking primary health care (Ekor, 2013). The global sales of herbal and traditional medicines rose from 15.2 billion United States dollars (USD) in 2001 to 35.8 billion USD in 2015 (Figure 1-1) with an average growth rate of approximately 6.3 percent per annum (Euromonitor, 2015a).

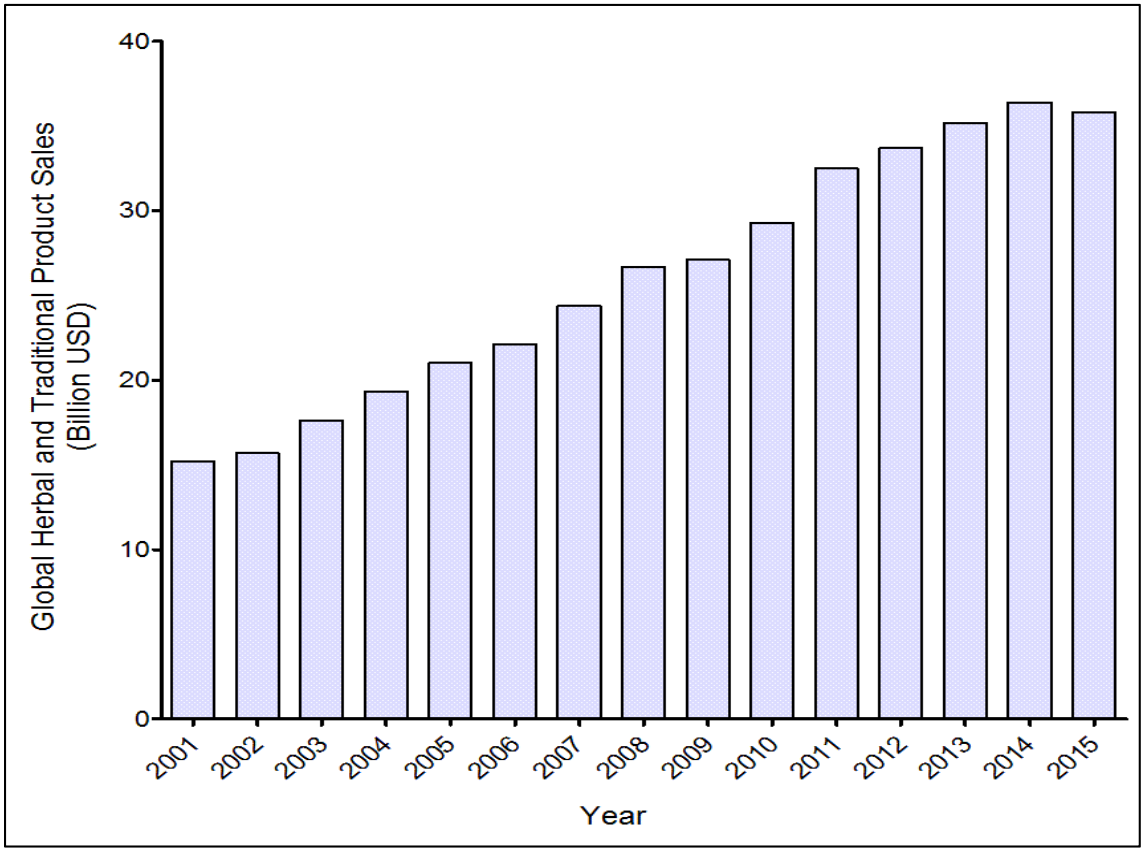

Figure 1-1: Global herbal and traditional product sales from year 2001 to year 2015 in billion USD (Euromonitor, 2015a).

The consumption of herbal and traditional medicine in the US rose from 3.2 billion USD in year 2001 to 4.6 billion USD in year 2015 with an annual sales growth rate of approximately 2.6 percent (Euromonitor, 2015b). In Canada, the average sales rose from

306.4 million USD in 2001 to 511.2 million USD in 2015 with an average growth rate of approximately 3.7 percent per annum (Euromonitor, 2015c).

The growth in use of traditional and herbal medicines in North America have been greatly influenced by traditional Chinese medicine, Indian Ayurvedic medicine, Perso-Arabic Unani medicine, Japanese Kampo medicine, European herbal medicine, and indigenous North American medicine. In the US, a survey of 2049 participants found that 12% of Americans use at least one herbal supplement per year (Foster et al., 2000). In the US, the top consumed herbal supplements are garlic, echinacea, ginseng, evening primrose oil, gingko biloba, goldenseal, St. John's wort, and saw palmetto (Hall and Nazir, 2005). In Canada, a survey of 1543 participants found that 11.2% of participants have used herbal supplements with garlic, echinacea, ginseng, evening primrose oil, and gingko biloba among the most consumed (Troppmann et al., 2002).

1.1.4. Regulation of natural health products in Canada

The inappropriate use of NHP and lack of proper regulation can result in serious health consequences. As a result, NHP including herbal medicines have been regulated under the "Natural and Non-prescription Health Products Directorate" since 2004 (Health Canada, 2015). Depending on the health benefit claims and overall risks, NHP are assessed under different pathways and steps from conventional drugs relying on evidence from animal studies, human clinical data, pharmacopoeias, and/or traditional resources to determine the safety and efficacy of such products.

In Canada, licensed NHP must be safe for sale over-the-counter (OTC) with an eight-digit Natural Product Number (NPN), Homeopathic Medicine Number (DIN-HM), or an Exemption Number (EN). Despite these regulatory efforts, Canadians experiencing adverse health effects due to NHP consumption appear to be on the rise. In year 2010, 15% of the NHP consumers have experienced side effects, or unwanted reactions, compared to 12% of consumers in year 2005 (Ipsos Reid, 2011).

1.2. Herb-Drug and Food-Drug Interactions

1.2.1. Drug pharmacokinetics and pharmacodynamics

In early drug development phases, the pharmacokinetic and pharmacodynamic profiles of a drug, and its metabolite(s), are studied prior to drug approval. Pharmacokinetics is the science which studies the absorption, distribution, metabolism, and excretion of a drug in the body after administration. Orally administrated drugs are absorbed *via* the enterocytes into the portal vein from the gut lumen followed by passage through the liver before being distributed to various parts of the body. Drug pharmacokinetics is influenced by many factors including age (Greenblatt et al., 1982), obesity (Abernethy and Greenblatt, 1982), gender (Fletcher et al., 1994), pregnancy (Krauer and Krauer, 1977), genetic polymorphism (Eichelbaum,1982), and ethnicity (Kalow, 1982). Disease state also plays an important role in the pharmacokinetics of a drug especially when key organs such as liver (Blaschke, 1977), kidneys (Reidenberg, 1977), heart (Benowitz and Meister, 1976), and thyroid gland (Shenfield, 1981) are not functioning normally. On the other hand, pharmacodynamics is the science which studies the relationship of drug, or its metabolite(s), concentration and its effect on organs, tissues, and cells. The effect, or response, is a result of the binding or blockage of the drug, or its metabolite(s), with target active sites including receptors and ion channels (Tomlin, 2010). Drug-drug, food-drug, and herb-drug interactions play an important role in altering the pharmacokinetics and/or pharmacodynamics profiles of an administrated drug in the body (Sørensen, 2002).

1.2.2. Cytochrome P-450 and drug metabolism

Living organisms use catabolic and anabolic enzymatic processes to maintain normal body functions. The enzymatic systems which are responsible for the metabolism of foreign compounds are divided into two groups or phases, namely phase-I (oxidation, reduction, and hydrolysis) and phase-II (conjugation). Phase-I reactions involve mainly the mixed-function oxidases (MFO) or cytochrome P-450 (CYP) enzymes (Beedham, 1997) with 57 different isoforms have been identified in humans (Guengerich, 2005). It is

estimated that more than 85 percent of drugs in use are metabolized by CYP enzymes (Figure 1-2) (Rendic and Di Carlo, 1997).

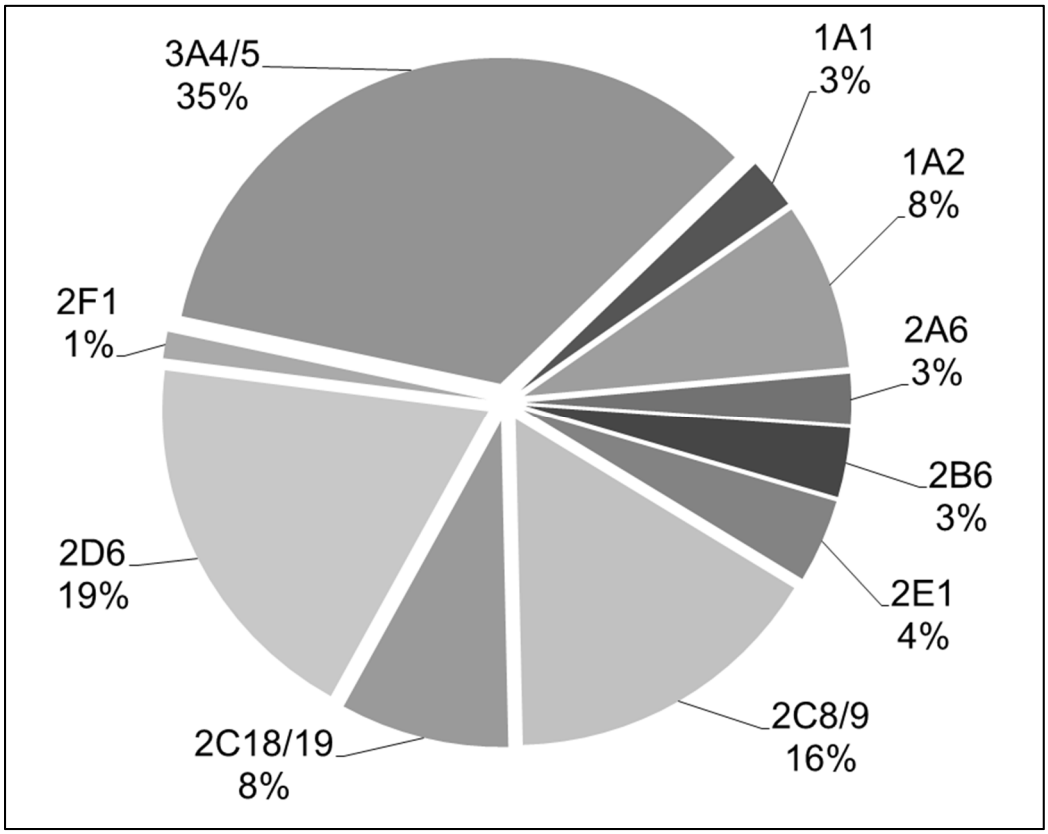

Figure 1-2: Pie-chart of involvement percentages for main CYP P-450 isoforms in human pharmaceutical drug metabolism.

The main locations of CYP enzymes are in the liver, intestine, kidney, and lung (Wilkinson, 2005). In eukaryotes, CYP are located mainly in the endoplasmic reticulum (ER) with the active site exposed at the cytosolic face of the ER membrane (Cribb et al., 2008). Upon oral drug consumption, the CYP enzymes in the liver and intestine are mainly responsible for the first-pass effect on blood drug levels. In addition to drug metabolism, CYP enzymes play a significant role in the metabolism of endogenous substrates including sterols, vitamins, fatty acids, and eicosanoids (Guengerich, 2005). In phase-II reactions, these intermediate metabolites may conjugate with endogenous chemicals to produce polar metabolites that can be easily eliminated. These phase-II reactions are mediated mainly by specific enzymes such as glutathione *S*-transferases, *UDP*-glucuronosyltransferases, sulfotransferases, and *N*-acetyltransferases (Kadlubar and Kadlubar, 2010).

1.2.3. Definition of drug-drug interactions

After oral administration, a drug is usually metabolized by CYP enzymes in the intestine and liver where drug-drug interactions (DDI) may occur in the presence of an inhibitor (Yeung et al., 2010). Human DDI is a clinical situation in which one drug, or perpetrator drug, interferes with the pharmacokinetics and/or pharmacodynamics of another drug, or victim drug. Pharmacokinetic-based DDI involve interferences in the absorption, distribution, metabolism, and/or excretion processes of the victim drug (Rodríguez-Fragoso et al., 2011). On the other hand, pharmacodynamic-based DDI occur at the receptor, signaling, and/or effector levels resulting in synergetic (supra-additive), additive (no interaction), and antagonism (infra-additive) effects (Hinder, 2011). In general, the outcome of such interference(s), in many cases, is serious including drug toxicity, ineffective drug treatment, and/or death (Juurlink et al., 2003).

1.2.4. Methods used to predict drug-drug interactions

Different approaches have been used to predict DDI including *in silico* and *in vitro-in vivo* extrapolation (*IVIVE*) mathematical models. *In silico* methodologies are computer-based approaches used to predict DDIs in early drug discovery and development. The main *in silico* methods used to predict DDIs include quantitative structure-activity relationship (QSAR) modelling, pharmacophore modelling, and docking modelling (Stoner et al., 2010). Along with *in silico* approaches, DDI continues to be detected and predicted using *in vitro* enzyme inhibition and induction data in combination with mathematical *IVIVE* models. Many models have been developed for *IVIVE* DDI prediction in mammals. The selection of a prediction model for a study relies on the mechanism of interaction, *i.e*. reversible inhibition, irreversible inhibition, and enzyme induction (Houston and Galetin, 2010).

The outcome of model prediction is mainly determined by the CYP enzyme selected for the study and the assay used to measure enzymatic activity. Based on the recent USFDA DDI guidelines, the prediction outcome generally classifies inhibitors into the following categories: strong inhibitors (≥ 5 fold change in area-under-curve (AUC) or > 80% reduction in clearance (CL)), moderate inhibitors (≥ 2 but < 5 fold change in AUC

or 50-80% reduction in CL), and weak inhibitors (≥ 1.25 but < 2 fold change in AUC or 20-50% reduction in CL) (USFDA, 2012).

1.2.5. Herb-drug and food-drug interactions can result in adverse health effects

Herb-drug interactions (HDI) and food-drug interactions (FDI) have become an important topic due to the popularity of NHPs in North America. However, it is difficult to predict HDI and FDI as plant-based food and drugs contain complex mixtures of bioactive components (Zhou et al., 2003). The following are examples of HDI and FDI: (a) concomitant consumption of ginkgo (*Ginkgo biloba*) and anticonvulsants, Depakote and Dilantin, resulted in fatal seizures (Kupiec and Raj, 2005); (b) consumption of St. John's wort (*Hypericum perforatum*) and immunosuppressive drug cyclosporine together increased the risk of rejection in organ transplant patients (Henderson et al., 2002). St. John's wort also reduced plasma concentrations of digoxin in cardiac patients when consumed concomitantly (Johne et al., 1999); and (c) consumption of grapefruit (*Citrus paradisi*) or grapefruit juice with drugs such as cyclosporine (Ducharme et al., 1995), midazolam (Kupferschmidt et al., 1995), triazolam (Hukkinen et al. 1995), felodipine and nifedipine (Bailey et al., 1998), and verapamil (Fuhr et al., 2002) significantly increased the oral bioavailability of these drugs. A few studies suggested that the inhibitory effect of grapefruit juice on intestinal CYP3A4 was mostly due to the presence of furanocoumarin constituents such as bergamottin and 6,7-dihydroxybergamottin in grapefruit juice (Schmiedlin-Ren et al., 1997; He et al., 1998).

1.3. Furanocoumarins

1.3.1. The function and structure of furanocoumarins

Furanocoumarins are produced by plants as a defense against various types of pathogens/predators ranging from microbes to mammals (Berenbaum, 1995). For example, the furanocoumarin angelicin, and its chemical derivatives, were reported to possess anti-fungal activity against *Candida albicans*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae,* and *Aspergillus niger* (Sardari et al., 1999). The

furanocoumarin 8-methoxypsoralen (8-MOP) exhibited anti-microbial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae* (Ojala et al., 2000). The seeds of garden angelica (*Angelica archangelica* L.) were used as a potent insecticide against the larvae of Egyptian cotton leafworm (*Spodoptera littoralis*) due to their furanocoumarin constituents (Pavela and Vrchotová, 2013). The Caucasian giant hogweeds (*Heracleum sosnowskyi* and *Heracleum mantegazzianum*) were reported to contain furanocoumarins that caused contact dermatitis and skin necrosis in humans (Rzymski et al., 2015).

The chemical structure of furanocoumarin consists of a furan ring fused with a coumarin molecule. Two common forms of furanocoumarins are observed: linear and angular furanocoumarins which are represented by the structures of psoralen and angelicin, respectively (Figure 1-3).

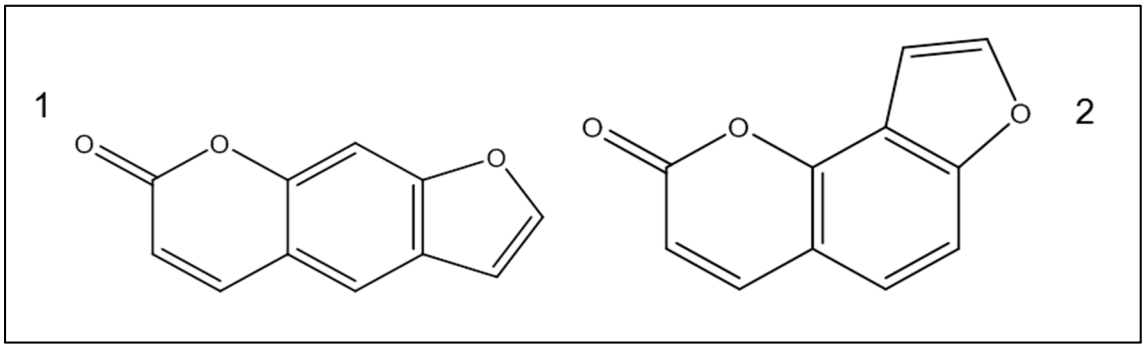

Figure 1-3: Chemical structures of the two furanocoumarins: psoralen (1) and angelicin (2).

Furanocoumarins are synthesized continuously by plants at low levels but are produced at much higher concentrations when plants are affected by disease and infection (Lord et al., 1988), ultra-violet light exposure and nutrient levels (Zangerl and Berenbaum, 1987), harvest location (Sigurdsson et al., 2012), seasonal changes (Zobel and Brown, 1990), region location (Yan et al., 2001), pollution (Dercks et al., 1990), storage conditions (Chaudhary et al., 1985), and fungicide exposure (Nigg et al., 1997).

The consumption of fruits and vegetables rich in furanocoumarins are known to cause interactions with therapeutic drugs in humans. The ‘grapefruit juice effect’ is caused by concomitant consumption of grapefruit juice and drug substrates of CYP3A4 (Bailey et

al., 1991). Furanocoumarin-containing herbs can be used as photosensitizing agents in the treatment of vitiligo, a skin pigment-related disease (El-Mofty, 1964).

1.3.2. Plant sources of furanocoumarins

Many furanocoumarin-containing plant families have considerable values in nutrition, medicine, and the economy. These include *Amaranthaceae, Apiaceae (Umbelliferae), Compositae (Asteraceae), Cyperaceae, Dipsacaceae, Goodeniaceae, Guttiferae (Clusiaceae), Leguminosae (Fabaceae* or *Papilionaceae), Moraceae, Pittosporaceae, Rosaceae, Rutaceae, Samydaceae, Solanaceae, and Thymelaeaceae* (Diawara and Trumble, 1997). Previous studies have shown that many species of plants, especially those from the *Apiaceous*, *Rutaceae, Leguminosae,* and *Moraceae* families, contain furanocoumarin bioactive such as bergamottin (He et al., 1998), epoxybergamottin (Wangensteen et al., 2003), 5,7-dihydroxybergamottin (Guo et al., 2000), 8-MOP (Poutaraud et al., 2000), 5-methoxypsoralen (5-MOP) (Yan et al., 2001), bergaptol (Lu et al., 2007), imperatorin (Wei and Ito, 2006), isoimperatorin (Wei et al., 2009), angelicin (Dong et al., 2003), heraclenin (Rastogi et al., 2007), heraclenol (Rawat et al., 2013), isopimpinellin (ISOP) (Song et al., 2012), psoralen (Dhalwal et al., 2007), oxypeucedanin (Chaudhary et al., 1986), oxypeucedanin hydrate (Piao et al., 2004), byakangelicol (Zhang et al., 2009), byakangelicin (Ngwendson et al., 2003), and phellopterin (Waksmundzka-Hajnos et al., 2004).

The *Apiaceae* plant family, also known as the carrot, celery, or parsley family, is an important source of food and medicine for humans. The species commonly used as food include celery (*Apium graveolens*) (Beier et al., 1983), parsley (*Petroselinum crispum*) (Beier et al., 1994), anise (*Pimpinella aniseum*) (Reichling and Galati, 2004), fennel (*Foeniculum vulgare*) (Zaidi et al., 2007), parsnip (*Pastinaca sativa*) (Lombaert et al., 2001), caraway (Carum carvi), coriander (*Coriandrum sativum*) (Ceska et al., 1987), and dill (*Anethum graveolens*) (Stavri and Gibbons, 2005). The species that are used as medicines include khella shaitani (*Ammi majus*) (Krivut and Perel'son, 1967), garden angelica (*Angelica archangelica*) (Härmälä et al., 1992), duhuo (*Angelica pubescens*) (Chen et al., 1995), baizhi (*Angelica dahurica*) (Baek et al., 2000), danggui (*Angelica sinensis*) (Noe, 1997, as cited in Al-Bareeq et al., 2010), shechuangzi (*Cnidium monnieri*)

(Yan et al., 2001), cow parsnip (*Heracleum maximum*) (O'Neill et al., 2013), lovage (*Levisticum officinale*) (Paszkiewicz et al., 2008), and khella (*Ammi visnaga*) (Sellami et al., 2013).

The *Rutaceae* family, also known as the rue or citrus family, include food such as grapefruit (Bailey et al., 1991), orange (*Citrus sinensis*), and mandarin (*Citrus reticulata*) (Peroutka et al., 2007), lime (*Citrus aurantifolia*) (Wagner et al., 2002), lemon (*Citrus limon*), clementine (*Citrus clementina*), bergamot (*Citrus bergamia*) (Dugrand et al., 2013), pomelo (*Citrus grandis*) (Saita et al., 2004), and bitter orange (*Citrus aurantium*) (Guo et al., 2001). The species used as medicines include common rue (*Ruta graveolens*) (Poutaraud et al., 2000), ruda (*Ruta chalepensİs*) (Günaydin and Savci, 2005), mountain rue (*Ruta montana*) (Benkiki et al., 2002), apple stone (*Aegle marmelos*) (Bhattacherjee et al., 2015), yuy (*Casimiroa tetrameria*) (Heneka et al., 2005), *Thamnosma rhodesica* (Ahua et al., 2004), Mojave desert rue (*Thamnosma montana*) (Kutney et al., 1973), Sandboegoe (*Thamnosma africana*) (Mafokane et al., 2006), and common prickly-ash (*Zanthoxylum americanum*) (Saqib et al., 1990). The *Leguminosae* plant family, commonly known as the legume, pea, or bean family, includes buguzhi (*Psoralea corylifolia*) (Ahandani et al., 2013). The *Moraceae* plant family, also known as the mulberry or fig family, includes the common figs (*Ficus carica*) (Rouaiguia-Bouakkaz et al., 2013) and *Dorstenia foetida* (Heinke et al., 2011).

1.3.3. How much furanocoumarins do we consume?

The average daily intake of furanocoumarins in US is estimated to be 1.3 mg per person (maximum daily intake, 2.5 mg) based on comsumption of lime, celery, parsley, parsnip, grapefruit, lemon, carrot, and orange (Wagstaff, 1991). In Germany, the average daily intake of furanocoumarins from non-flavoured and flavoured food is 0.6 mg per person (maximum daily intake, 2.3 mg) (Guth et al., 2011). In the United Kingdom (UK), the estimated furanocoumarin daily intake is 1.2 mg per person (Committee on Toxicity (COT) 1996, as cited in Guth et al., 2011). Based on the US, UK, and Germany exposure data, average daily intake of furanocoumarins is estimated at 1.0 mg per person (maximum daily intake is estimated at 2.4 mg per person).

1.3.4. Health effects due to furanocoumarin consumption

The consumption of linear and angular furanocoumarins (Figure 1-3) is associated with many health effects. Furanocoumarins in common herbs and food are potent, irreversible inhibitors of P-450 CYP enzymes, and may interfere with the oral clearance (CL) of therapeutic drugs in humans. For example, psoralen was shown to be an irreversible inhibitor of CYP1A2 (Zhuang et al., 2013) and CYP2B6 (Ji et al., 2015); angelicin was shown to be an irreversible inhibitor of CYP1A2 (Zhuang et al., 2013) and CYP2B6 (Lu et al., 2015); 8-MOP was shown to be an irreversible inhibitor of CYP2A6 (Koenigs et al., 1997) and CYP2B1 (Koenigs and Trager, 1998); 5-MOP exhibited irreversible inhibition for CYP3A4 (Zaidi et al., 2007); ISOP was characterized as an irreversible inhibitor of CYP1A2 (Kang et al., 2011); isoimperatorin was found to be an irreversible inhibitor of CYP2B6 (Cao et al., 2015).

Furthermore, angelicin, psoralen, 8-MOP and 5-MOP are able to cause phototoxic dermatological reactions in humans and animals after consuming or contacting dermally with plant species belonging to the *Apiaceae*, *Moraceae*, and *Rutaceae* families (Quinn et al., 2014). The mechanism of action is attributed to the ability of the furanocoumarins to intercalate with nucleic acids in the DNA of skin cells. Upon exposure to ultra-violet light, the reaction is activated and adducts are formed resulting in cell apoptosis and cell proliferation (Gasparro et al., 1998). Psoriasis patients undergoing Psoralen-Ultra-Violet-A (PUVA) treatments by consuming 8-MOP and/or 5-MOP have a higher chance of developing squamous cell carcinomas (Stern and Lange, 1988; Lindelöf et al., 1991; Stern and PUVA Follow-up, 2012).

1.4. Caffeine

1.4.1. The structure and biological effects of caffeine

Caffeine (1,3,7-trimethylxanthine) is a white, crystalline, purine alkaloid, first isolated from Arabian Mocha beans by Friedlieb Ferdinand Runge and Johann Wolfgang von Goethe in 1819 (Weinberg and Bealer, 2001). Similar to nicotine and morphine, caffeine is considered a secondary metabolite synthesized by plants to act as a natural

pesticide against plant-feeding insects (Nathan, 1984). In addition, plants belonging to the genera of *Coffee* and *Citrus* are found to produce enough caffeine in their nectar to alter the behaviour of pollinators by enhancing the memory of reward (Chittka and Peng, 2013). Thus, insect pollinators such as bees prefer nectar that contains caffeine, in low levels, over sucrose-enriched nectar despite the relatively bitter taste of caffeine (Wright et al., 2013).

The chemical structure of caffeine is similar to a part of the adenosine molecule (Figure 1-4). Thus, caffeine is able to occupy the adenosine receptors thereby blocking the inhibitory effect of adenosine on central nervous system (CNS). By antagonizing the CNS effect of adenosine, caffeine is able to cause stimulatory effect in humans (Biaggioni et al., 1991). As such, caffeine and caffeinated beverages are used frequently by night-shift workers, long-haul drivers, security guards, and students to seek the anti-fatigue effects. Caffeine is also a principal component in a number of OTC medications (House and Palmentier, 2004), and is increasingly being sold as a fitness and muscle-building dietary supplement (Jabbar and Hanly, 2013).

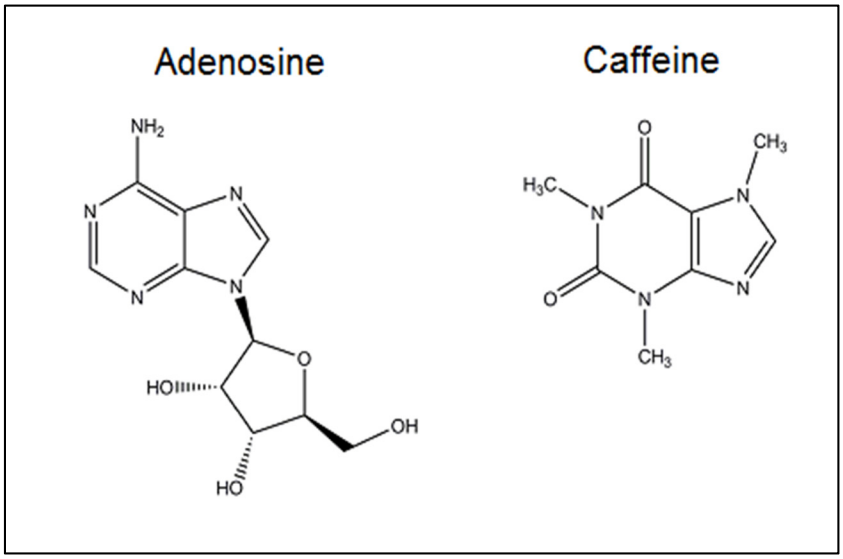

Figure 1-4: Chemical structures of caffeine and adenosine.

1.4.2. Natural sources of caffeine

Caffeine and other purine alkaloids, such as theobromine and theophylline, are naturally-occurring substances found in many widely-consumed plant genera including

Camellia, *Coffea*, *Cola*, *Ilex*, *Theobroma*, *Herrania*, and *Paullinia* (Ashihara et al., 2011). The genus *Camellia* has numerous species that contain caffeine and are widely used including the common tea (*C.sinensis*) which usually contains up to 2.8% dry leaf weight of caffeine, *C.assamica* (assam tea) which contains up to 2.4% dry leaf weight of caffeine, *C.taliensis* which contains up to 2.5% dry leaf weight of caffeine, and *C.kissi* which contains <0.02% dry leaf weight of caffeine (Nagata, 1986). The genus *Coffea* has numerous species that contain caffeine including the popular *C.arabica*, also known as arabica coffee, which contains up to 1.2% dry seed weight of caffeine, *C. canephora*, also known as robusta coffee, which contains up to 2.4% dry seed weight of caffeine, *C.liberica*, also known as Barako coffee, which contains up to 1.4% dry seed weight of caffeine, *C.stenophylla* which contains up to 1.7% dry seed weight of caffeine, *C.congensis* which contains up to 2.0% dry seed weight of caffeine, *C.eugeniodes* which contains up to 0.4% dry seed weight of caffeine, *C.kapakata* which contains up to 0.7% dry seed weight of caffeine, *C.salvatrix* which contains up to 0.7% dry seed weight of caffeine, and *C.racemosa* which contains up to 0.8% dry seed weight of caffeine (Mazzafera and Carvalh, 1991).

The genus *Cola* including the popular Kola nut (*C.nitida*) is native to tropical Africa and used as an ingredient in drinks and medication. It was later introduced to Central and South America. *C.nitida* contains up to 1.8% dry seed weight of caffeine (Belliardo et al., 1985). *C.acuminata* and *C.anomala* also contain caffeine but in smaller amounts (Niemenak et al., 2008). The genus *Ilex* includes a popular drink in South America known as yerba mate tea, which is made from the leaves of *I.paraguariensis*. The leaves of mate contain up to 2% dry weight of caffeine (Filip et al., 1998). The genus *Theobroma* includes a popular species of *T.cacao* that is commonly used for cocoa, cocoa butter, and chocolates, and contains up to 0.8% dry fruit weight of caffeine (Senanayake and Wijesekera, 1971). The *T.bicolor* and *T.angustifolium* also contain caffeine but to a lesser extent than *T.cacao* (Sotelo and Alvarez, 1991). The genus *Herrania* found in Central and South America includes the species of *H.albiflora*, *H.balaensis*, *H.cuatrecasana*, *H.nitida*, and *H.purpurea*. The Herrania species contains pure alkaloids but only trace amounts of caffeine in their seeds (Hammerstone et al., 1994). The genus *Paullinia* includes another popular drink in South America known as guarana, which is made from the seeds of *P.cupana* which contains up to 7% dry seed weight of caffeine (Pagliarussi et al., 2002).

Guarana is a popular ingredient in soft drinks and energy boosters, and is considered the main caffeine source for South American natives. The *P.yoco* is a related species which contains up to 0.5% dry cortex weight of caffeine (Weckerle et al., 2003).

1.4.3. How much caffeine do we consume?

Canadians are among the top world consumers of caffeine-containing products (Figure 1-5). The average consumption of caffeine in Canada is 308 mg/person/day while the average consumption in the world is 82 mg/person/day based on the combined consumption of coffee, tea, and cocoa (FAOSTAT, 2009).

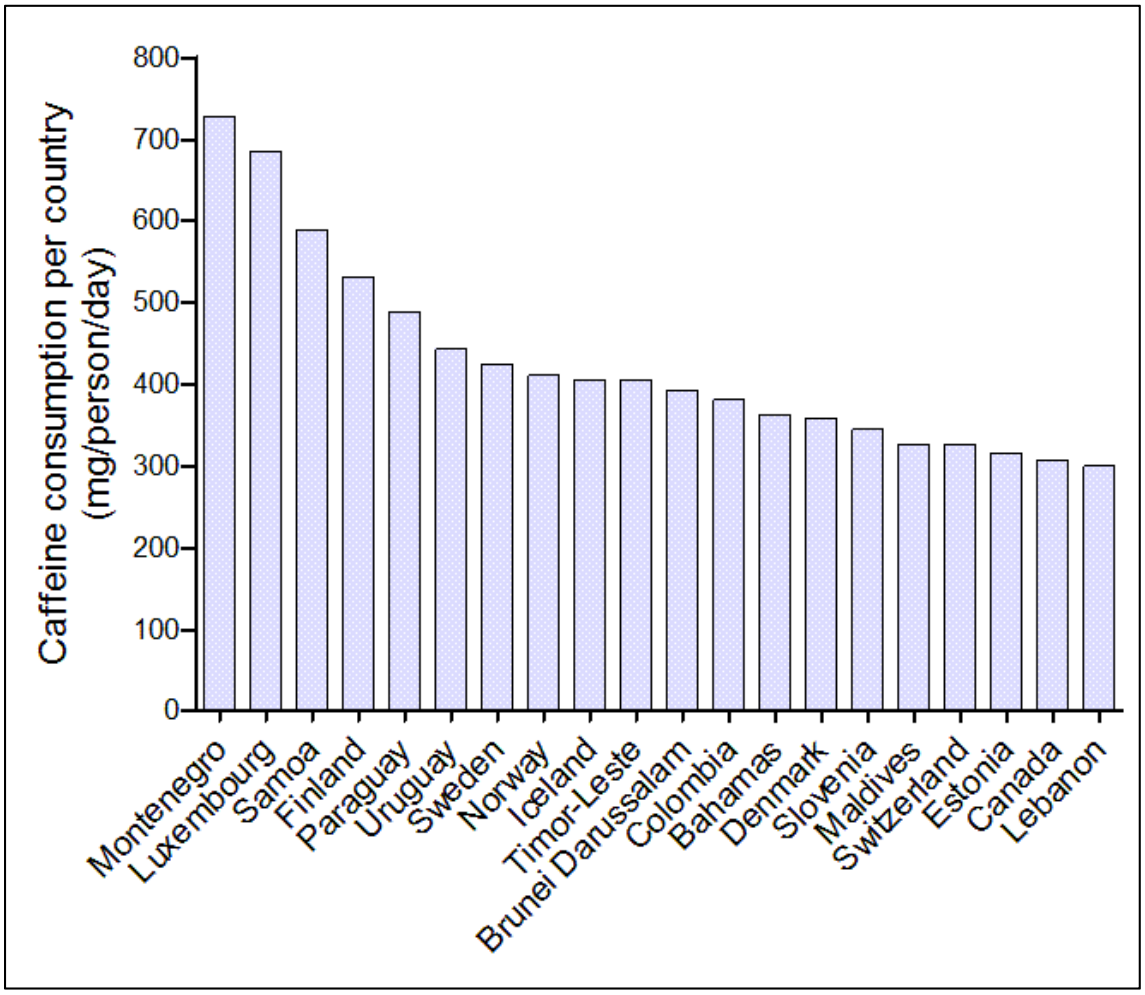

Figure 1-5: Top 20 countries of caffeine consumption based on the combined consumption of coffee, tea, and cocoa per capita per day.

Based on the 2008 Statistics Canada report, consumption of caffeinated beverages such as coffee, tea, and carbonated-soda by Canadian adults, 19 years and over, is ranked second after water and before juices, milk and alcoholic drinks resulting in 20% of men and 15% of women, aged 31 to 70 years, exceeding the maximum recommended safe caffeine consumption level set by Health Canada at 400 mg per day (Garriguet, 2008). Coffee, tea, cocoa, and soft drinks have a caffeine content of 29-176 mg, 8-91 mg, 6-40 mg, and 13-35 mg per 150 mL serving, respectively (Barone and Roberts, 1996).

1.4.4. The pharmacokinetics of caffeine in humans

After oral consumption of caffeinated drinks or food, caffeine is completely and rapidly absorbed and distributed to almost all organs and tissues in humans with minimal first-pass effect (Blanchard and Sawers, 1983). Caffeine is found to cross the blood-brain, blood-placenta, and blood-testis barriers; it is detected in plasma, saliva, cerebral spinal fluid (Tin et al., 1979), semen (Beach et al., 1984), amniotic fluid (Sommer et al., 1975), breast milk (Tyrala and Dodson, 1979), meconium (Madej, 2010), hair (Mizuno et al., 1996), and urine (Axelrod and Reichenthal, 1953).

Caffeine in the plasma reaches its peak concentration in about 30-60 min after oral administration. The elimination half-life of caffeine varies from 2.7 to 9.9 h, indicating large variation in elimination (Blanchard and Sawers, 1983). Caffeine metabolism is influenced by the following factors including ethnicity (Grant et al., 1983), gender (Carrillo and Benitez, 1996), menstrual cycle (Lane et al., 1992), age and puberty (Lambert et al., 1986), oral contraceptive consumption (Patwardhan et al., 1980), pregnancy (Parsons and Pelletier, 1982), liver disease (Desmond et al., 1980), and smoking (Parsons and Neims, 1978).

Caffeine is extensively metabolized with 3% or less being excreted unchanged in the urine (Tang-Liu et al., 1983). In contrast to laboratory animals, the main route of caffeine metabolism in humans (70-80%) is CYP1A2-mediated 3-*N*-demethylation to paraxanthine (Kot and Marta, 2008a). The formation of minor metabolites such as theobromine, theophylline, and 1,3,7-trimethyluric acid involve CYP2C8/9, 2E1, and 3A4

(Figure 1-6) (Tang-Liu et al., 1983; Lelo et al., 1986; Gu et al., 1992; Kot and Marta, 2008b). As such, caffeine is used as a probe drug to determine *in vivo* CYP1A2 activity in the liver (Doehmer et al., 1992; Miners et al., 1996). Paraxanthine, the major metabolite of caffeine, can be further metabolized to 1-methylxanthine, 1-methyluric acid, 5-acetylamino-6-formylamino-3-methyluracil, and 1,7-dimethyluric acid (Lelo et al., 1989). Neonates cannot demethylate caffeine and, until about three months of age, caffeine is eliminated primarily by renal excretion although it is suggested that caffeine C-*8*-hydoxylation by CYP3A4 is the main metabolic pathway until caffeine demethylation matures with age (Cazeneuve et al., 1994).

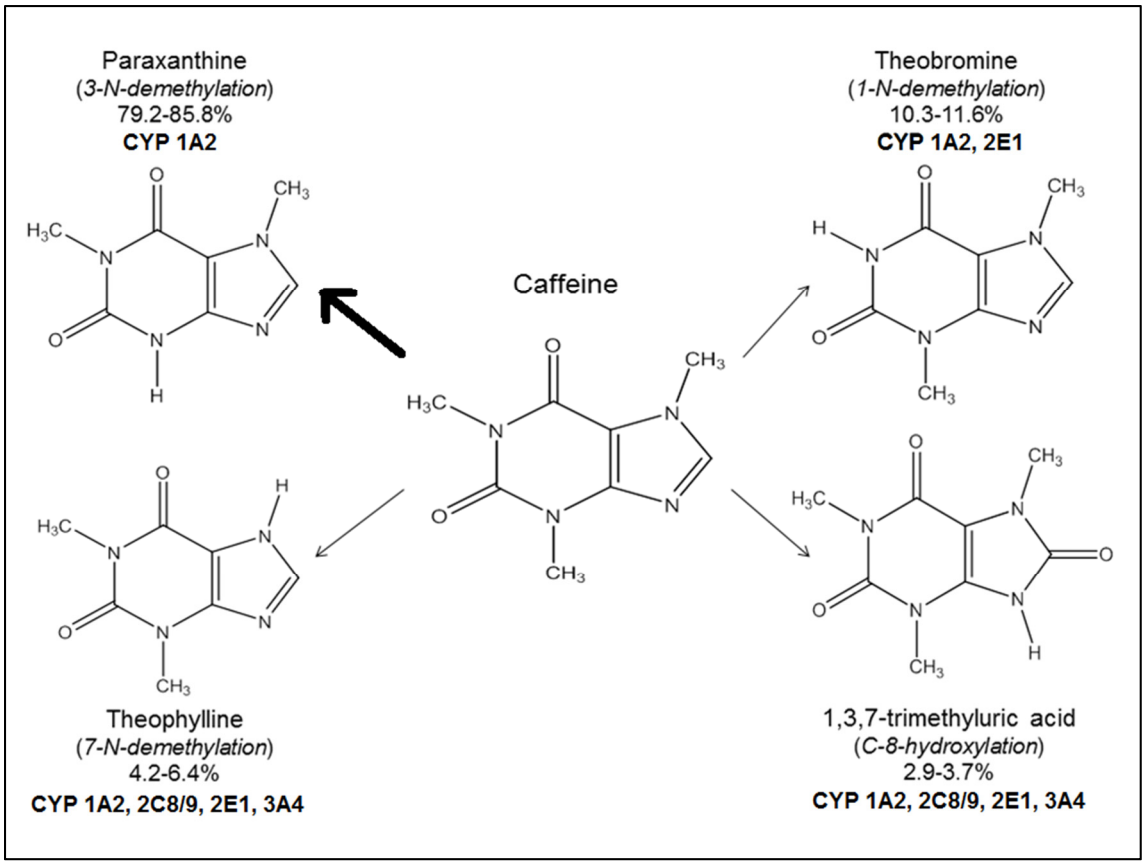

Figure 1-6: The primary metabolites of caffeine in humans.

1.4.5. Health effects due to an increase in caffeine consumption

The following is a summary of reported adverse health effects due to frequent caffeine consumption in sensitive individuals including: (a) cardiovascular effects such as increases in heart rate (Robertson et al., 1981), blood cholesterol (Thelle et al., 1983), and

blood pressure (Robertson et al., 1978) are often reported, (b) behavioral effects including depression (Uhde et al., 1984), mood change (Goldstein et al., 1965), and headache (Shirlow and Mathers, 1985) also are reported, (c) reproductive effects may include infertility (Wilcox et al., 1988), low birth weight (Mau and Netter, 1974), intrauterine growth retardation (Kuzma and Sokol, 1982), preterm birth (Furuhashi et al., 1985), spontaneous abortion and stillbirth (Weathersbee et al., 1977), sudden infant death syndrome (Ford et al., 1998), breech presentation (Barr and Streissgnth, 1991), persistent cryptorchidism (Mongraw-Chaffin and Cohn, 2008), and congenital lip deficiency (Chen et al., 2012), and (d) bone calcium imbalance resulted from caffeine consumption includes low bone density (Rapuri et al., 2001) and fractures (Kiel et al., 1990).

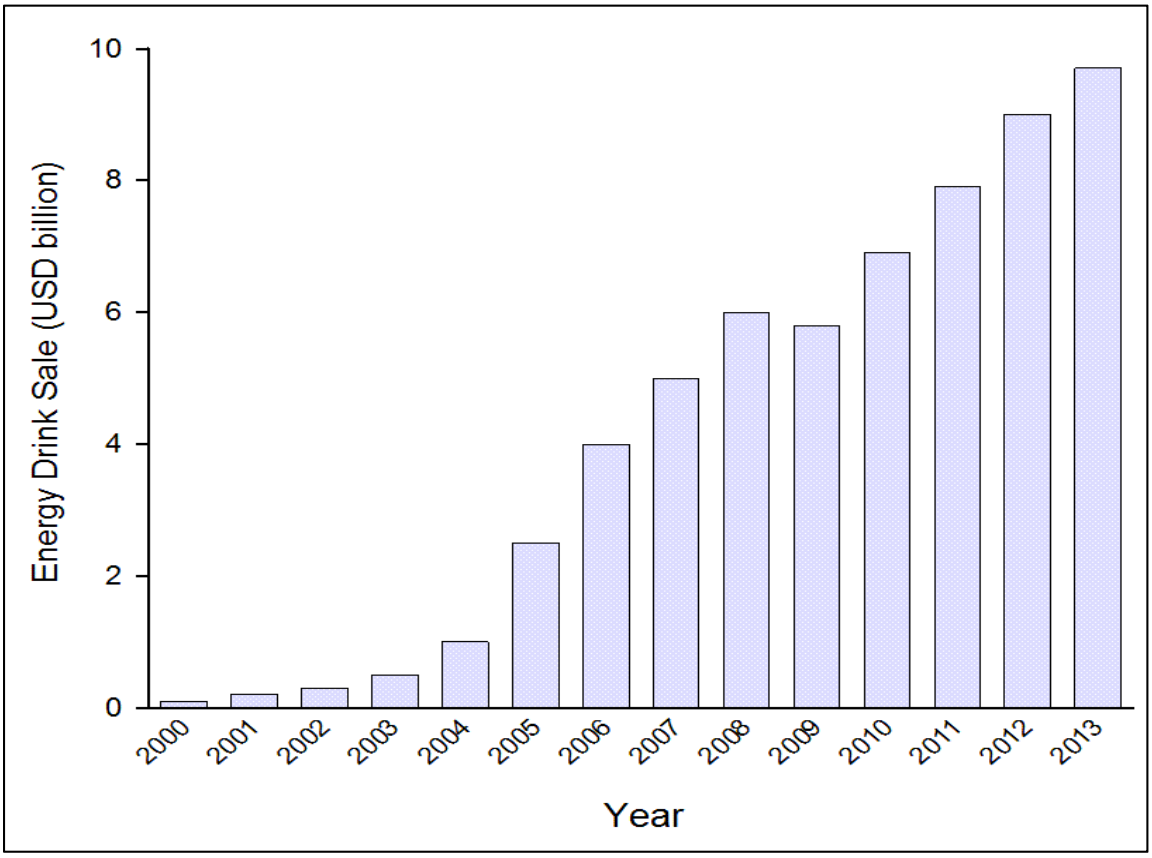

Figure 1-7: Energy drink sales in the US (Euromonitor, 2015d)

In the last decade, the introduction of energy drinks has increased the intake of caffeine by adolescents and children. Incidents of abuse, overdose, and adverse health effects have risen sharply after the introduction of caffeine supplements and fortified caffeinated drinks (SAMHSA, 2013) (Figures 1-7 and 1-8). Based on the 2014 US Drug

Abuse Warning Network (DAWN) report, approximately 1 in 10 energy drink-related emergency department visits resulted in hospitalization (SAMHSA, 2014). The increase in the number of reported caffeine intoxication cases has lead a few countries, including Lithuania and United Arab Emirates, to ban energy drink sales to adolescents (Thrastardottir, 2014).

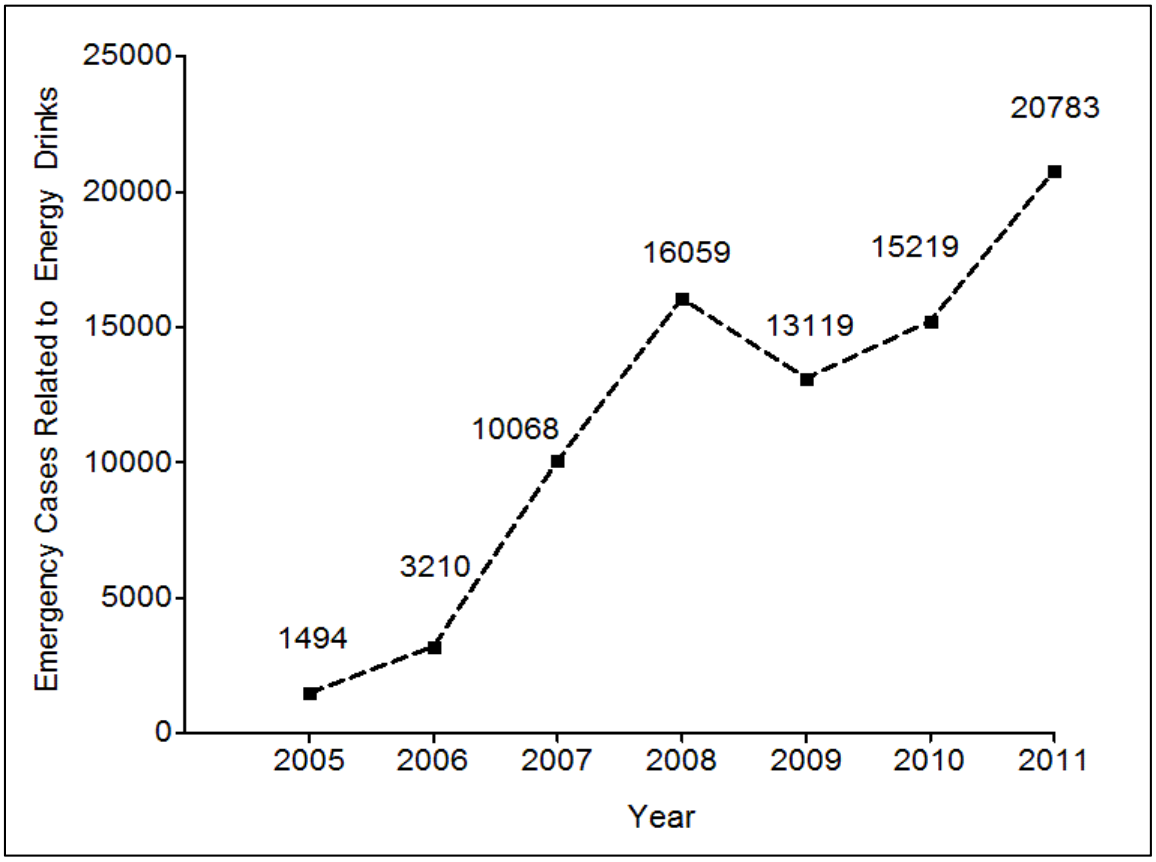

Figure 1-8: The number of reported emergency cases related to caffeinated energy drink consumption in the US (including all age groups and gender) (SAMHSA, 2013).

The ingestion of more than 1 g of caffeine is reported to be associated with acute caffeine overdose symptoms such as vomiting, abdominal pain, tremor, agitation, fluctuating level of consciousness, rigidity, and seizures (Holmgren et al., 2004). Caffeine overdose is also associated with rhabdomyolysis and subsequent acute renal failure (Wrenn and Oschner, 1989). Although rare, deaths have been reported with oral doses of more than 3 g of caffeine (Riesselmann et al., 1999). Caffeine overdose can result in stimulatory CNS effects which might lead to seizures, respiratory arrest, and death

(Kerrigan and Lindsey, 2005). Caffeine also stimulates the myocardium resulting in tachycardia, arrhythmia, and ventricular fibrillation leading to hypotension and sudden cardiac arrest (Dimaio and Garriott, 1974). Caffeine has been detected in the blood, urine, liver, kidney and gastric contents of dead victims with reported post-mortem caffeine blood concentrations exceeding 80 mg/L (Dimaio and Garriott, 1974; Leson et al., 1988; Riesselmann et al., 1999; Holmgren et al., 2004; House and Palmentier, 2004; Barbera et al., 2013; Jabbar and Hanly, 2013).

1.5. Research Objectives

Pharmacokinetic interaction between grapefruit juice and drugs metabolized by CYP3A4 has been studied extensively and health care providers are warned that such interactions might lead to serious health consequences in patients. However, little or no information is available on potential interactions between other furanocoumarin-containing herbs/food and pharmaceuticals, although it is increasingly clear that the furanocoumarins in numerous herbs/food might play an important role in herb-drug and food-drug interactions. The overall objectives of this thesis were: (a) to compare the pharmacokinetics of caffeine in humans before and after pre-treatment with the extract from a furanocoumarin-containing food/herb, (b) to elucidate the mechanism(s) of herb-caffeine interaction using *in vitro* incubations with pure furanocoumarin chemicals and human liver microsomes (HLMs), and (c) to predict *in vivo* furanocoumarin-caffeine (herb-drug interactions) in humans based on *in vitro* caffeine metabolism inhibition data and an integrated dose of the furanocoumarin mixture in the liver of humans.

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Chapter 2.

8-methoxypsoralen, 5-methoxypsoralen, and Isopimpinellin Concentrations in Extracts From the *Apiaceae* and *Rutaceae* Families of Plants

2.1. Abstract

Background: Previous studies have shown that the furanocoumarin chemicals in grapefruit play important roles in food-drug interactions (FDI). It is possible that the furanocoumarin constituents in herbal products may also inhibit the metabolism of conventional drugs after co-administration in humans.

Objectives: To identify and quantify furanocoumarin bioactive in selected food and herbs using gas chromatography mass spectrometry (GC-MS) and high performance liquid chromatography with ultra-violet detector (HPLC-UV) methods.

Methods: Ethanolic and aqueous extracts from 29 plant products belonging to the *Apiaceae, Lamiaceae, Leguminosae*, and *Rutaceae* families of plants were screened and quantified for furanocoumarin chemicals using GC-MS and HPLC-UV.

Results: 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and isopimpinellin (ISOP) were detected in the following herbal extracts: *Ammi majus* L. seeds, *Angelica archangelica* L. roots, *Angelica pubescens* Maxim. roots, *Apium graveolens* L. seeds, *Apium graveolens* L. flakes, *Cnidium monnieri* (L.) Cusson fruits, *Petroselinum crispum* (Mill.) Fuss leaves, *Pimpinella aniseum* L. seeds, and *Ruta graveolens* L. leaves. The amounts of furanocoumarins detected ranged from 0.016 to 11.468 mg/g dry weight of the plant products.

Conclusions: The results showed that linear furanocoumarins such as 8-MOP, 5-MOP, and ISOP were mainly found in the *Apiaceae* and *Rutaceae* families of plants.

2.2. Introduction

Furanocoumarins are an important group of phytochemicals found in the plant families of *Amaranthaceae, Apiaceae (Umbelliferae), Compositae (Asteraceae), Cyperaceae, Dipsacaceae, Goodeniaceae, Guttiferae (Clusiaceae), Leguminosae (Fabaceae or Papilionaceae), Moraceae, Pittosporaceae, Rosaceae, Rutaceae, Samydaceae, Solanaceae,* and *Thymelaeaceae* (Diawara and Trumble, 1997). Furanocoumarins such as psoralen, angelicin, 8-MOP and 5-MOP can cause phototoxic dermal reactions in humans and animals upon contact (Quinn et al., 2014). In Middle-Eastern traditional medicine, a combination of sunlight exposure and *Ammi majus* consumption is used to treat vitiligo, a skin disease related to the loss of pigment (El-Mofty, 1964). Thus, consumption of fruit and vegetables rich in furanocoumarin chemicals, *e.g.,* grapefruit, are known to interfere with the metabolism of drugs that are substrates of CYP3A4 isozyme (Schmiedlin-Ren et al., 1997) although grapefruit juice does not appear to have any effect on the pharmacokinetics of caffeine in humans (Maish et al., 1996).

Previous studies have shown that furanocoumarin constituents in many plant products are potent inhibitors of the cytochrome (CYP) P-450 system; these are capable of decreasing the systemic clearance of prescription drugs in humans after co-administration (Zhou et al., 2003). For example, bergamottin (He et al., 1998), 5,7-dihydroxybergamottin (Bellevue et al., 1997), and 5-MOP (Zaidi et al., 2007) are potent, irreversible inhibitors of CYP3A4 isozyme. 8-MOP also has been shown to be an irreversible inhibitor of CYP2A6 (Koenigs et al., 1997) and CYP2B1 (Koenigs and Trager, 1998) isozymes. ISOP (Kang et al., 2011), psoralen and angelicin (Zhuang et al., 2013) also are irreversible inhibitors of CYP1A2 isozyme

The core structure of furanocoumarins consists of a furan ring fused with a coumarin molecule. The simplest linear and angular furanocoumarin structures are represented by psoralen and angelicin, respectively (Figure 2-1). Numerous studies have detected the linear and angular furanocoumarins in herbal products, vegetables, and fruit using chromatographic techniques such as thin-layer chromatography (TLC) (Ivie, 1978; Cieśla et al., 2008); HPLC with UV detector (Beier et al., 1994; Kamiński et al., 2003; Frérot and Decorzant, 2004), fluorescent detector (FLD) (Frérot and Decorzant, 2004),

and MS detector (Dercks et al., 1990; Frérot and Decorzant, 2004; Peroutka et al., 2007). GC with MS detector (Beier et al., 1994; Peroutka et al., 2007) and flame ionization detector (FID) (Cardoso et al., 2000) were also used.

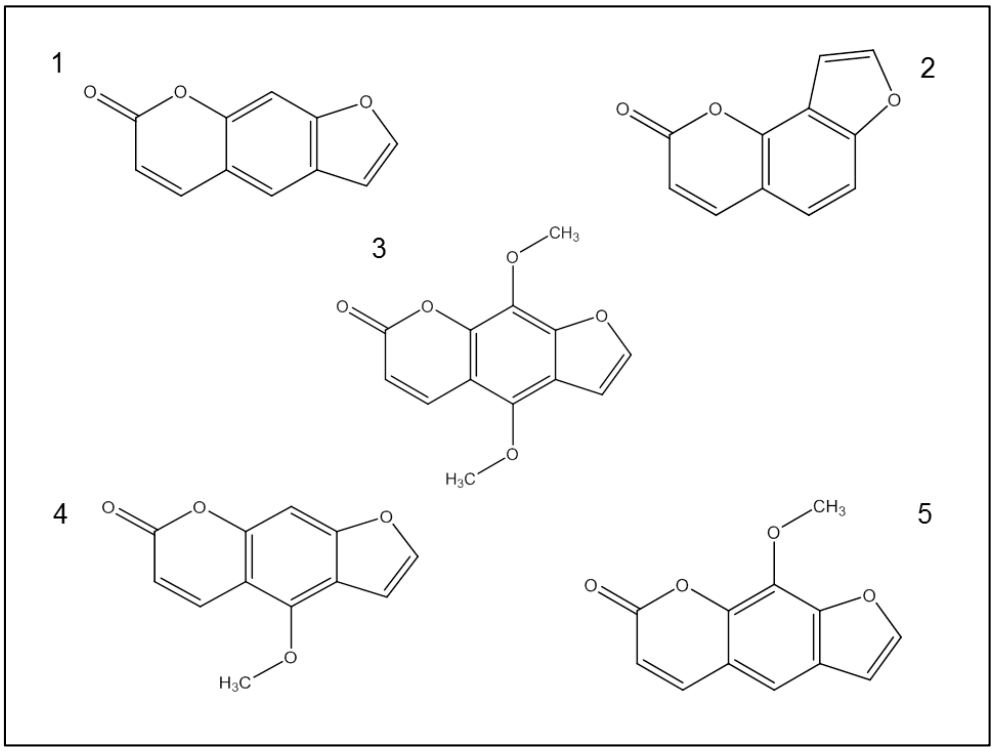

Figure 2-1: Chemical structures of psoralen (1), angelicin (2), ISOP (3), 5-MOP (4), and 8-MOP (5). All these are linear furanocoumarins except angelicin which is an angular furanocoumarin.

In this study, the furanocoumarins in 29 selected food and herbal products were identified and quantified using GC-MS and HPLC-UV. GC-MS was used to screen for both linear and angular furanocoumarins in the ethanolic extracts of these food and herbal products. The most common furanocoumarins in the ethanolic extracts were further quantified using HPLC-UV. Aqueous extracts were prepared according to the traditional method of preparing decoctions. The furanocoumarins in the aqueous extracts were quantified using HPLC-UV.

2.3. Materials and Supplies

2.3.1. Sources of herbs and food

Food and herbal products from the *Apiaceae*, *Lamiaceae*, *Leguminosae*, and *Rutaceae* families of plants were obtained from commercial sources in Canada, USA, and Jordan (refer to Appendix A for more information about the suppliers and origins of the food and herbs). The selection of food and herbal products was based on previous reports on the presence of furanocoumarins in these plant products. To the best of our knowledge, the plant products have been authenticated by the suppliers. They are also preservative-free, grown organically and/or wild-crafted. The food and herbal products were obtained in dry form to facilitate weighing and analysis.

2.3.2. Chemicals and instrumentation

Acetonitrile (≥99.9%), ethanol (99.9%), 8-MOP (≥98.0%), and 5-MOP (99.0%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). ISOP (98.3%) was obtained from ChromaDex (Irvine, CA, USA). Ultrapure water was produced using a Millipore system (Billerica, MA, USA) with a minimum resistivity of 18.0 MΩ•cm at 25°C. Screening for furanocoumarins in the herbal extracts was performed using a GC-MS system comprised of an Agilent 6890 GC, 5973 MS, 7683B auto sampler, and controlled by ChemStation software (version D.01.02.16) (Santa Clara, CA, USA). Selected linear furanocoumarins were quantified in aqueous herb extracts using an Agilent 1090 HPLC, 79880A UV, 1046A FLD, and controlled by ChemStation software (version A.10.02).

2.4. Experimental Methods

2.4.1. Screening analysis for linear and angular furanocoumarins in the ethanolic extracts of herbs/food using GC-MS.

Ethanol is used to extract the plant products because furanocoumarins are soluble in this organic solvent. It should be noted that ethanol also is used to prepare traditional decoction and tincture (Duke et al., 2002).

Table 2-1: List of 29 selected food and herbal products from the *Apiaceae, Lamiaceae, Leguminosae, and Rutaceae* plant families for this study.

Botanical Name	Common Name	Plant Family	Plant Part
Ammi majus L.	Khella Shaitani	*Apiaceae*	Seeds
Ammi visnaga L.	Khella	*Apiaceae*	Seeds
Angelica archangelica L.	Garden Angelica	*Apiaceae*	Roots
Angelica dahurica (Hoffm.) Maxim	Bai Zhi	*Apiaceae*	Roots
Angelica pubescens Maxim	Du Huo	*Apiaceae*	Roots
Angelica sinensis (Oliv.) Diels	Dong Gui	*Apiaceae*	Roots
Anethum graveolens L.	Dill	*Apiaceae*	Leaves
Anethum graveolens L.	Dill	*Apiaceae*	Seeds
Anthriscus cerefolium Hoffm.	Chervil	*Apiaceae*	Leaves
Apium graveolens L.	Celery	*Apiaceae*	Seeds
Apium graveolens L.	Celery	*Apiaceae*	Flakes
Carum carvi L.	Caraway	*Apiaceae*	Seeds
Citri reticulatae Blanco	Chen Pi	*Rutaceae*	Peels
Cnidium monnieri (L.) Cusson	Shi Chuang Zi	*Apiaceae*	Seeds
Coriandrum sativum L.	Coriander	*Apiaceae*	Seeds
Cuminum cyminum L.	Cumin	*Apiaceae*	Seeds
Foeniculum vulgare Mill.	Fennel	*Apiaceae*	Seeds
Levisticum officinale W.D.J.Koch	Lovage	*Apiaceae*	Root
Levisticum officinale W.D.J.Koch	Lovage	*Apiaceae*	Leaves
Ligusticum chuanxiong S.H.Qiu , Y.Q. Zeng, K.Y.Pan , Y.C.Tang & J.M.Xu	Chuan Xiong	*Apiaceae*	Roots
Ligusticum porteri J.M.Coult. & Rose	Osha	*Apiaceae*	Roots
Ligusticum sinense Oliv.	Gao Ben	*Apiaceae*	Roots
Ocimum basilicum L.	Basil	*Lamiaceae*	Leaves
Pastinaca sativa L.	Parsnip	*Apiaceae*	Roots
Petroselinum crispum (Mill.) Fuss	Parsley	*Apiaceae*	Leaves
Petroselinum crispum (Mill.) Fuss	Parsley	*Apiaceae*	Roots
Pimpinella aniseum L.	Anise	*Apiaceae*	Seeds
Psoralea corylifolia L.	Bu Gu Zhi	*Leguminosae*	Seeds
Ruta graveolens L.	Common Rue	*Rutaceae*	Leaves

The different food and plant products were minced separately and reduced to fine powder using a Salton food processor (Dollard-des-Ormeaux, QC, Canada) model CG-1174. Precisely 2.0 g of dry herb/food powder was weighed using a Sartorius analytical balance (Goettingen, Germany) model 2004 MP and mixed with 200.0 mL ethanol. The ethanolic mixture was sonicated using a Branson sonicator (Shelton, CT, USA) for 3 h under atmospheric pressure with a measured temperature range between 40-60ºC. The extracts were cooled to room temperature. Ethanol was added back to the mixture to replace for the volume lost during extraction. The mixture was filtered by a Millipore Millex-LG filter unit (0.2 µm), and analyzed by a GC-MS system. Furanocoumarins in the extracts were identified and quantified using the GC-MS method of Peroutka et al. (2007) with modifications. The GC-MS was equipped with an Agilent HP-5 MS column (30 m × 0.25 mm, 0.25 µm film thickness). The following temperature program was used to conduct the analysis: 75ºC (0 - 2 min), 75-250ºC (2 - 20 min), 250 - 280^{0}C (20 - 25 min), and 280ºC (25 – 30 min). The volume of injection was 1.0 µL in split-less mode. Helium was used as the carrier gas at a constant flow rate set at 0.5 mL/min. The MS was set at full scan mode from 50 to 600 *m/z* range. The remaining method settings and zone temperatures were similar to that of the published method.

2.4.2. Quantitative analysis of 8-MOP, 5-MOP, and ISOP in the aqueous extracts of herbs using HPLC-UV.

The powdered forms of foods or herbal product were also mixed with 600.0 mL of tap water and boiled under atmospheric pressure with occasional stirring on a Fisher Thermix hot plate model 11-493 (Hampton, NH, USA) until half of the volume was evaporated. Once cooled to room temperature, the aqueous mixture was filtered by a Millipore Millex-LG filter unit (0.2 µm). The filtrate was analyzed by the HPLC-UV. Table 2-2 summarizes the amounts of herbal products used in the extraction. In general, they were the administered doses in previous herbal studies.

The HPLC-UV method used to analyze 8-MOP, 5-MOP, and ISOP in the aqueous extracts was modified from the procedure of Frerot and Decorzant (2004). The furanocoumarins were separated by gradient elution. The mobile phase consisted of various proportions of acetonitrile (A) in water at different HPLC run times: 0-35 min (A, 5-

100%), 35-40 min (A, 100%), and 40-45 min (A, 100-5%). The injection volume was 5.0 µL and the flow rate of the mobile phase was 1.0 mL/min at room temperature. The UV detector wavelength was set at 310 nm, with 550 nm as the reference wavelength. The furanocoumarins were separated using two HPLC phenyl-based reverse-phase columns from Phenomenex (Torrance, CA, USA): a Kinetex pentafluorophenyl (PFP) column (250 x 4.6 mm, 5 µm particle size) and a Kinetex phenyl-hexyl (P-H) column (250 x 4.6 mm, 5 µm particle size). The Agilent Zorbax XDB reverse-phase C-18 column (250 x 4.6, 5 µm particle size) was also used to compare the analytical results.

Table 2-2: The amounts of herbs used to prepare aqueous extracts for the pharmacokinetic studies.

Botanical Name	Reported Single Dose Range (g)	Amount Used in Present Study (g)	Reference
A.majus seeds	6.0 - 12.0 (dry)	6.0 (dry)	Lerner et al. (1953)
A.archangelica roots	0.5 - 4.5 (dry)	4.5 (dry)	Duke et al. (2002)
A.pubescens roots	Up to 10.0 (dry)	12.0 (dry)	Jianhua et al. (2009)
A.graveolens seeds	Up to 100 (fresh)	10.0 (dry)	Rastmanesh and Baer (2011)
A.graveolens flakes	1.0 – 13.0 (dry)	10.0 (dry)	Duke et al. (2002)
C.monnieri fruits	3.0 – 10.0 (dry)	3.0 (dry)	Jianhua et al. (2009)
P.crispum leaves	0.5 – 20.0 (dry)	10.0 (dry)	Duke et al. (2002)
P.aniseum seeds	Up to 50 (fresh)	10.0 (dry)	Rastmanesh and Baer (2011)
R.graveolens leaves	0.5 – 10.0 (dry)	3.0 (dry)	Duke et al. (2002)

2.4.3. Calibration curves and data analysis

The furanocoumarins in the ethanolic extracts of foods and herbs were measured semi-quantitatively based on a one-point standard concentration; 3140, 303, and 153 µg/mL for 8-MOP, 5-MOP, and ISOP, respectively. Individual furanocoumarins in the aqueous extracts were determined quantitatively using calibration curves prepared from a range of standard concentrations: 8-MOP (6.13 – 196.25 µg/mL), 5-MOP (4.74 – 151.67 µg/mL), and ISOP (2.4 – 153.3 µg/mL). The concentrations of 8-MOP, 5-MOP, and ISOP in the herbal extracts were determined by linear regression analysis using Microsoft Excel software version 2010 (Redmond, WA, USA). The limit of detection (LOD) and limit of quantification (LOQ) of the furanocoumarins were calculated from the calibration curve based on DIN 32645 method (DIN, 1994) using B.E.N. software version 2 (Herbold and

Schmitt, 2000). 8-MOP, 5-MOP, and ISOP concentrations were expressed as the mean ± standard deviation (SD) of HPLC-UV results from a minimum of three separate extractions.

2.5. Results and Discussion

2.5.1. GC-MS screening results

The presence of linear and angular furanocoumarins was screened in 29 food and herbal products from the *Apiaceae*, *Lamiaceae*, *Leguminosae*, and *Rutaceae* families of plants using GC-MS. Furanocoumarins were detected in the following herbal extracts: *A.majus* roots, *A.archangelica* roots, *A.pubescens* roots, *A.graveolens* seeds and flakes, *C.monnieri* fruits, *L.officinale* roots, *P.crispum* leaves, *P.aniseum* seeds, *P.corylifolia* fruits, and *R.graveolens* leaves. The most common furanocoumarins detected were 8-MOP, 5-MOP, and ISOP (Table 2-3). These findings were consistent with the report that 8-MOP and 5-MOP were the most common furanocoumarins in these plants (EMEA, 2007).

The furanocoumarins were identified initially based on the retention times of standard chemicals and mass spectra search in the GC-MS database. The retention time of 8-MOP, 5-MOP, and ISOP were 10.24, 10.35, and 11.20 min, respectively (Figures 2-2 and 2-3). The mass spectra of 8-MOP and 5-MOP showed the base peak of 216 m/z followed by ion 173 *m/z*. On the other hand, ISOP had a base peak at 231 *m/z* followed by ion 246 *m/z* (refer to Appendices B to D for more information). Each peak was identified in the chromatograms using the National Institute of Standards and Technologies (NIST) library database search function. However, due to the complex nature of herbal matrices, ions 216 *m/z* and 246 *m/z* were used to measure the abundance of 8-MOP, 5-MOP, and ISOP in the food and herbal extracts (Figures 2-2 to 2-3).

Table 2-3: Semi-quantitative results for 8-MOP, 5-MOP, and ISOP in ethanolic extracts of selected plant products using GC-MS.

Botanical Name	Plant part	Linear Furanocoumarins Content (µg/g) Dry Weight						
		8-MOP		5-MOP		ISOP		Total
A.majus	Seeds	2349.0	a	380.9	a	5553.7	a	8283.6
A.archangelica	Roots	73.3	a	180.5	a	463.8	a	717.6
A.pubescens	Roots	16.4	a	11.5	a	n.d.	c	28.0
A.graveolens	Seeds	4.3	b	7.3	b	16.2	b	27.8
A.graveolens	Flakes	7.4	b	3.0	b	8.2	b	18.6
C.monnieri	Fruits	442.1	a	252.5	a	1480.4	a	2175.1
P.crispum	Leaves	n.d.	c	14.3	b	n.d.	c	14.3
P.aniseum	Seeds	5.8	a	n.d.	c	n.d.	c	5.8
R.graveolens	Leaves	458.7	a	171.2	a	43.6	a	673.4

[a] Identification confirmed by retention time and mass spectrum

[b] Identification confirmed by retention time only.

[c] n.d. = not detected.

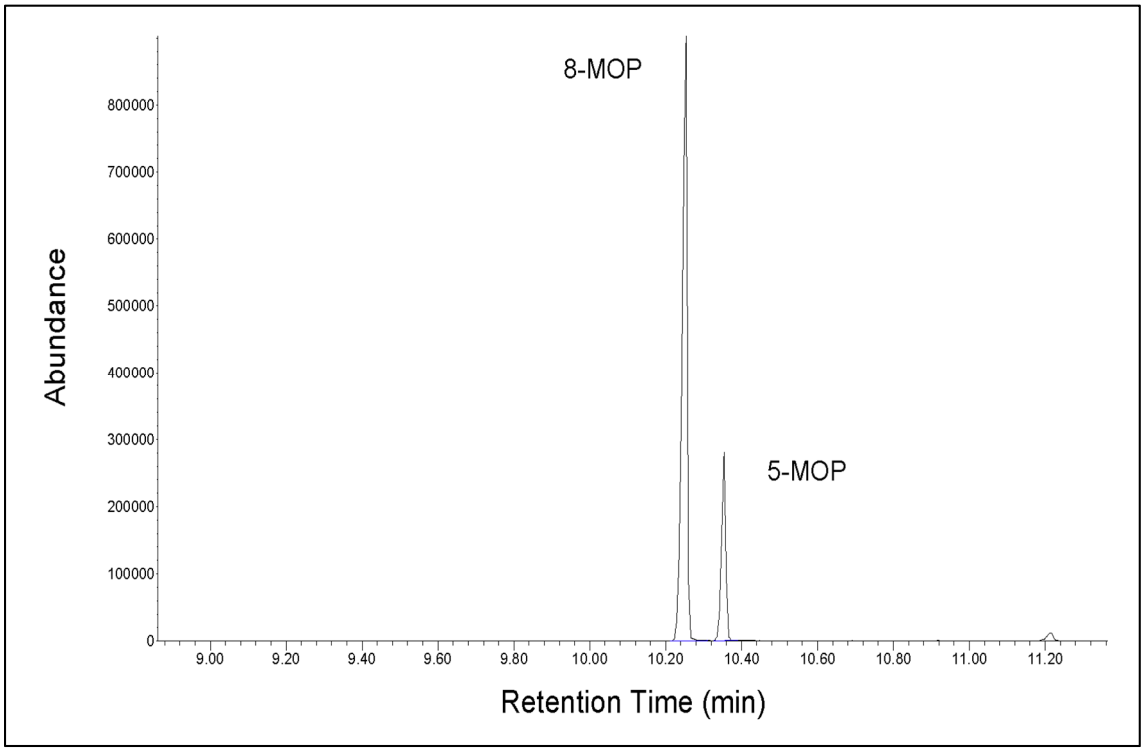

Figure 2-2: GC-MS chromatogram of extracted ion 216 for 8-MOP and 5-MOP.

Several minor and less common furanocoumarins were also detected in the ethanolic extracts of herbs. For example, *A.archangelica* roots contained the angular furanocoumarin pimpinellin with retention time 10.69 min and a NIST match score of 98%. Also detected were the linear furanocoumarins of sphondin, oxypeucedanin hydrate, neobyakangelicol, and byakangelicin with retention times at 10.34, 14.34, 14.83, and 15.70 min and NIST match scores of 95, 93, 94, and 98%, respectively. The angular furanocoumarin, isobergapten was detected in *A.archangelica* and *L.officinale* roots with retention times 10.2 and 10.19 min and NIST match scores of 91 and 93%, respectively. The angular furanocoumarin, angelicin was detected in *A.archangelica* roots and *P.corylifolia* fruits with 8.93 and 8.91 min retention times, respectively and a NIST match score of 97% for both herbs. The linear furanocoumarin, psoralen was detected in *P.corylifolia* fruits and *R.graveolens* leaves with retention times of 9.19 and 9.18 min, respectively and NIST match scores of 98 and 96% respectively. Refer to Appendices E-K for more information on the chemical structures and fragmentation ions for the detected furanocoumarins in this study.

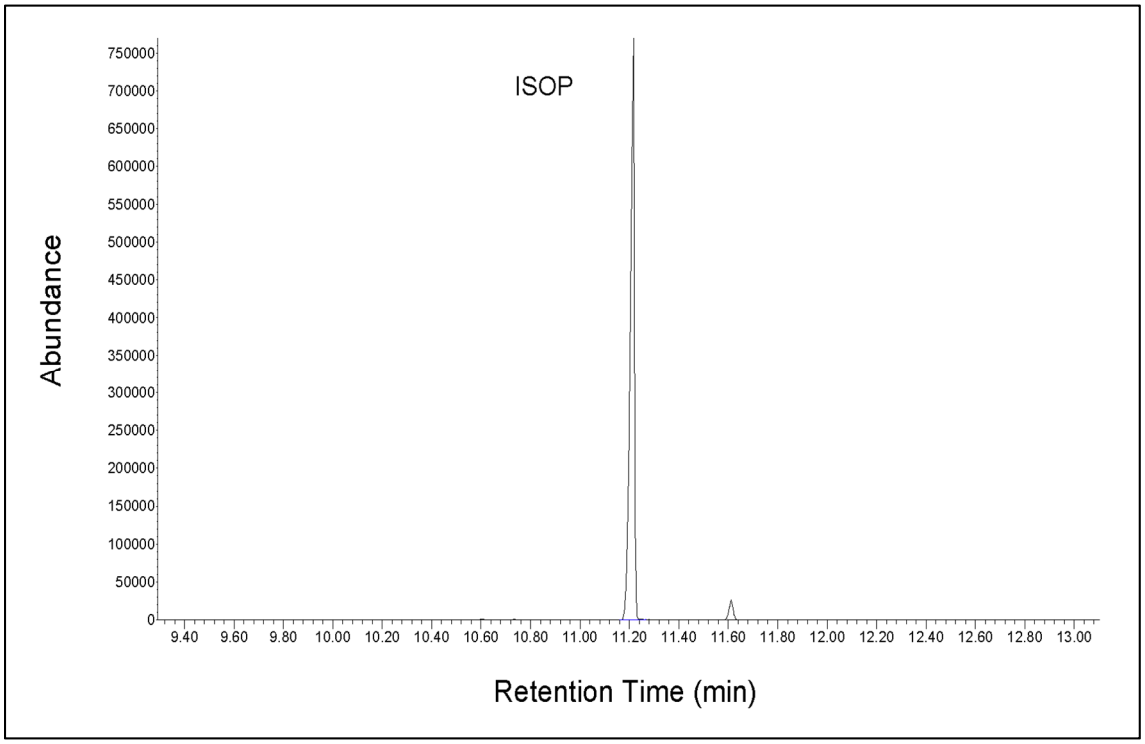

Figure 2-3: GC-MS chromatogram of extracted ion 246 for ISOP.

2.5.2. HPLC-UV quantitative results

In addition to GC-MS screening, 8-MOP, 5-MOP, and ISOP concentrations in the aqueous extracts of the plant products were quantified using HPLC-UV. The HPLC-UV was used to measure 8-MOP, 5-MOP, and ISOP concentrations in the aqueous extracts to avoid intensive sample preparation before analysis. The main reason of extracting the herbal products with water was to mimic the traditional method of decoction preparation for pharmacokinetic interaction studies in Chapter 3. Nevertheless, using a HPLC gradient elution method consisting of acetonitrile and water, the PFP column successfully separated 8-MOP, ISOP, and 5-MOP with retention times of 16.30, 17.23, 17.87 min, respectively (Figure 2-4). In contrast, the P-H column could only partially separate 8-MOP, 5-MOP, and ISOP with retention times at 16.53, 17.44, and 17.50 min, respectively (Figure 2-5). The C-18 column was unable to separate angelicin from 8-MOP. It also did not separate 5-MOP from ISOP despite using different solvents and time programming in the gradient elution (data not shown).

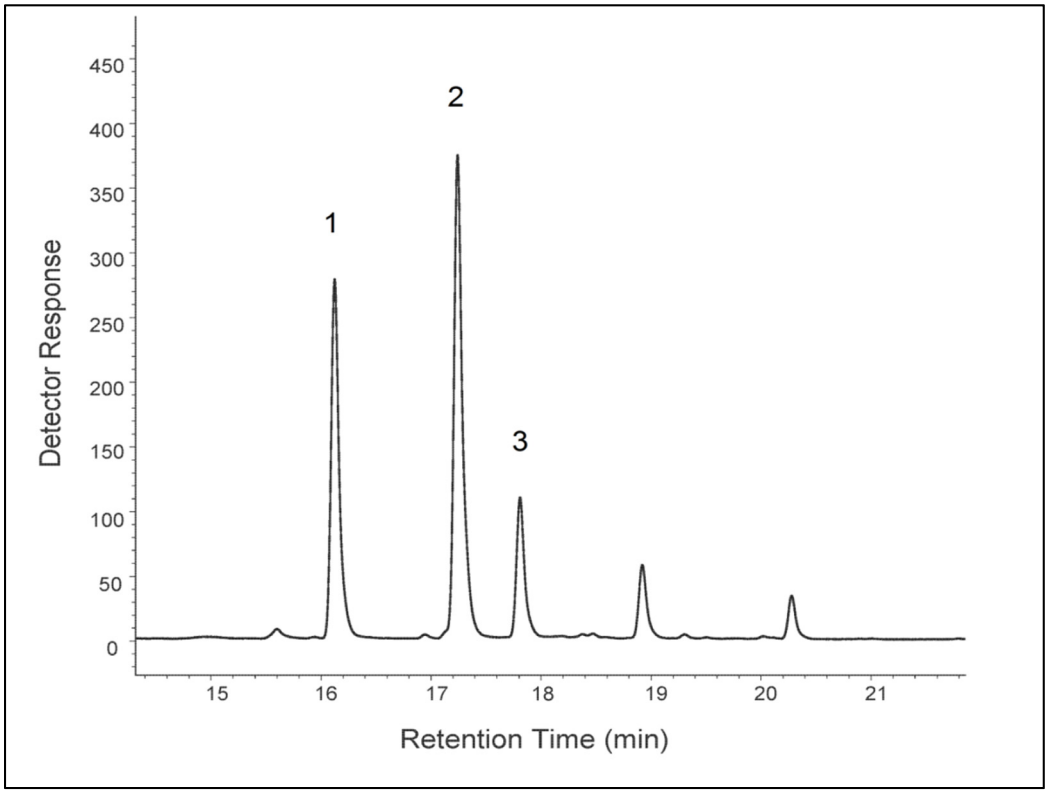

Figure 2-4: HPLC-UV chromatogram of linear furanocoumarins using the PFP column. Peak 1 represents 8-MOP; peak 2 represents ISOP; peak 3 represents 5-MOP.

Previous HPLC methods for the separation of 8-MOP, 5-MOP, and ISOP have been reported. For example, Beier (1985) reported the separation of three furanocoumarins using a reverse-phase phenyl column and an isocratic system of methanol and water (44/56 v/v) with retention times between 5 and 10 min. Liu et al. (2004) separated the three furanocoumarins using a gradient method with methanol-acetonitrile-water and reverse-phase C-18 column with retention times between 5 and 10 min. Li and Chen (2005) used a gradient method of methanol and water with reverse-phase C-18 column and managed to separate the three furanocoumarins with retention times ranging between 2 and 4 min. Although these methods have successfully separated 8-MOP, 5-MOP, and ISOP in *C.monnieri* and *A.graveolens* extracts, they could not be used in the present study due to the complex chemical mixtures of other herbs. For example, *A.archangelica* root contained other chemicals that interfered with the analysis of furanocoumarins.

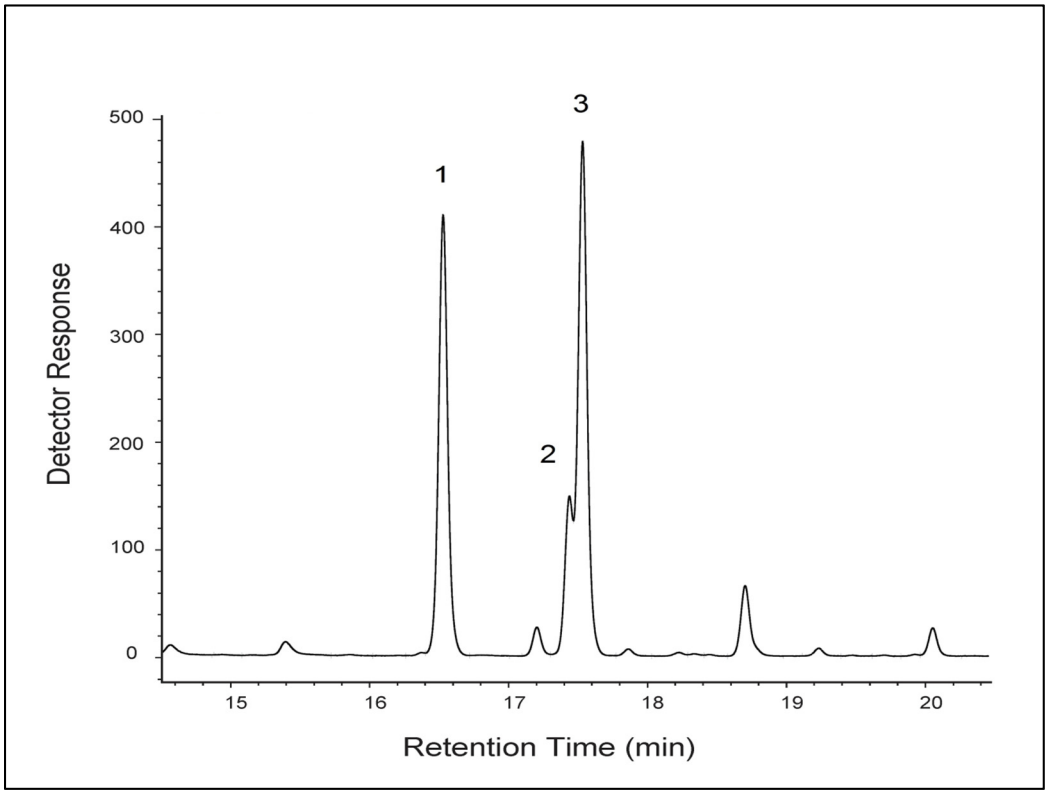

Figure 2-5: HPLC-UV chromatogram of linear furanocoumarins using the P-H column. Peak 1 represents 8-MOP; peak 2 represents 5-MOP; peak 3 represents ISOP.

Wszelaki et al. (2011) used a gradient system of formic acid-acetonitrile-water (0.1/5/95 v/v/v) and formic acid-acetonitrile (0.1/100 v/v) with a reverse-phase C-18 (1.9 µm particle size) column to separate the three furanocoumarins in *A.archangelica* root with retention times between 5 and 7 min for 8-MOP, 5-MOP, and ISOP. Frerot and Decorzant (2004) also separated the furanocoumarins using a gradient system of water-acetonitrile-tetrahydrofuran (85/10/5 v/v/v) and acetonitrile-methanol-tetrahydrofuran (65/30/5) with reverse-phase C-18 (3.1 µm particle size) column to separate 15 furanocoumarins in citrus oils with retention times between 20 and 25 min for 8-MOP, 5-MOP, and ISOP. Although both methods were able to separate the furanocoumarins in complex matrices, they could not be implemented by the HPLC system due to the elevated flow back pressure.

Table 2-4: Analytical limits for the detection of individual linear furanocoumarins using HPLC-UV with PFP column.

Furanocoumarin	LOD	LOQ
	µg/mL	
8-MOP	7.8	29.1
5-MOP	2.1	7.8
ISOP	6.3	23.3

Using UV spectra to identify furanocoumarins was not feasible as linear and angular furanocoumarins exhibit very similar UV spectra (Frerot and Decorzant, 2004). This is especially problematic when several furanocoumarins co-elute with one another (data not shown). In addition to the retention time of reference materials, the identities of 8-MOP, 5-MOP, and ISOP were confirmed by spiking the extracts with small amounts of pure standards. Although the observed maximum absorbance for the furanocoumarins in this study was between 218 and 223 nm, the UV wavelength of 310 nm was chosen for the detection and quantification of 8-MOP, 5-MOP, and ISOP as the sensitivity was reasonable at that specific wavelength (Table 2-4), and it reduced the spectral interferences from the herbal extract matrices. The use of HPLC-FLD was also explored to detect linear and angular furanocoumarins because they are fluorescent compounds but such attempt was not successful. The FLD was not useful in the present study, a finding which is in agreement with the results of Frerot and Decorzant (2004). The presence of furanocoumarins in the herbal extracts supports the findings of previous

studies that furanocoumarins, such as those in this study, do not appear to be degraded by conventional cooking or traditional decoction preparation methods (Ivie et al., 1981).

The furanocoumarin levels of this study (Table 2-5) are in agreement with those reported in previous studies. For example, Duke (1988) have reported the content of 8-MOP in seeds of *A.majus* seeds to range from 2.3 to 5.8 mg/g and we have found 3.2 mg/g in this study. The content of 5-MOP is reported to range from 0.4 to 3.1 mg/g which also is in agreement with our result of 0.72 mg/g. Krivut and Perel'son (1967) have determined that the content of ISOP in *A.majus* seeds range from 0.47 to 0.64% which are comparable to our result of 0.75%. The later study also has reported 8-MOP and 5-MOP contents range from 0.18 to 0.37% and 0.21 to 0.24%, respectively, which are in agreement with our results of 0.32 and 0.07%, respectively.

Table 2-5: 8-MOP, 5-MOP, and ISOP contents of furanocoumarin-containing herbs based on aqueous extraction.

Botanical Name	Plant part	Linear Furanocoumarins Content (µg/g dry weight) [a]									
		8-MOP			5-MOP			ISOP			Total
A.majus [b]	Seeds	3213.5	±	219.7	717.2	±	6.5	7537.2	±	1492.9	11467.8
A.archangelica [b]	Roots	651.4	±	51.8	392.5	±	208.0	606.0	±	131.9	1650.0
A.pubescens [c]	Roots	25.6	±	20.0	32.5	±	20.0	n.d. [d]			58.1
A.graveolens [b]	Seeds	21.0	±	4.4	16.9	±	6.0	236.5	±	22.4	274.4
A.graveolens [b]	Flakes	12.1	±	1.6	243.2	±	39.7	9.5	±	0.9	264.9
C.monnieri [b]	Fruits	707.1	±	78.8	466.8	±	95.4	1788.1	±	152.3	2962.0
P.crispum [b]	Leaves	n.d. [d]			34.4	±	10.6	n.d. [d]			34.4
P.aniseum [b]	Seeds	15.8	±	5.8	n.d. [d]			n.d. [d]			15.8
R.graveolens [b]	Leaves	1342.4	±	135.7	534.0	±	120.6	294.9	±	49.5	2171.2

[a] Results are expressed as mean ± SD.

[b] Based on three separate aqueous extracts.

[c] Based on five separate aqueous extracts.

[d] n.d. = not detected.

Numerous studies have identified the presence of 8-MOP, 5-MOP, and ISOP in *A.archangelic*a roots (Härmälä et al., 1992; Eeva et al., 2004). Chalchat and Garry (1993) quantified 5-MOP in pentane extracts of *A.archangelica* roots at 8.8% dry weight which is much higher than our result of 0.04% dry weight in water. Based on our best effort, we have not been able to find any reports on the contents of 8-MOP and ISOP in *A.archangelica* roots. In this study, we have determined the contents of 8-MOP and ISOP, for the first time, in *A.archangelica* roots to be 0.07% and 0.06% dry weight, respectively.

Previous studies have reported that about 5 ng/g dry weight of 8-MOP and 5-MOP were found in *P.aniseum* seeds using ultrasensitive bioassays (Ceska et al., 1987). In contrast, we have found much higher 8-MOP contents (15.8 µg/g dry weight) and no 5-MOP in *P.aniseum* seeds. Milesi et al. (2001) reported the contents of 8-MOP, 5-MOP, and ISOP in *R.graveolens* leaves to be 1.0 ± 0.6 mg/g, 1.6 ± 0.5 mg/g, and 0.1 ± 0.1 mg/g dry weight, respectively. We also have detected these linear furanocoumarins at comparable concentrations of 1.34 mg/g, 0.53 mg/g, and 0.30 mg/g dry weight, respectively. Beier et al. (1983) detected 8-MOP, 5-MOP, and ISOP in the leaves of healthy *A.graveolens* from three different locations in the United States; total furanocoumarin content, based on 8-MOP, 5-MOP, and ISOP, was about 0.73 ng/g fresh weight. Diawara et al. (1995) also reported finding of 8-MOP and 5-MOP in various parts of healthy *A.graveolens;* total linear furanocoumarins found ranged from 0.8 to 49.84 µg/g fresh weight. We have found much larger amounts of linear furanocoumarins in the present study (265 µg/g dry weight) probably because our results are based on the dry weights of leaves and stems.

Chen et al. (1995) reported the detection of 8-MOP and 5-MOP in the extract of *A.pubescens* roots. Lin et al. (2009) reported the presence of 5-MOP in *A.pubescens* roots with an average concentration of 7.4 µg/g dry weight. However, they did not find any 8-MOP in the *A.pubescens* root extracts. We were able to find both 8-MOP and 5-MOP in *A.pubescens* root extract at 26 and 33 µg/g dry weight, respectively. Beirer et al. (1994) found 8-MOP, 5-MOP, and ISOP in dried *P.crispum* leaves ranging from 5.3-53.0 µg/g, 56.7-146.7 µg/g, and 15.7-79.8 µg/g dry leaves weight, respectively. In contrast, we only found 5-MOP in *P.crispum* leaves at 34.0 µg/g dry leaves weight. Yan et al. (2001) analyzed 53 samples of *C.monnieri* fruits from various locations in China and reported 8-

MOP, 5-MOP, and ISOP concentrations ranging from 0.03-0.30%, 0.01-0.26%, and 0.02-0.32% dry weight, respectively. Our results show an average of 0.07%, 0.05%, and 0.18% dry weight for 8-MOP, 5-MOP, and ISOP, respectively.

Worth noting, we were unable to detect any furanocoumarins in the ethanolic and aqueous extracts of other plant products which have been shown to contain such chemicals in published studies. These herbal products include *A.visnaga* seeds (Sellami et al., 2013), *A.dahurica* roots (Zhang et al., 2009), *A.sinensis* roots (Noe, 1997, as cited in Al-Bareeq et al., 2010), *Citrus* peels (Siskos et al., 2008). *F.vulgare* seeds (Zaidi et al., 2007), *Anethum graveolens* leaves (Szopa and Ekiert, 2015), *C.carvi* and *C.sativum* seeds (Ceska et al., 1986), and *P.sativa* root (Lombaert et al., 2001). An explanation for the discrepancy in results between this study and other studies is not readily available but maybe related to the following effects on furanocoumarin content in plants: diseases and infection (Lord et al., 1988), ultra-violet light, nutrient levels (Zangerl and Berenbaum, 1987), geographical locations (Sigurdsson et al., 2012), seasonal changes (Zobel and Brown, 1990), pollution levels (Dercks et al., 1990), fungicide (Nigg et al., 1997), and storage conditions (Chaudhary et al., 1985).

2.6. Conclusions

In summary, 8-MOP, 5-MOP, and ISOP are among the most common furanocoumarin constituents in plant products chosen for this study. The aforementioned furanocoumarins are well known inhibitors of hepatic mixed-function oxidases. Results of the studies show the HPLC phenyl-based PFP column provided the best separation for 8-MOP, 5-MOP, and ISOP in aqueous herbal extracts. The HPLC method used in the present study is simple and cost-effective. The mobile phase consists of a solution of acetonitrile and water. The method also can be used to separate other furanocoumarin constituents in herbal extracts with only minor adjustment(s) of flow rate, solvent, and/or gradient elution programing. It is important to identify and quantify the furanocoumarin bioactive(s) in herbs before embarking on a pharmacokinetic interaction study in humans as the contents and composition of furanocoumarins vary greatly in the plant products.

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Chapter 3.

Reduction of Caffeine Clearance in Humans after Consuming Herbal Extracts from the *Apiaceae* and *Rutaceae* Plant Families

3.1. Abstract

Background: Caffeine is one of the most widely used drugs in the world. A majority of the absorbed caffeine is metabolized by hepatic P-450 cytochrome (CYP450) 1A2 before elimination. Previous *in vitro* and *in vivo* studies have shown that the furanocoumarins in foods especially those in grapefruit, bitter orange, and pomelo are potent mechanism-based inhibitors of human CYP450 enzymes.

Objectives: The goals of this study were (a) to study the pharmacokinetics of caffeine in humans before and after pre-treatment with an extract from a furanocoumarin-containing herb, and (b) to determine if herbal pretreatment would significantly reduce the oral clearance (CL) of caffeine with a concomitant increase area under the concentration-time curve (AUC) in the plasma.

Methods: This was a randomized, unblinded, and crossover pharmacokinetic study. Volunteers from Metro Vancouver, British Columbia, Canada were given an oral dose of caffeine tablets (200.0 mg) with and without pre-treatment of an aqueous extract from the following plant products: *Ammi majus* L. seeds, *Angelica archangelica* L. roots, *Angelica pubescens* Maxim. roots, *Apium graveolens* L. seeds, *Apium graveolens* L. flakes, *Cnidium monnieri* (L.) Cusson fruits, *Petroselinum crispum* (Mill.) Fuss leaves, *Pimpinella aniseum* L. seeds, or *Ruta graveolens* L. leaves.

Results: Consumption of *A.majus*, *A.archangelica*, *A.pubescens*, *C.monnieri*, and *R.graveolens* significantly increased the AUCR of caffeine in the volunteers by 4.3, 2.3, 1.7, 2.2, and 1.3 fold, respectively. The aqueous extracts of *A.graveolens* (both seeds and flakes), *P.crispum*, and *P.aniseum* did not seem to alter the pharmacokinetics of caffeine in volunteers significantly.

Conclusions: Results of these studies showed that co-administration of caffeine and furanocoumarin-containing herbs significantly inhibited caffeine metabolism resulting in increases in caffeine plasma concentrations. As such, caffeine sensitive individuals should avoid consuming caffeine with drugs which were metabolized by CYP1A2 enzyme.

3.2. Introduction

Caffeine is probably the most popular stimulant drug; it is consumed by millions of people around the world. Caffeine, a secondary metabolite synthesized by plants, acts as a natural defense against plant-feeding insects (Levinson, 1976). Caffeine is found naturally in many widely consumed plants under the genera of *Camellia*, *Coffea*, *Cola*, *Ilex*, *Theobroma*, *Herrania*, *Paullinia,* and *Citrus maxima* (Ashihara et al., 2011). Caffeine is an antagonist of the neuromodulator, adenosine, and causes stimulatory effects in humans (Biaggioni et al., 1991). Previous studies have concluded that caffeine is an addictive drug which may lead to withdrawal symptoms and side effects such as fatigue, anhedonia, sleepiness, headaches, anxiety, decreased motor behavior, increased heart rate, increased muscle tension, tremor, nausea, and vomiting (Fredholm et al., 1999). Many studies have also associated frequent caffeine consumption with adverse health effects involving the cardiovascular, reproductive, and central nervous systems (Dews, 1982).

Caffeine is used clinically to treat neonatal respiratory depression, obesity, postprandial hypotension, migraine and non-migraine headaches, and pain (Sawynok, 1995). Caffeine is rapidly and completely absorbed after oral administration; it peaks in plasma at 30-60 min after oral consumption. The reported elimination half-life ranges from 2.7 to 9.9 h indicating significant variability in humans (Blanchard and Sawers, 1983). Caffeine pharmacokinetics can be influenced by numerous factors such as ethnicity, age, gender, pregnancy, obesity, extent of physical exercise, alcohol use, diet, medications, liver diseases, and smoking (Arnaud, 2011). Caffeine undergoes minimal first-pass effect after ingestion and is distributed rapidly to plasma, saliva, cerebral spinal fluid, and urine (Carrillo and Benitez, 2000). Several studies have shown good correlation between blood levels and salivary concentrations of caffeine in humans (Newton et al., 1981; Zylber-Katz et al., 1984; Carrillo et al., 2000; Perera et al., 2010). As a result, saliva has become the

body fluid of choice to study the pharmacokinetics of caffeine in humans as it is a non-invasive and convenient method of sample collection (Suzuki et al., 1989).

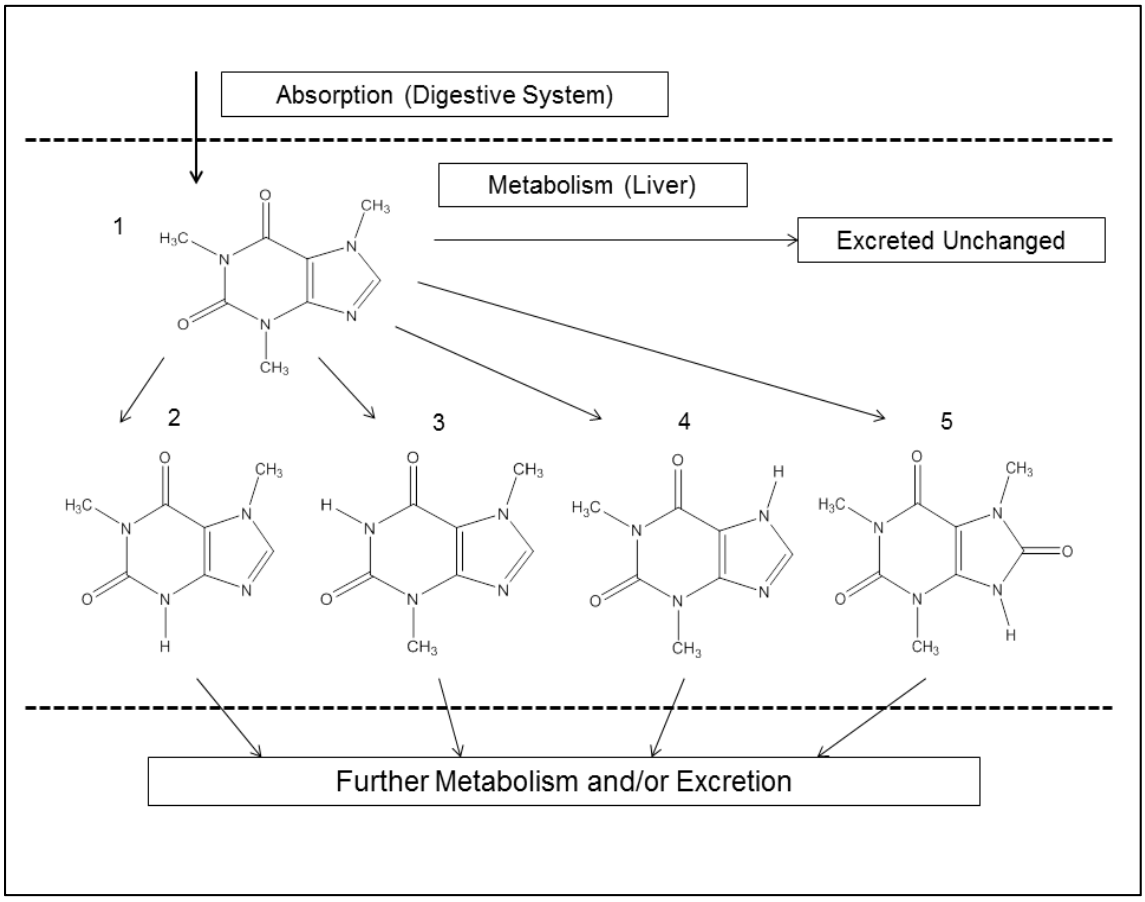

Figure 3-1: Major caffeine (1) metabolites of paraxanthine (2), theobromine (3), theophylline (4), and 1,3,7-trimethyluric acid (5).

In humans, caffeine is extensively metabolized and only 3% or less being excreted unchanged in the urine (Tang-Liu et al., 1983). The main route of caffeine metabolism in human is by 3-*N*-demethylation to produce paraxanthine mediated by CYP1A2. Other minor pathways of metabolism involve isoforms such as CYP2C8/9, 2E1, and 3A4 (Figures 1-6 and 3-1) (Gu et al., 1992; Kot and Marta, 2008). Therefore, caffeine is used as a probe drug for *in vivo* CYP1A2 activity with the relative ratios of urinary metabolites used as an indicator of the flux through different parts of the pathway (Doehmer et al., 1992; Miners and Birkett, 1996).

Natural health products (NHP) are believed to be safe and harmless (Ipsos Reid, 2011). Plants are the most common form of NHP and are frequently consumed concomitantly with modern medicines (Fugh-Berman, 2000). A plant product usually contains a complex mixture of phytochemicals that may interfere with drug metabolism (Hu et al., 2012). In many situations, such interference may result in toxicity and unwanted consequences and is referred to as herb-drug or food-drug interactions. Similar to drug-drug interactions, herb-drug and food-drug interactions mainly involve altering the pharmacokinetics of the victim drug *via* reversible and/or irreversible inhibition of the CYP450 enzyme(s) (Li et al., 1997).

Linear and angular furanocoumarins are important groups of phytochemicals in the plant families of *Apiaceae, Leguminosae,* and *Rutaceae* (Berenbaum, 1983). The core chemical structure of furanocoumarins consists of a furan ring fused with a coumarin molecule; the simplest linear and angular furanocoumarins are the psoralen and angelicin, respectively (Figure 2-1). Human consumption of vegetables from the *Apiaceae* plant family has led to a reduction in *in vivo* CYP1A2 activity (Lampe et al., 2000; Peterson et al., 2006). Previous *in vivo* studies have also shown that 8-MOP and 5-MOP can significantly reduce oral CL of caffeine in psoriasis patients (Mays et al., 1987; Bendriss et al., 1996). Recent *in vitro* studies have shown that psoralen and angelicin (Zhuang et al., 2013), ISOP (Kang et al., 2011), imperatorin and isoimperatorin (Cao et al., 2013), and bergamottin (Lim et al., 2005) are irreversible inhibitors of CYP1A2 enzyme. Compared to the reversible inhibitors, irreversible inhibitors often result in a stronger and long-lasting inhibition for the prescription drug since the metabolic activity can only be replenished by *de novo* synthesis of the enzyme (Ghanbari et al., 2006).

In Chapter 2, we screened 29 food and herbal products belonging to the *Apiaceae, Lamiaceae, Leguminosae, and Rutaceae* families of plants for the presence of furanocoumarins. We found significant quantities of 8-MOP, 5-MOP, and/or ISOP in the aqueous extracts of *A.majus* seeds, *A.archangelica* roots, *A.pubescens* roots, *A.graveolens* seeds and flakes, *C.monnieri* fruits, *P.crispum* leaves, *P.aniseum* seeds, and *R.graveolens* leaves. The main objective of this study was to determine if 8-MOP, 5-MOP, and ISOP constituents in the aforementioned herbal extracts could significantly alter

the CYP1A2 activity in volunteers after pretreatment with herbal extracts at doses similar to those used in traditional medicines.

3.3. Materials and Supplies

3.3.1. Sources of herbs

Nine herbal products were selected from a list of 29 foods and herbs in Chapter 2 to study potential herb-caffeine interaction in humans. The 9 herbal products that contained significant levels of 8-MOP, 5-MOP, and/or ISOP were *A.majus* seeds, *A.archangelica* roots, *A.pubescens* roots, *A.graveolens* seeds, *A.graveolens* flakes, *C.monnieri* fruits, *P.crispum* leaves, *P.aniseum* seeds, and *R.graveolens* leaves. These herbs were obtained commercially from suppliers in Canada and USA (refer to Appendix A for more information about the suppliers and origins). The herbs were authenticated by the suppliers and shown to be free of pesticides and preservatives.

3.3.2. Chemicals and instrumentation

Acetonitrile (≥99.9% purity), benzotriazole (99.0% purity), caffeine (≥99.0% purity), and ethyl acetate (≥99.7% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid (≥99.7% purity) was obtained from Anachemia (Rouses Point, NYC, USA). Nitrogen gas (N_2) (≥99.9% purity) were obtained from Praxair (Danbury, CT, USA). Ultrapure water was produced using a Millipore system (Billerica, MA, USA) with a minimum resistivity of 18.0 MΩ•cm at 25°C. The chromatographic system comprised of an Agilent high-performance liquid chromatography (HPLC) (Santa Clara, CA, USA) consisted of a 1090 series II Liquid chromatography, a 79880A ultra-violet detector (UV), and an automatic sampler. The HPLC system was controlled by ChemStation version A.10.02 software.

3.4. Experimental Methods

3.4.1. Volunteer selection

An advertisement requesting volunteers to participate in this study was announced at different locations in Vancouver. The volunteers had to meet the following eligibility criteria: 1) between the ages of 20-35 years, 2) no history of recreational drug use in the last three months, 3) no history of heavy alcohol consumption in the last three months, 4) no history of major heart or liver medical conditions, 5) non-smoker, and 6) not-pregnant nor breast-feeding. Successful volunteers were selected based on their medical histories and were briefed to familiarize with the study design, required experiments, obligations, significance of the study, and possible consequences. Neither monetary compensation nor benefits were offered. The study protocol was reviewed and approved by Simon Fraser University Office of Research Ethics under permit number 2012s0565 (refer to Appendices L and M for more information about the advertisement flyer and consent form).

3.4.2. Herbal extract preparation

The amounts of herbal products used in the present study were based on the doses recommended by herbalists and traditional medicine practitioners. These are summarized in Table 2-2. The herbal products were extracted by water as aqueous extracts resembled the traditional method of decoction preparation. The herbs were first powdered in a Salton food processor model CG-1174 (Dollard-des-Ormeaux, QC, Canada) and mixed with 600 mL of tap water. The herbal mixture was then boiled on a Fisher Thermix 11-493 model hot plate (Hampton, NH, USA) under atmospheric pressure. When the liquid was reduced to about one half of the original volume, the herbal mixture was removed from the hot plate and left to cool for 10-20 min. The total extraction time was approximately 3 to 4 h including the cooling time. Once cooled, the herbal mixture was filtered by a metal sieve. The filtrate was put into a pre-washed glass bottle and kept at 2-8^{0}C until use.

3.4.3. *In vivo* studies

The pharmacokinetic study was an open-labeled, randomized, and crossover design. A minimum of three volunteers were used for each herb. The study consisted of two phases: Phase I study was used to establish the baseline plasma caffeine levels in the volunteers before they were treated by the herbal extracts. Phase II study measured the magnitude of caffeine metabolism inhibition in the volunteers after they were treated by the herbal extracts. The volunteers were instructed to refrain from consuming caffeine-containing food and drinks for a minimal period of 12 hours before starting the experiment. Additionally, the volunteers were asked to avoid consuming specific foods or herbs that were known to interfere with caffeine metabolism during the course of the studies (refer to Appendix N for the list of interfering herbs and food provided to the volunteers). The volunteers were also instructed to avoid heavy meals but encouraged to drink plenty of water throughout the sampling period.

3.4.3.1 Phase I: caffeine baseline study

The volunteers were supplied with a study kit containing AdremPharma WakeUps™ caffeine tablets (Scarborough, ON, Canada), labelled vials, and supplementary study forms. The 200.0 mg caffeine dose was the maximum single dose recommended by the supplier and considered to be safe by the European Food Safety Authority (EFSA) (Bull et al., 2014). Fourteen vials with the following labeled time points were supplied: pre-dose (0), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 12.0, and 24.0 h. Sampling methods and time points were based on previous caffeine pharmacokinetic studies in humans (Mays et al., 1987; Bendriss et al., 1996). Volunteers were instructed to collect 0.5-1.0 mL of saliva samples at each time point into the supplied vials. The volunteers were also requested to document the sampling time using the supplementary study forms. After sampling, the volunteers were instructed to store the saliva samples in the dark at freezing temperatures until sample submission (refer to Appendix O for a scanned copy of phase I supplementary study form).

3.4.3.2 Phase II: herb-caffeine interaction study

In phase II of the study, volunteers were instructed to consume an herbal extract 3 h before repeating the caffeine pharmacokinetic study but used different time points of saliva sampling. The volunteers were given another study kit containing caffeine tablets, herb extract, labeled vials, and supplementary study forms. Fourteen vials were labeled with the following time points: pre-dose (0), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 36.0, and 48.0 h. The remaining instructions were similar to that of phase I study (refer to Appendix P for a scanned copy of phase II supplementary form).

3.4.4. Saliva sample extraction procedure

Caffeine concentrations in the saliva samples were quantified according to the HPLC method reported by Perera et al. (2010) with modifications. The saliva samples were thawed at room temperature, mixed by vortex for 30 seconds, and then centrifuged for 10.0 min at approx. 4000 xg. Exactly 200.0 µL aliquot of each saliva sample was removed and spiked with 100.0 µL of benzotriazole (50.0 µg/mL), the internal standard (ISTD). The spiked samples were extracted once with 4.0 mL of ethyl acetate by vortex for 2 min followed by centrifugation for 5 min at approx. 4000 xg. The organic layer was removed and evaporated down to dryness using a gentle stream of purified N_2 gas. The residues were reconstituted in 150.0 µL of mobile phase by vortex and 100.0 µL was injected into the HPLC-UV system.

3.4.5. Caffeine measurement in saliva extract

Caffeine and ISTD were separated by an Agilent Zorbax XDB reverse-phase C-18 column (250 x 4.6 mm, 5 µm particle size) at room temperature. Isocratic elution was carried out using a mobile phase consisting of water, acetonitrile, and acetic acid (80:19:1 v/v/v) at a flow rate of 1.5 mL/min. The mobile phase was first degassed by a Sigma-Aldrich nylon membrane filter (pore size 0.22 µm) before use. The UV detector of the HPLC was set at 170 nm. The total analysis time was 20.0 min. Caffeine concentrations in the saliva samples were determined using a multi-level calibration curve of caffeine/ISTD peak area ratios versus caffeine concentrations ranging from 0.11 to 13.63 µg/mL. The limit of detection (LOD) and limit of quantification (LOQ) of caffeine in the

analytical method were calculated from the calibration curve based on the DIN 32645 method (DIN, 1994) using B.E.N. software version 2 (Herbold and Schmitt, 2000).

3.4.6. Pharmacokinetic parameters and statistical analysis

A plasma caffeine concentration-time curve was plotted for each volunteer using Prism GraphPad software version 5.04 (San Diego, CA, USA). The following pharmacokinetic parameters were determined from the concentration-time curve: T_{max} (time to reach peak plasma level) and C_{max} (concentration of peak plasma level) were determined by direct observation of the data. $AUC_{0\text{-}inf}$ (area under plasma concentration-time curve from zero to infinity) and $AUC_{0\text{-}last}$ (area under plasma concentration-time curve from zero to last time point) were determined by non-compartmental analysis using PK Solver 2.0 software (Zhang et al., 2010). The $AUC_{0\text{-}inf}$ was determined using the log-linear trapezoidal rule from dosing time to last time point and extrapolated to infinity by dividing the last concentration by the elimination rate constant (*k*). CL was determined by dividing the dose with $AUC_{0\text{-}inf}$. The AUCR was calculated by dividing the $AUC_{0\text{-}inf}$ with prior furanocoumarin exposure by $AUC_{0\text{-}inf}$ without prior exposure.

Student's paired *t*-test (1908), two-tailed and 95% confidence interval (CI) was used to compare if the caffeine concentration-time curves before and after pre-treatment with an herbal extract was significantly different using Microsoft Excel software version 2010 (Redmond, WA, USA). The *P* value of 0.05 or less was considered to be statistically significant.

3.5. Results and Discussion

The HPLC method used to measure caffeine concentration in human saliva was modified from Perara et al. (2010). Method modification involved an increase in the volume of saliva used in the extraction from 100.0 µL to 200.0 µL. The caffeine dose administered to the volunteers was also increased from 100.0 mg to 200.0 mg in order to increase the success of caffeine detection by the HPLC-UV method. The published LOD and LOQ by Perara et al. (2010) was 15 and 50 ng/mL respectively. The LOD and LOQ values in this study were comparable at 11.4 and 43.1 ng/mL respectively. Thus, the modified caffeine

measurement method allowed the detection of caffeine in saliva sample at post-dosing time-points of 36 and 48 h in this study. The retention times for caffeine and ISTD were 10.3 and 12.8 min, respectively (Figure 3-2).

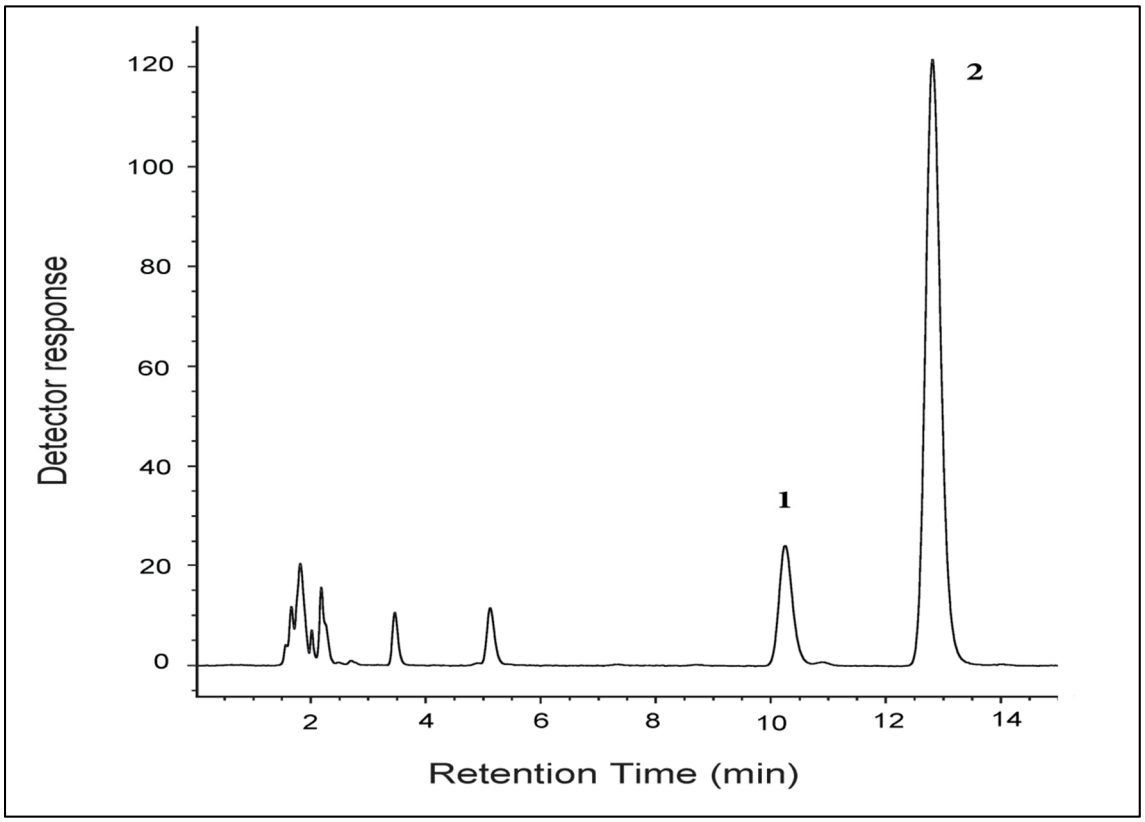

Figure 3-2: Separation and detection of caffeine (1) and ISTD (benzotriazole) (2) in human saliva extract by HPLC-UV.

Several saliva samples were extracted and analyzed in triplicates to determine if the modified HPLC method was reproducible. The average intra-assay coefficient of variation (CV) values were lower than 10% for C_{max} and $AUC_{0\text{-}last}$ measurements which are within acceptable limits (Shultheiss and Stanton, 2009) (refer to Appendix Q for more detailed information). Based on the reproducibility results, no sample clean-up or preparation steps, such as deproteinization, was necessary for caffeine analysis.

3.5.1. Plasma caffeine baseline measurement studies

In response to the advertisement, 35 volunteers agreed to participate in this study (Table 3-1). However, 9 volunteers have completed phase I only. Many applicants have

inquired but chosen not to participate in this study for the following stated reasons: no monetary compensation, undesired deprivation of caffeinated drinks and food, fear of health consequences due to caffeine consumption, fear of health consequences due to herb extract consumption, complexity of study design, requirement of extra time and commitment, and inability to provide the required minimal volume of saliva sample.

Table 3-1: A summary of volunteer participation and withdrawal reasons for the present herb-caffeine interaction study.

Category	Number of Volunteers
Total Participants [a]	35
Completed Phase I only (partial completion) [b]	9
Completed Phase I and II (data shown in this study)	19
Completed Phase I and II (data not shown in this study)	7
Reasons for partial completion [b]	
Sensitive to caffeine consumption	1
Sensitive to herb extract consumption	1
Deprivation of caffeinated food and drinks	1
Noncompliance to study requirements	2
Other reason(s)	4

[a] Volunteers withdrawn from the study prior to or during phase I are not included.

[b] Volunteers that completed phase I but not phase II.

Consenting volunteers were briefed on the study requirements and obligations, and provided with the study kits after they were determined eligible. After collecting the saliva samples from the volunteers, the saliva samples were extracted and analyzed as described earlier. Caffeine concentrations in saliva were converted to plasma concentration using a 0.79 conversion factor (Zylber-Katz et al., 1984; Fuhr et al., 1993).

3.5.2. *In vivo* herb-caffeine interaction studies

After completing phase I study, the volunteers were prepared for phase II study which involved pretreating the volunteers with an aqueous herbal extract 3 h prior to caffeine administration. Results of the studies revealed that 5 out of the 9 herbal extracts tested significantly reduced the CL of caffeine in the volunteers with concomitant increases in AUC values ($P \leq 0.05$).

Figures 3-3 to 3-7 show the effects of pretreating the volunteers with *A.majus* seeds, *A.archangelica* roots, *A.pubescens* roots, *C.monnieri* fruits, or *R.graveolens* leaves. The increases in AUC ranged from 1.3 to 4.3 fold (Tables 3-3 and 3-4). For example, *A.majus* seeds pretreatment significantly increased the mean caffeine AUC by 4.3 fold; it also increased the mean T_{max} from 0.8 to 2.4 h and increased mean C_{max} by 16.6% but reduced mean caffeine CL by 77.3% (Fig 3-3, Tables 3-2 and 3-3). Pretreatment with *A.archangelica* roots increased the mean AUC by 2.3 fold and mean T_{max} from 0.8 to 1.3 h; it reduced mean caffeine CL by 53.4% and had no effect on mean C_{max} value (Figure 3-4, Tables 3-2 and 3-3). Pretreatment with *A.pubescens* roots significantly reduced mean caffeine CL by 33.8%; it significantly increased mean C_{max} by 22.0% and increased the mean AUC by 1.7 fold but the mean T_{max} was insignificantly reduced from 0.9 to 0.7 h (Figure 3-5, Tables 3-2 and 3-3). Preatment with *C.monnieri* fruits increased the mean AUC of caffeine by 2.2 fold; it also increased the mean T_{max} from 0.7 to 1.8 h and reduced the mean CL by 60.3% but had no effect on mean C_{max} value (Figure 3-6, Tables 3-2 and 3-3). Pretreatment with *R.graveolens* leaves significantly increased the mean AUC of caffeine by 1.3 fold; it also increased mean T_{max} from 0.6 to 1.0 h and mean C_{max} by 12.1% but reduced mean caffeine CL by 33.8%. (Figure 3-7, Tables 3-2 and 3-3).

The remaining 4 of the 9 herbs had no significant effect on the systemic CL of caffeine in the volunteers (P>0.05) (Figures 3-8 to 3-11). For example, pretreatment with *A.graveolens* seeds increased the mean AUC by 1.1 fold but the increase was statistically insignificant; it also increased the mean T_{max} from 0.7 to 1.2 h but reduced C_{max} by 8.9% and CL by 18.2% (Figure 3-8, Tables 3-2 and 3-3). Pretreatment with *A.graveolens* flakes increased mean T_{max} from 0.8 to 1.5 h; it reduced mean caffeine CL by 17.2% but had no effect on C_{max} and AUC values (Figure 3-9, Tables 3-2 and 3-3). Consumption of *P.aniseum* seeds significantly increased mean T_{max} from 0.6 to 1.1 h; it insignificantly reduced mean C_{max} value by 16.9% and mean CL by 7.3% but had no effect on AUC value (Figure 3-10, Tables 3-2 and 3-3). Consumption of *P.crispum* leaves increased mean AUC by 1.4 fold but the increase was statistically insignificant; it also increased C_{max} value by 5.0% and reduced CL by 40.1% but had no effect on T_{max} value (Figure 3-11, Tables 3-2 and 3-3).

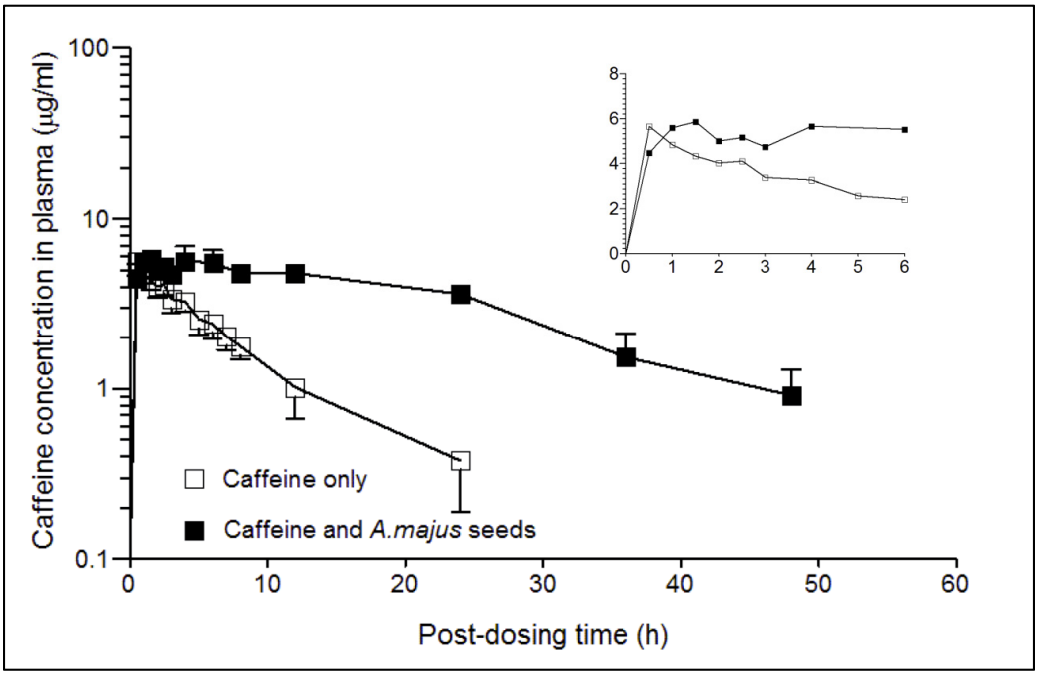

Figure 3-3: Caffeine time-concentration profile for volunteer plasma with and without pretreatment of *A.majus* seeds (*n* = 6). Insert represents linear-linear plot of the data to show the C_{max} more clearly.

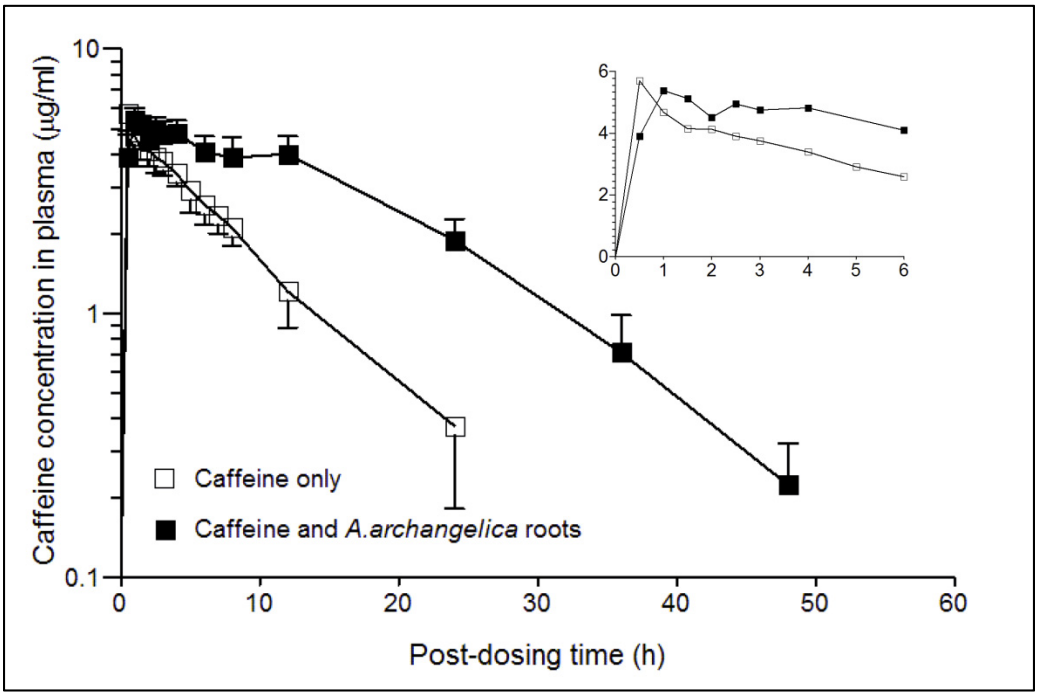

Figure 3-4: Caffeine time-concentration profile for volunteer plasma with and without pretreatment of *A.archangelica* roots (*n* = 6). Insert represents linear-linear plot of the data to show the C_{max} more clearly.

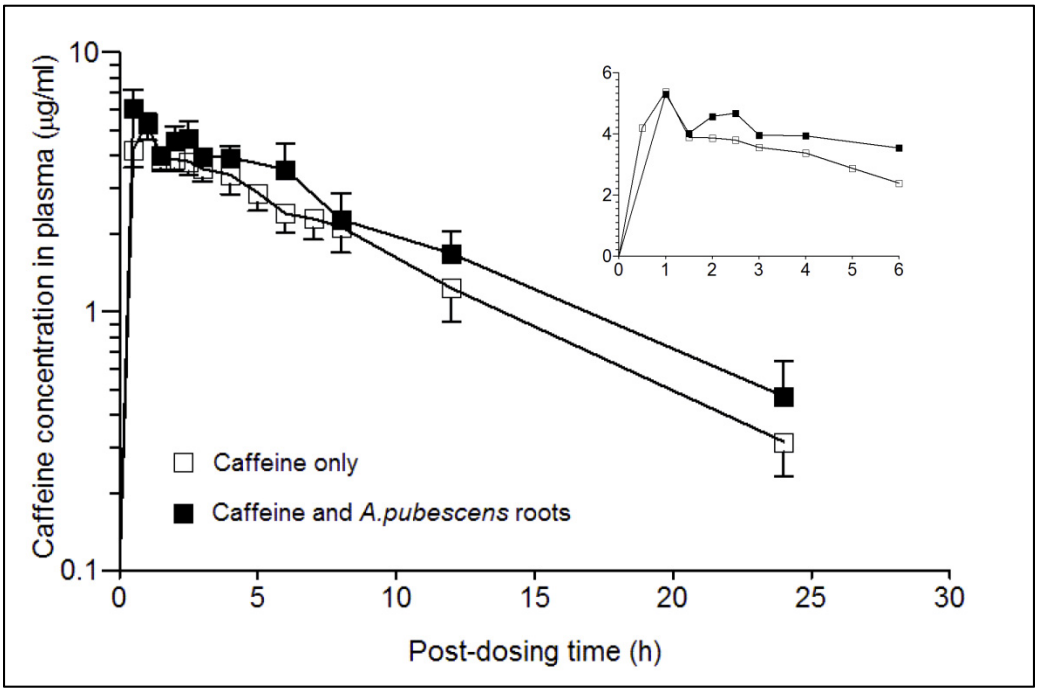

Figure 3-5: Caffeine time-concentration profile for volunteer plasma with and without pretreatment of *A.pubescens* roots (*n* = 5). Insert represents linear-linear plot of the data to show the C_{max} more clearly.

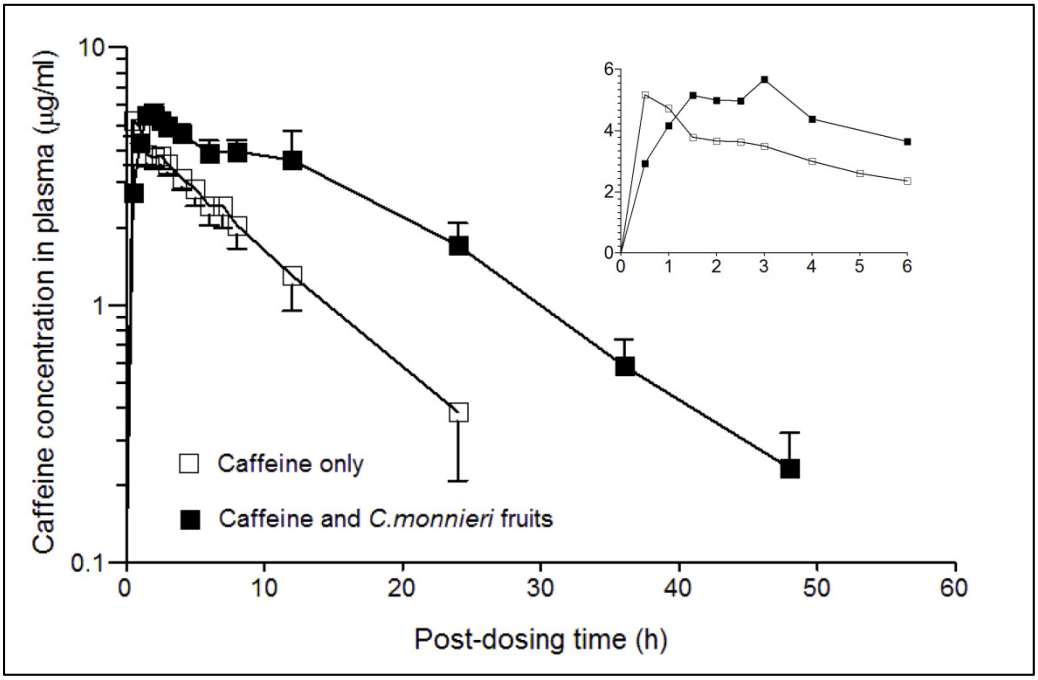

Figure 3-6: Caffeine time-concentration profile for volunteer plasma with and without pretreatment of *C.monnieri* fruits (*n* = 5). Insert represents linear-linear plot of the data to show the C_{max} more clearly.

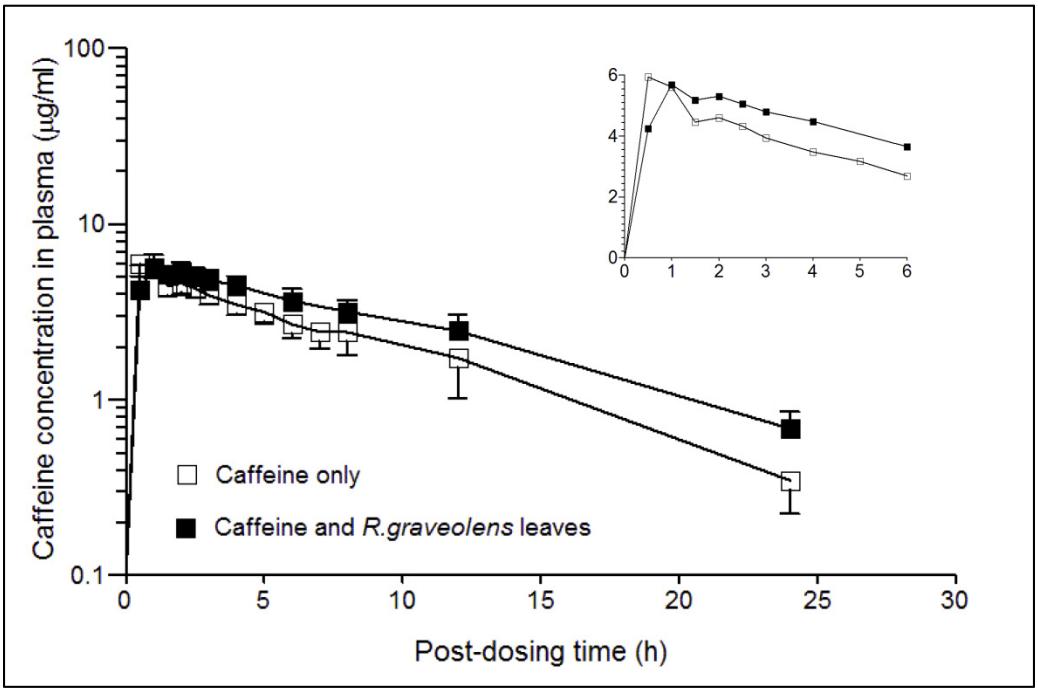

Figure 3-7: Caffeine time-concentration profile for volunteer plasma with and without pretreatment of *R.graveolens* leaves (*n* = 6). Insert represents linear-linear plot of the data to show the C_{max} more clearly.

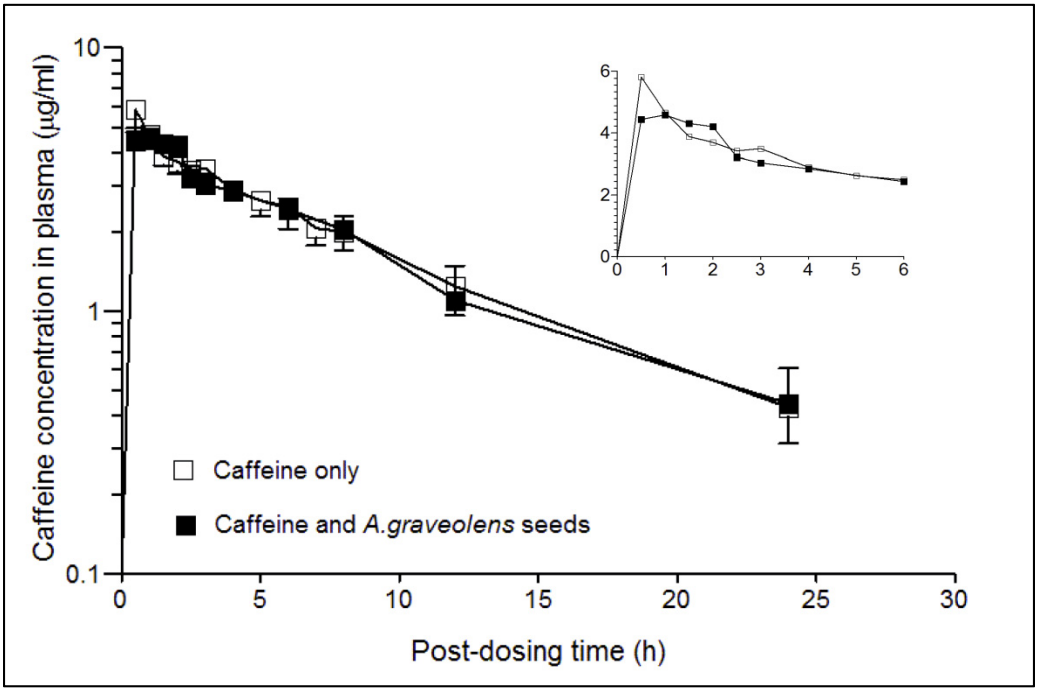

Figure 3-8: Caffeine time-concentration profile for volunteer plasma with and without pretreatment of *A.graveolens* seeds (*n* = 6). Insert represents linear-linear plot of the data to show the C_{max} more clearly.

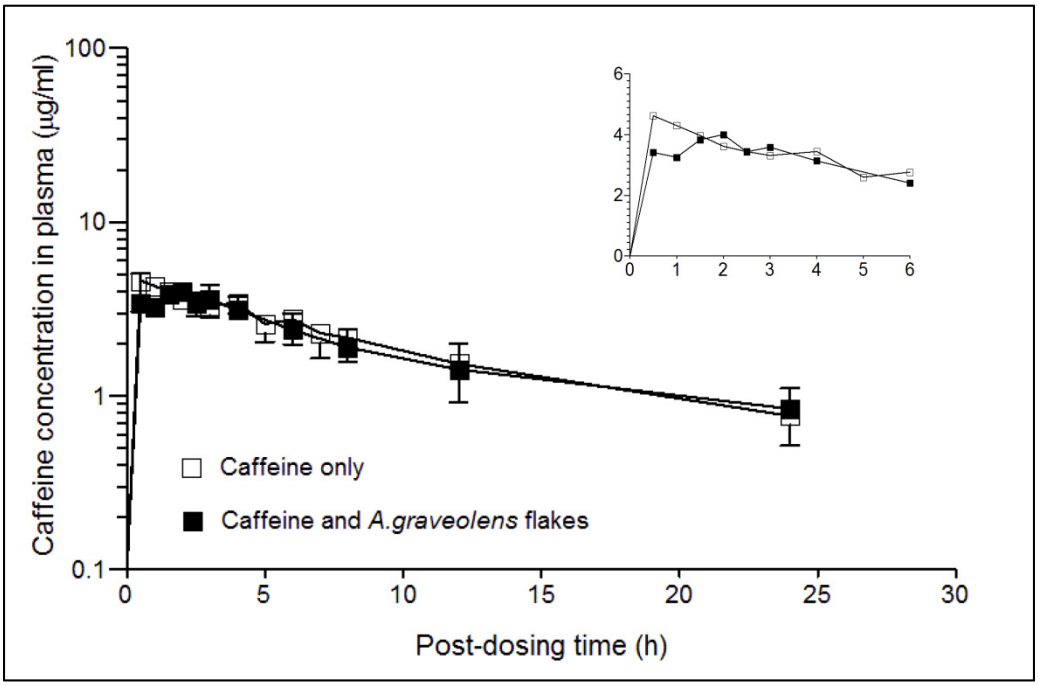

Figure 3-9: Caffeine time-concentration profile for volunteer plasma with and without pretreatment of *A.graveolens* flakes (*n* = 3). Insert represents linear-linear plot of the data to show the C_{max} more clearly.

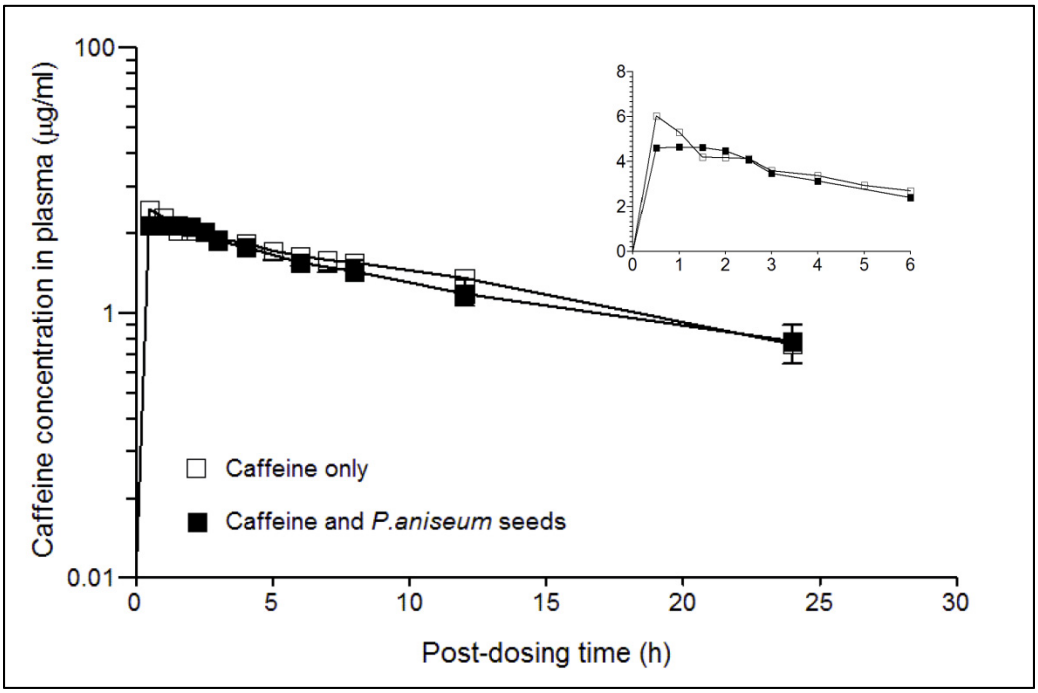

Figure 3-10: Caffeine time-concentration profile for volunteer plasma with and without pretreatment of *P.aniseum* seeds (*n* = 6). Insert represents linear-linear plot of the data to show the C_{max} more clearly.

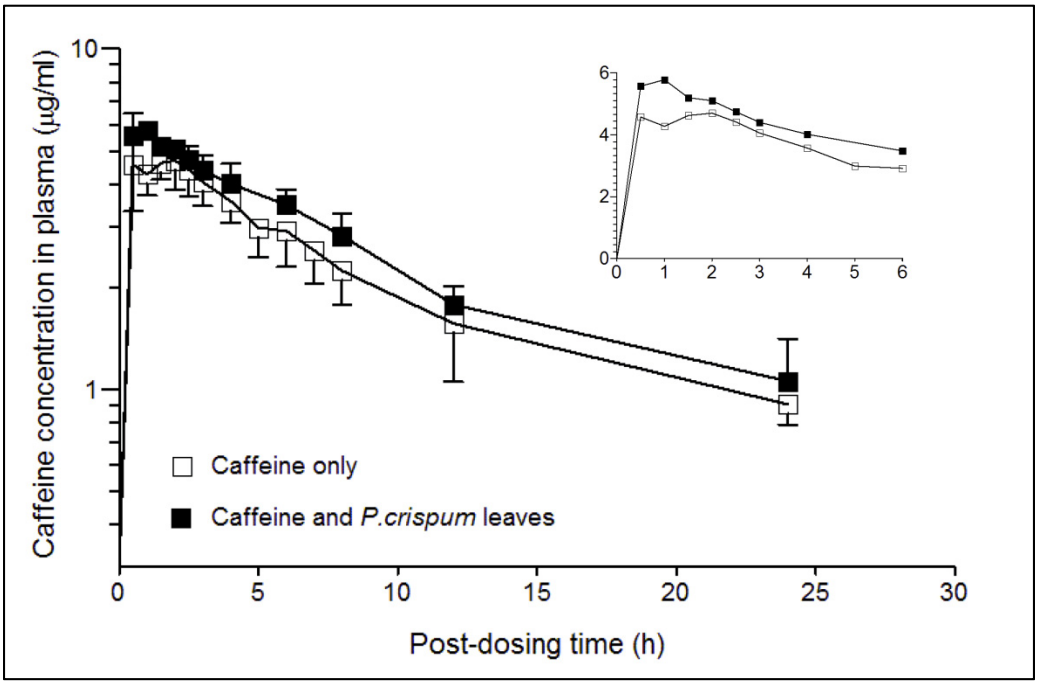

Figure 3-11: Caffeine time-concentration profile for volunteer plasma with and without pretreatment of *P.crispum* leaves (n = 4). Insert represents linear-linear plot of the data to show the C_{max} more clearly.

Table 3-4 compares the pharmacokinetic parameters in the present study with previously published studies of Mays et al. (1987), Caraco et al. (1995), Bendriss et al. (1996), and Kamimori et al. (2002). Mays et al. (1987) dosed psoriasis volunteers with 200.0 mg of crushed caffeine tablets in an aqueous suspension and obtained a T_{max} value of 1.0 ± 0.5 h. In contrast, healthy volunteers in this study were dosed with intact caffeine tablets and obtained comparable T_{max} value of 0.8 ± 0.4 h. These results should address the concern that caffeine absorption in the present study might be slow, or limited, due to the administration of intact tablets and confirm previous reports that caffeine pharmacokinetics does not depend on its dosage form (Perara et al. 2012). The mean CL values in the present study were lower than those in the aforementioned studies perhaps due to the difference in the measurement of caffeine concentrations in the saliva and plasma. In the present study, caffeine concentration in the saliva was divided by 0.79 to obtain plasma concentration (Zylber-Katz et al., 1984; Fuhr et al., 1993).

Table 3-2: Pharmacokinetic parameters of caffeine in volunteers before pretreatment with furanocoumarin-containing herbs.

Botanical Name	*n*	Plasma Caffeine Pharmacokinetic Parameters for Untreated Volunteers [a]																	
		T_{max} h			C_{max} µg/mL			$AUC_{0\text{-last}}$ µg/mL*h			$AUC_{0\text{-inf}}$ µg/mL*h			CL mL/min		
A.majus seeds	6	0.8	±	0.4	5.4	±	2.1	35.0	±	16.6	40.4	±	17.8	95.6	±	39.5
A.archangelica roots	6	0.8	±	0.4	5.4	±	1.8	36.8	±	16.4	43.3	±	17.4	89.0	±	40.3
A.pubescens roots	5	0.7	±	0.3	5.9	±	1.3	39.6	±	15.3	41.8	±	16.5	93.7	±	45.5
A.graveolens seeds	6	0.7	±	0.3	6.1	±	2.1	38.3	±	16.4	42.9	±	17.6	91.3	±	42.5
A.graveolens flakes	3	0.8	±	0.2	5.4	±	1.8	42.9	±	21.2	50.1	±	27.9	89.9	±	64.9
C.monnieri fruits	5	0.7	±	0.2	6.0	±	1.4	41.3	±	17.3	46.1	±	22.3	90.2	±	49.1
P.crispum leaves	4	1.2	±	0.7	6.0	±	1.5	44.5	±	20.6	53.0	±	25.9	82.5	±	56.3
P.aniseum seeds	6	0.6	±	0.2	6.5	±	1.8	48.8	±	27.7	55.8	±	31.8	80.8	±	49.8
R.graveolens leaves	6	0.6	±	0.2	6.6	±	1.8	47.8	±	27.4	50.9	±	30.6	85.7	±	47.0

[a] Results are expressed as mean ± SD.

Table 3-3: Pharmacokinetic parameters of caffeine in volunteers after pretreatment with furanocoumarin-containing herbs.

Botanical Name	*n*	Plasma Caffeine Pharmacokinetic Parameters for Treated Volunteers [a]														
		T_{max} h			C_{max} µg/mL			$AUC_{0\text{-last}}$ µg/mL*h			$AUC_{0\text{-inf}}$ µg/mL*h			CL mL/min		
A.majus seeds	6	2.4	±	2.1	6.3	±	2.2 [c]	151.0	±	53.2 [d]	174.6	±	83.5 [d]	21.7	±	6.9 [d]
A.archangelica roots	6	1.3	±	0.3 [d]	5.4	±	1.3	97.2	±	47.4 [d]	100.3	±	51.0 [d]	41.4	±	20.6 [d]
A.pubescens roots	5	0.9	±	0.9	7.2	±	1.0 [c]	56.7	±	21.9 [b]	70.4	±	43.5	62.0	±	34.1 [c]
A.graveolens seeds	6	1.2	±	0.7 [b]	5.6	±	1.2	45.5	±	19.0	48.2	±	18.2	74.7	±	18.0
A.graveolens flakes	3	1.5	±	1.3	5.4	±	1.1	49.6	±	20.6	52.2	±	23.6	74.4	±	36.3
C.monnieri fruits	5	1.8	±	0.3 [d]	5.9	±	0.9	98.6	±	28.8 [d]	101.8	±	30.0 [d]	35.8	±	13.2
P.crispum leaves	4	1.1	±	0.9	6.3	±	0.9	58.6	±	18.0 [b]	73.1	±	21.8	49.4	±	17.3
P.aniseum seeds	6	1.1	±	0.6	5.4	±	1.2	51.7	±	23.5	54.0	±	24.4	74.9	±	37.3
R.graveolens leaves	6	1.0	±	0.8	7.4	±	2.4	66.7	±	33.0 [c]	68.6	±	34.5 [c]	56.8	±	20.5 [b]

[a] Results are expressed as mean ± SD.

[b] Insignificant in comparison to untreated volunteers ($0.05<P\leq0.10$).

[c] Significant in comparison to untreated volunteers ($0.01<P\leq0.05$).

[d] Highly significant in comparison to untreated volunteers ($P\leq0.01$).

Additionally, we assumed the saliva/plasma concentration ratio is similar in all sampling time-points and volunteers. Furthermore, previous studies have reported different saliva/plasma concentration ratios. For example, Newton et al. (1981) has reported a slightly lower saliva/plasma caffeine ratio of 0.74. Cook et al. (1976) and Parsons and Neims (1978) have reported a saliva/plasma caffeine ratio of 1.02 and 0.90, respectively. Clearly the saliva/plasma concentration ratio used has introduced some uncertainty in calculating caffeine pharmacokinetic parameters in the present study.

Previous studies indicated that smoking significantly induces caffeine CL in humans (Brown et al., 1988; Vistisen et al., 1991; Benowitz et al., 2003). However, we did not detect significant differences between smokers and non-smokers in caffeine CL in this study. On the contrary, two smoking volunteers had much lower CL values, 20.6 and 59.4 mL/min respectively, than the non-smoker volunteers. In the baseline pharmacokinetic study, the calculated median caffeine CL is 69.2 mL/min (see Appendix R for more information). This also has been reported by Kalow and Tang (1991) that the highest caffeine CL is found in the non-smokers rather than smokers. These results seem to suggest factors other than tobacco smoke are more potent inducers of CYP1A2 enzyme. For example, it has been reported that certain diets such as charcoal-grilled beef (Kall and Clausen, 1995), high temperature pan-fried beef (Sinha et al., 1994), and broccoli (Lampe et al., 2000) are potent inducers for CYP1A2 enzyme in humans.

The following are some of the study limitations: (a) the most obvious limitation is the dose of the herbal extract is not normalized by BW and this has contributed to the observed interindividual variability of interaction results. The reason why BW is not used to standardize the dose is because herbal products are seldom consumed based on BW; (b) the exclusion of females, children, seniors, and caffeine-sensitive individuals (*e.g.* cardiovascular disease patients, pregnant women, *etc.*) from the volunteer pool is another limitation of this study. As such, the results reflect only herb-caffeine interactions within a subpopulation of healthy males between 21 and 40 years of age.

Table 3-4: Pharmacokinetic parameters of caffeine in humans with no furanocoumarin pretreatment.

Parameter	Unit	Human Caffeine Pharmacokinetic Studies					
		This Study [a c]	Mays et al. (1987) [b d]	Caraco et al. (1995) [a c]	Bendriss et al. (1996) [c d]	Kamimori et al. (2002) [a c]	
n		19	5	14	8	12	
Dose	mg	200	200	200	200	200	
Dose form		Intact tablet	Crushed tablet	Capsule	n.p. [d]	Capsule	
Sample Type		Saliva	Plasma	Plasma	Plasma	Plasma	
T_{max}	h	0.8 ± 0.4	1.0 ± 0.5	n.p. [e]	n.p. [e]	2.0 ± 1.0	
C_{max}	µg/mL	6.3 ± 1.7	4.3 ± 0.4	n.p. [e]	n.p. [e]	4.1 ± 1.9	
$AUC_{0\text{-}inf}$	µg/mL*h	52.6 ± 20.2	34.0 ± 6.0	40.4 [f]	24.0 ± 9.0	33.5 ± 13.7	
CL	mL/min	73.1 ± 30.8	110.0 ± 17.0	82.6 ± 34.0	158.3 ± 63.3	129.6 [g]	

[a] Based on healthy volunteers.

[b] Expressed as geometric mean ± standard error of mean.

[c] Expressed as mean ± standard deviation (see Appendix R for more information).

[d] Based on psoriasis volunteers.

[e] n.p. = not provided.

[f] Calculated from published data based on AUC = Dose/CL.

[g] Calculated from published data based on average body weight of volunteers (75.5 kg).

In the present study, a single dose of herbal extract was given orally to the volunteers 3 h before caffeine administration. Since the herbal extracts inhibited caffeine metabolism significantly, the furanocoumarins in the herbal extracts must have been distributed to the liver rapidly after absorption. These results are consistent with previous studies that the T_{max} of 8-MOP and 5-MOP are usually around 3 h post-dosing. For example, Walther et al. (1992) compared the pharmacokinetics of three 8-MOP formulas in 88 patients receiving Psoralen and Ultra-Violet A (PUVA) and found the T_{max} range from 0.9 ± 0.4 h to 2.0 ± 0.6 h. Siddiqui et al. (1984) also compared the pharmacokinetics of 8-MOP in microenema, hard and soft gelatin capsule dose forms, in 351 PUVA patients and found the T_{max} to range from 0.6 ± 0.2 h to 2.3 ± 0.9 h. Bonnot et al. (1994) have analyzed 8-MOP in 475 serum samples for PUVA patients and reported an average T_{max} of 2.0 ± 0.9 h. Stolk et al. (1981) analyzed 5-MOP in 10 PUVA patients receiving different dose levels and reported a T_{max} of 3.0 ± 0.6 h. Shephard et al. (1999) also analyzed 5-MOP in 7 PUVA and healthy individuals and reported an average T_{max} of 2.8 ± 0.8 h. Bendriss et al. (1996) analyzed 5-MOP in 7 PUVA patients with an average T_{max} of 6.0 h (range 3.0-8.0 h). Thus, there is substantial interindividual variability for 8-MOP and 5-MOP pharmacokinetics in humans. We were unable to find any pharmacokinetic studies for ISOP in humans. However, in laboratory animal studies, the reported T_{max} for ISOP after administering an oral dose of *Toddalia asiatica* L. (Lam) herb extract is about 1.3 ± 0.3 h (Liu et al., 2012). Li et al. (2014) also have reported a T_{max} of 1.1 ± 0.3 h after an oral dose of *Cnidium monnieri* herb extract.

Results of the study show pharmacokinetic interaction does not occur between caffeine and celery, parsley, or anise suggesting consumption of fresh celery, fresh parsley, and dried anise does not interfere with the CL of caffeine in humans. However, each herb has been purchased from just one supplier; the same herb from a different supplier may provide different pharmacokinetic results. For example, disease-resistant celery has been shown to contain unusually high contents of furanocoumarins (Berkley et al., 1986). Diawara et al. (1993) also has found higher levels of 8-MOP and 5-MOP in University of California (UC) bred l*Fusarium*-resistant celery than the standard cultivar, Tall Utah 52-70R. Nigg et al. (1997) have reported Florida 296 breed celery contains four times more psoralen, 8-MOP, 5-MOP, and ISOP than Florida 2-14 Florimart, M9, M68, and Junebell breed lines. Beier et al. (1994) have compared the contents of linear

furanocoumarins in four varieties of dried parsley leaves and found that one of these brands has much higher psoralen, 8-MOP, 5-MOP, and ISOP levels than the others.

3.6. Conclusions

In the present study, we have demonstrated, for the first time, the consumption of *A.majus* seeds*, A.archangelica* roots*, C.monnieri* fruits*, A. pubescens* roots*,* and *R.graveolens* leaves can significantly alter caffeine CL in healthy subjects at doses commonly used by humans. Inhibition of caffeine metabolism by a single oral dose of the aforementioned herbs suggests irreversible enzyme inhibition of CYP1A2 enzyme. Further studies are required to characterize the interaction mechanism(s) and explain the observed findings. As caffeine is mainly metabolized by CYP1A2 in humans, our results indicate that care must be exercised when furanocoumarin-rich herbs are co-administered with pharmaceutical drugs which are CYP1A2 substrates. The results also support developing regulation and proper labeling for herbs containing inhibitors of CYP450 enzymes.

3.7. References

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Chapter 4.

In Vitro Cytochrome 1A2 Inhibition by Plant Extracts, 8-methoxypsoralen, 5-methoxypsoralen, and Isopimpinellin in Human Liver Microsomes

4.1. Abstract

Background: Human cytochrome P-450 (CYP450) system plays an important role in environmental chemical and pharmaceutical drug biotransformation. Many plants from the *Apiaceae* and *Rutaceae* families are potent inhibitors of CYP450 enzymes. Indeed, pharmacokinetic interactions between co-administered caffeine and furanocoumarin-containing plants have been demonstrated in Chapter 3.

Objectives: The main goals of this study were: (a) to study the inhibitory potency of pure furanocoumarins, *i.e.* 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and isopimpinellin (ISOP), and extracts of *A.majus* L. and *A.archangelica* L. on CYP1A2 enzyme using multi-donor, pooled human liver microsomes (HLM), (b) to estimate an integrated dose for the furanocoumarin mixture in *A.majus* and *A.archangelica* herbs, and (c) to elucidate the mechanism of caffeine metabolism inhibition using pure 8-MOP, 5-MOP, and ISOP in HLM.

Results: 8-MOP, 5-MOP, and ISOP standards exhibited concentration-, NADPH-, and time-dependent inhibition (TDI) of caffeine metabolism. The IC_{50}, K_I, and k_{inact} for 8-MOP were 0.09 ± 0.05 μM, 0.78 ± 0.38 μM, and 0.17 ± 0.01 min^{-1}, respectively. The IC_{50}, K_I, and k_{inact} for 5-MOP were 0.13 ± 0.11 μM, 3.72 ± 3.66 μM, and 0.35 ± 0.12 min^{-1}, respectively. The IC_{50}, K_I, and k_{inact} for ISOP were 0.29 ± 0.22 μM, 4.48 ± 0.56 μM, and 0.65 ± 0.03 min^{-1}, respectively.

Conclusions: Linear furanocoumarins such as 8-MOP, 5-MOP, and ISOP were potent mechanism-based inactivators (MBI) of CYP1A2 enzyme. The integrated doses of the furanocoumarin mixtures in *A.majus* and *A.archangelica* were estimated to be 7.5 and 1.8 mg 8-MOP equivalent/g dry herb, respectively. Moreover, the inhibitory potencies of the

herbal extracts were equal to the sum of inhibitory potencies of individual furanocoumarins in the mixture.

4.2. Introduction

Numerous enzymes are used to catalyze essential biochemical reactions to maintain life and health in animals and plants. Upon exposure to foreign compounds, the CYP450 enzymes in living organisms are able to convert these chemicals into more hydrophilic metabolites through a series of metabolic processes. The phase-I enzymes responsible for xenobiotic metabolism are known as mixed-function oxidases or CYP450 enzymes with more than 50 isozymes identified in humans to-date (Guengerich, 2005). It is estimated that only 6 of these CYP450 isozymes are responsible for the metabolism of about 60% of the prescription drugs (Venkatakrishnan et al., 2001). The CYP450 enzymes are heme-containing proteins that oxidize substrates into metabolites in the presence of oxygen and NADPH (Figure 4-1) (Guengerich, 2001). The main locations of the CYP450 enzymes are in the liver, intestine, kidney, and lung (Wilkinson, 2005). Upon oral administration of drugs, the CYP450 enzymes in the liver and intestine are responsible for the first-pass effect and may cause drug-drug interactions (DDI), food-drug interactions (FDI), or herb-drug interactions (HDI) in the presence of CYP450 inhibitors including food/herbal products.

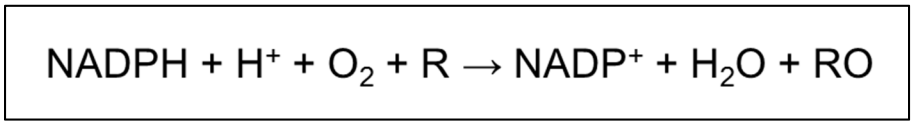

Figure 4-1: The oxidative metabolic pathway mediated by CYP450 enzymes for drug substrate (R) metabolism.

In humans, CYP1A2 is an important phase-I isoenzyme, which accounts for approximately 13% of the total hepatic CYP pool (Shimada et al., 1994). Despite the small percentage in the CYP450 pool, CYP1A2 plays an important role in the metabolism of many pharmaceuticals which include acetaminophen (Laine et al., 2009), caffeine (Kot and Daniel, 2008a), clozapine (Pirmohamed et al., 1995), cyclobenzaprine (Wang et al., 1996), duloxetine (Lantz et al., 2003), fluvoxamine (Spigset et al., 2001), mexiletine (Labbe et al., 2003), nabumetone (Turpeinen et al, 2009), olanzapine (Ring et al., 1996),

ondansetron (Dixon et al., 1995), propranolol (Masubuchi et al., 1994), riluzole (Sanderink et al., 1997), ropivacaine (Oda et al., 1995), theophylline (Ha et al., 1995), tizanidine (Granfors et al., 2003), triamterene (Fuhr et al., 2005), verapamil (Kroemer et al.,1993), *R*-warfarin (Kaminsky and Zhang, 1997), zileuton (Machinist et al., 1995), and zolmitriptan (Wild et al., 1999).

Human CYP1A2 is inducible upon exposure to polyaromatic hydrocarbons (Conney, 1982), *Brassicaceae* vegetables such as broccoli (Kall et al., 1996), and cigarette smoke (Kalow and Tang, 1991). The importance of CYP1A2 is the bioactivation of procarcinogens such as heterocyclic amines (Boobis et al., 1994), mycotoxins (aflatoxin B_1) (Gallagher et al., 1996), and polycyclic aromatic hydrocarbons (Shimada et al., 2001) into reactive carcinogenic intermediates. Thus, regular consumption of *Apiaceae* vegetables, which contain CYP1A2 inhibitors, is believed to reduce the risk of cancer by inhibiting the biotransformation of procarcinogens (Peterson et al., 2006).

The mechanism of CYP450 inhibition generally can be classified as reversible, quasi-irreversible, and irreversible inhibition with the former further classified as competitive and non-competitive inhibition. Competitive inhibition occurs when a chemical occupies the active site of the enzyme, thus, obstructing the enzyme active site from binding with the substrate. In contrast, non-competitive inhibition occurs when a xenobiotic inhibitor does not compete with the substrate for a specific enzyme active site. Irreversible inhibition often involves the metabolic bioactivation of a drug into reactive intermediates which forms irreversible adducts with the active site amino acids in the apoprotein, quasi-irreversible binding to the prosthetic heme iron atom (metabolite-inhibitor complex, or MIC), direct alkylation or arylation of the porphyrin framework of the heme, and/or degradation the prosthetic heme to products that modifies the protein resulting in enzyme destruction (Correia and de Montellano, 2005). In contrast to reversible inhibition, irreversible inhibition of CYP450 destructs the enzyme function permenantly and resynthesis is required to replace the destructed enzymes.

Linear furanocoumarins are derivatives of a simple furanocoumarin, psoralen which consists of a furan ring attached to a coumarin molecule (Figure 2-1). Previous studies have shown that psoriasis patients undergoing 8-MOP or 5-MOP treatments have

significantly lower oral clearance of caffeine (Mays et al., 1987; Bendriss et al., 1996). 8-MOP, 5-MOP, and ISOP have also been shown to be irreversible inhibitors of CYP450 and recombinant CYP450 (rCYP450) enzymes in *in vitro* studies. For example, 8-MOP is a potent MBI of CYP2A6 in HLM (Koenigs et al., 1997) and in reconstituted purified CYP2B1 enzyme (Koenigs and Trager, 1998). 5-MOP is a MBI for CYP3A4 in HLM (Zaidi et al., 2007) and ISOP is a MBI for rCYP1A2 (Kang et al., 2011). Recently, psoralen and angelicin have also been found to be TDI of CYP1A2 in rat and human liver microsomes, respectively (Zhuang et al., 2013).

Previous *in vitro* and *in vivo* studies have shown that the initial step of 8-MOP and 5-MOP metabolism involves oxidative attack of the furan ring to form electrophilic, reactive metabolite(s) (Figure 4-2) which bind covalently with CYP450 proteins (Fouin-Fortunet et al., 1986; Tinel et al., 1987; Koenigs and Trager, 1998; Kolis et al., 1979; Schmid et al., 1980; John et al., 1992).

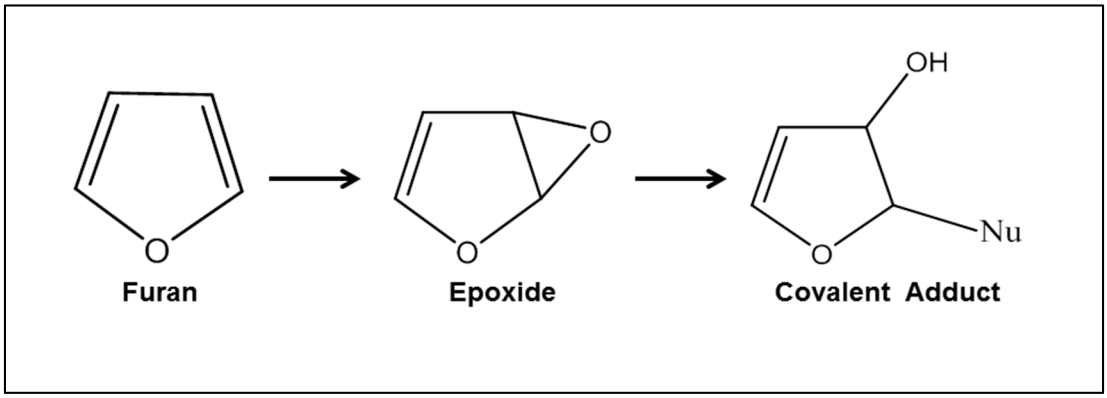

Figure 4-2: Main metabolic pathway of furan ring *via* epoxidation and epoxide ring opening by nucleophilic (Nu) amino acid residue in CYP450 enzyme resulting in its inactivation (Taxak et al., 2013).

However, little or no information is available on the k_{inact} and K_I values of caffeine metabolism by 8-MOP, 5-MOP, and ISOP in HLM. The objectives of the present study were: (a) to determine the inactivation constants for 8-MOP, 5-MOP, and ISOP on caffeine metabolism inhibition in HLM, (b) to provide experimental evidence on ^{14}C-8-MOP adduct formation with HLM and rCYP1A2, and (c) to compare the integrated doses of the furanocoumarin bioactive in *A.majus* and *A.archangelica* herbs using two different chemical mixture assessment approaches: the concentration/dose addition approach and the whole-mixture dose equivalent approach.

4.3. Materials and Supplies

4.3.1. Chemicals

8-MOP (≥98.0% purity), 5-MOP (99.0% purity), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH) (≥97.0% purity), and caffeine (≥99.0% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). ISOP was obtained from ChromaDex (Irvine, CA, USA) (98.3% purity) and Sigma-Aldrich (≥95.0% purity). Trichloroacetic acid (TCA) (≥99.0 purity), acetone (>99.5% purity), dipotassium phosphate (K_2HPO_4) (≥60.0 purity), and monopotassium phosphate (KH_2PO_4) (≥60.0 purity) were obtained from Anachemia (Rouses Point, NYC, USA). Methanol (≥99.9% purity) and acetonitrile (≥99.9% purity) were obtained from Thermo Fisher Scientific (Hampton, NH, USA).

Radiolabelled caffeine (3-methyl-^{14}C) with specific activity of 50-60 mCi/mmol (1.85-2.22 GBq/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). 8-MOP (methyl-^{14}C) with specific activity of 40-60 mCi/mmol (1.48-2.22 GBq/mmol) was obtained from ViTrax Radiochemicals (Placentia, CA, USA). Scintillation cocktail fluids were obtained from PerkinElmer (Waltham, MA, USA) and Amersham Biosciences (Piscataway, NJ, USA). Dimethyl sulfoxide (DMSO) (spectral grade) was obtained from Caledon (Georgetown, ON, Canada). Nitrogen (N_2) (≥99.9% purity) and carbon monoxide (CO) (≥99.5% purity) gases were obtained from Praxair (Danbury, CT, USA). Ultrapure water was produced using a Millipore system (Billerica, MA, USA) with a minimum resistivity of 16.0 MΩ•cm at 25°C.

4.3.2. Microsomes and enzyme

Multi-donor (50 donors) HLM were obtained commercially from BD Gentest (Franklin Lakes, NJ, USA; catalog number 452161 and lot numbers of 99268 and 18888), Xenotech (Lenexa, KS, USA; catalog number H0610 and lot number 1210267), and GIBCO Thermo Fisher Scientific (Waltham, MA, USA; catalog number HMMCPL and lot number PL050B). Baculosomes reagent plus human rCYP1A2 was obtained

commercially from Life Technologies (Carlsbad, CA, USA; catalog number P2792 and lot number 49239).

4.4. Experimental Methods

4.4.1. ^{14}C-Caffeine demethylation incubation assay

Probe substrates such as caffeine (Bloomer et al., 1995), phenacetin (Butler et al., 1989), 7-ethoxyresorufin (Bourdi et al., 1990), and theophylline (Bachmann et al., 2003) were commonly used to assess *in vitro* CYP1A2 enzyme activity. The radiometric caffeine method of Bloomer et al. (1995) was adopted with modification for the present study. Briefly, the incubation mixture consisted of non-labelled caffeine (82.0 µM), radiolabelled caffeine (0.2 µCi), NADPH (1.34 mM), potassium phosphate buffer (50.0 mM, pH 7.4), and 2.0 µL of inhibitor dissolved in DMSO. The final incubation volume was 200.0 µL. The volume of DMSO added did not exceed 1.0% of the incubation volume. The incubation mixture was pre-warmed for 5.0 min before the addition of 0.2 mg HLM to initiate the reaction. The incubation was conducted in a Thermo Scientific Precision Dubnoff metabolic incubator (Waltham, MA, USA) with a shaking rate of 60 cycles per min at 37.0^{0}C. The total incubation time was 10.0 min. The reaction was terminated with the addition of 50.0 µL of ice-chilled 10% TCA. The incubation mixture was kept on ice and centrifuged for 10.0 min at approx. 4000 xg. The radiolabeled metabolites in the supernatant were separated using Sigma-Aldrich Superclean ENVI-Carbon solid phase extraction (SPE) tubes (3.0 ml, 0.25 g, 80-100 mesh).

The SPE column was previously conditioned with 2.0 mL of methanol and 2.0 mL of water under gravity-driven flow. Once conditioned, 200-300 µL of the supernatant from the incubation mixture was gently applied onto the SPE cartridge. The formed formaldehyde and formic acid were eluted under gravity-driven flow with 2.0 mL of water, and were collected in scintillation vials as described by Bloomer et al. (1995) (Figure 4-3). The eluents were added to 15.0 mL of scintillation cocktail, vigorously mixed, and counted using a Hidex 300 SL Automatic Liquid Scintillation Beta Counter (Turku, Finland) or Beckman LS 6500 Scintillation Beta Counter (Brea, CA, US).

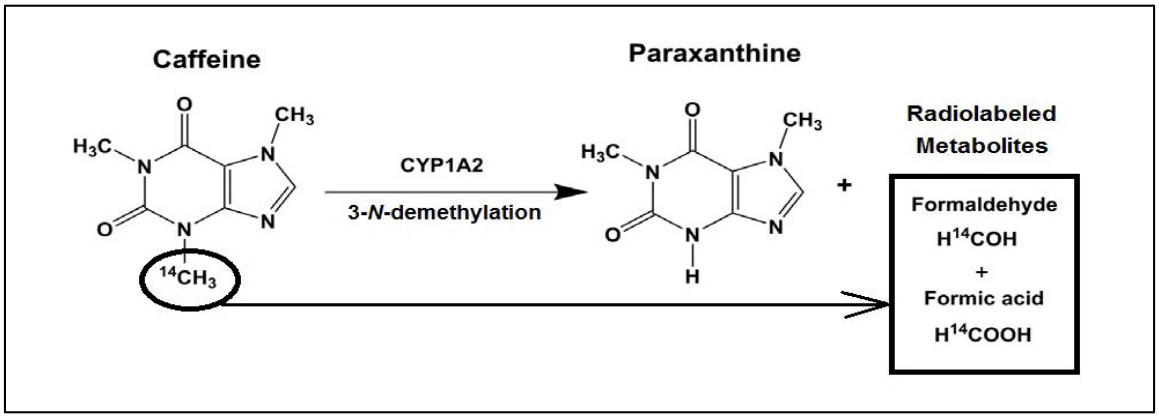

Figure 4-3: Radiolabeled caffeine metabolism *via* 3-*N*-$^{14}CH_3$ removal.

4.4.2. IC_{50} determination

Pure furanocoumarins were incubated separately with HLM (BD Gentest lot numbers of 99268/18888 for the first incubation; GIBCO, lot number PL050B for the second and third incubation) to measure the IC_{50} of CYP1A2 inhibition. In these studies, 8-MOP, 5-MOP, and ISOP standards were dissolved in DMSO and added to the incubation mixture. The final concentrations of 8-MOP in the incubation mixture ranged from 0.01-11.12 µM; 5-MOP ranged from 0.06-7.10 µM; and ISOP ranged from 0.01-38.45 µM. The final incubation volume was 200.0 µL. Final DMSO concentration did not exceed 1.0% of incubation volume. The logarithm (log_{10}) of furanocoumarin concentrations were plotted against the percentage CYP1A2 inhibition. The IC_{50} were determined using the best-fit, non-linear regression analysis of GraphPad Prism Software version 5.04 (San Diego, CA, USA).

4.4.3. Irreversible K_I and k_{inact} determination

The IC_{50} values of 8-MOP, 5-MOP and ISOP were used as guides to select the concentration range for the K_I and k_{inact} experiments. Thus, furanocoumarin standards were dissolved individually in DMSO to prepare a range of standard solutions: 1.07-17.14 µM for 8-MOP, 1.13-18.07 µM for 5-MOP, and 0.47-3.75 µM for ISOP. K_I and K_{inact} determination required prior knowledge of k_{obs} values which was first measured using the dilution incubation procedure (Obach et al., 2010). Thus, the first step involved pre-incubating HLM (GIBCO, lot number PL050B) with an initial concentration of 8-MOP, 5-

MOP, and ISOP of 0.7 μM, 1.77 μM, and 3.75 μM, respectively to determine the pre-incubation times. The selected pre-incubation times were: 0.5, 1.0, 1.5, and 2.0 min for 8-MOP and 5-MOP, and 0.5, 1.0, 2.0, and 3.0 min for ISOP. The pre-incubation step included HLM (1.2 mg), NADPH (8.48 mM), potassium phosphate buffer (50.0 mM, pH 7.4), and a furanocoumarin (12.0 μL). The total incubation volume was 190.0 μL. The second dilution incubation step involved removing an aliquot of the first incubation mixture and diluting it by more than 10-fold with potassium phosphate buffer (50.0 mM, pH 7.4) and used it in a second set of incubations containing NADPH (0.84 mM), non-labeled caffeine (50.0 μM), and radiolabel caffeine (0.2 μCi). The total incubation volume was 320.0 μL.

The remaining incubation and SPE steps were similar to those described earlier in the demethylation incubation assay (see 4.4.1.) except the volume of supernatant added to the SPE cartridge which was 300.0 μL instead of 200.0 μL. The k_{obs} values were obtained using linear regression analysis of a plot of natural logarithm (Ln) of remaining enzyme activity percentage *versus* preincubation times. The absolute k_{obs} values were then plotted against furanocoumarin inhibitor concentrations to calculate the K_I and k_{inact} values using non-linear regression analysis based on Equation 4-1:

Equation 4-1: Plotting equation for K_I and k_{inact} determination (Mayhew et al., 2000)

$$k_{obs} = \frac{k_{inact} \times [I]}{[I] + K_I}$$

where k_{obs} is the observed first-order decay rate constant (min^{-1}), k_{inact} is the maximal rate of inactive enzyme formation (min^{-1}) at saturating inhibitor concentrations, [I] is the initial concentration (μM) of the inhibitor in the incubation medium, and K_I is the inhibitor concentration based on k_{obs} equal half of measured k_{inact} value.

4.4.4. ^{14}C-8-MOP adduct formation with HLM and rCYP1A2

We also investigated if radiolabeled 8-MOP (Figure 4-4) formed irreversible adducts with HLM and rCYP1A2. The binding was investigated using different incubation conditions including omission of NADPH cofactor (-NADPH), reduction of oxygen

concentration (+N_2), increase in carbon monoxide concentration (+CO), and HLM deactivation by heat (+90ºC) to ascertain mechanism-based inhibition.

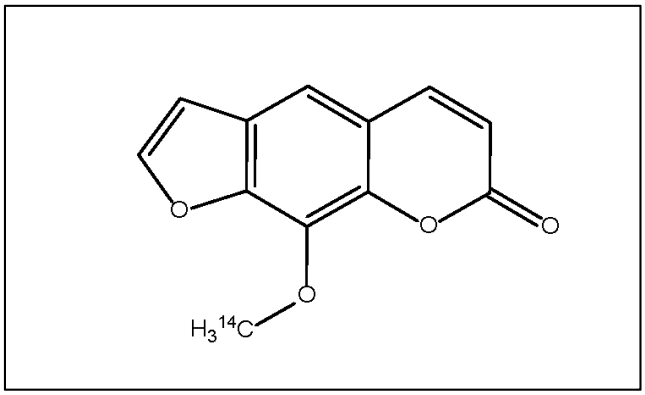

Figure 4-4: Chemical structure of 8-MOP with ^{14}C positioned at the methoxy functional group.

4.4.4.1 NADPH-dependent experiment

The purpose of this experiment was to determine if adduct formation depended on the availability of NADPH cofactor in the incubation mixture. The study was conducted using the procedure of Jollow et al. (1973) which included two sets of test tubes representing the control reaction (+NADPH) and test reaction (-NADPH). Each test tube included HLM (1.0 mg) (GIBCO, lot number PL050B) or human rCYP1A2 enzyme (1.1 mg), ^{14}C-8-MOP (0.2 µCi), potassium phosphate buffer (50.0 mM, pH 7.4), NADPH (1.0 mM) for control reaction, or substituted with potassium phosphate buffer (50.0 mM, pH 7.4) for blank reaction, with total incubation volume of 1.34 mL. The incubation mixture was pre-warmed for a minimum of 5.0 min and started with the addition of HLM or rCYP1A2.

The incubation was carried out in a metabolic incubator set at 60 cycles per min at 37ºC. At the end of a 10.0 min incubation period, the reaction was terminated by the addition of 1.0 mL of ice-chilled 20% TCA and kept on ice. After centrifugation for 10.0 min at approx. 4000 xg, the supernatant was separated and discarded. The remaining microsomal pellet was washed in sequence with 1.0 mL of methanol, 1.0 mL of acetonitrile, and 1.0 mL of acetone. Each solvent wash was carried out by a 5.0 min vortex mixing followed by a 10.0 min centrifugation at approx. 8000 xg. Three cycles of solvent wash were used for each of the microsomal pellets. Upon the last washing, the pellet was

reconstituted in 0.5 mL of potassium phosphate buffer (50.0 mM, pH 7.4) and transferred to a scintillation vial. The pellet suspension was further diluted by 15.0 mL of scintillation solution, vigorously mixed, and counted.

4.4.4.2 Oxygen-dependent experiment

The purpose of this experiment was to demonstrate the dependency of adduct formation on the availability of O_2 in the incubation mixture. The experiment included two sets of test tubes representing the control reaction ($-N_2$) and test reaction ($+N_2$). For the reduction of O_2 levels in the test reaction tubes, pure N_2 gas was bubbled in the test reaction mixture for 60.0 min, flushed with N_2, and capped with parafilm. The control reaction test tubes were treated similarly without N_2 treatment. The remaining incubation conditions were similar to that of -NADPH experiment.

4.4.4.3 Carbon monoxide-binding experiment

The purpose of this experiment was to demonstrate whether the presence of CO decreased 8-MOP adduct formation with HLM. The +CO experiment included two sets of test tubes representing the control reaction (-CO) and test reaction (+CO). For the binding of CO with the CYP enzyme, the test reaction tubes were treated with CO gas for 60.0 min, flushed with CO gas, and capped with parafilm. The control reaction test tubes were treated similarly without CO gas pretreatment. The remaining incubation conditions were similar to that of -NADPH experiment.

4.4.4.4 HLM-deactivation experiment

The purpose of this experiment was to detect non-specific binding in the incubation mixture. The heat experiment included two sets of test tubes representing the control reaction (without +90ºC pretreatment) and test reaction (with +90ºC pretreatment). For the measurement of non-specific binding, the test reaction tubes were heated for 60.0 min at 90ºC. Once the test tubes were cooled, ^{14}C-8-MOP (0.2 µCi), and NADPH (1.0 mM) were added. The control reaction test tubes were treated similarly without heat pretreatment.

The remaining incubation conditions were similar to that of NADPH-dependent experiment.

4.4.5. Integrated dose/concentration calculation for a mixture of furanocoumarins

The integrated dose/concentration of a mixture of furanocoumarins was estimated by summation of individual 8-MOP, 5-MOP, and ISOP concentrations in the herbal extract. Two different chemical mixture assessment models, namely the concentration/dose addition (CA) approach (Safe, 1998; ATSDR, 2004) and the whole-mixture (WM) approach (Hertzberg et al., 2000; Lorazen et al., 2004) were used to calculate the dose/concentration equivalent of 8-MOP for each of the *Ammi majus* L. seed and *Angelica archangelica* L. root extracts as these herbs contained significant amounts of 8-MOP, 5-MOP, and ISOP (see Chapter 2).

4.4.5.1 Whole-mixture (WM) approach

The WM approach is a common method of evaluating chemical mixture as a single entity without prior knowledge of individual chemical concentration in such mixture (Hertzberg et al., 2000) (Equation 4-2). Thus, a stock solution of *A.majus* seeds (or *A.archangelica* roots) was prepared by precisely weighing 6.0 g of *A.majus (*or 9.0 g *A.archangelica*) powder and mixed with 600 mL of tap water. The mixtures were boiled separately for approximately 3-4 h on a Fisher Thermix 11-493 model hot plate (Hampton, NH, USA). When half of the volume was evaporated, the herbal preparation was cooled to room temperature and filtered with a metal sieve. Exactly 20.0 mL aliquot of the filtrate was removed, put into a glass tube and evaporated down to dryness in a Jouan RC10.22 model vacuum concentrator (Saint-Herblain, France). The remaining residues were redissolved in 2.0 mL DMSO. Serial dilutions were prepared by mixing 0.5 mL of the filtrate with 0.5 mL of DMSO. This procedure was repeated to yield 6 serial concentrations (100%-1.56%) relative to the full strength of each herbal extract. The dilution series were incubated separately with HLM (Xenotech, lot number 1210267), ^{14}C-caffeine, non-labelled caffeine, and NADPH. The incubation conditions and data analysis were similar to those described to determine the IC_{50} values of furanocoumarin standards. The IC_{50}

values of the herbal extract expressed in dilution factor were determined. Using the IC_{50} values of 8-MOP and herbal extracts; we were able to calculate the total dose/concentration for the furanocoumarin mixtures as follows:

Equation 4-2: The WM approach equation (Lorazen et al., 2004)

$$\text{Dose/Concentration Equivalents} = \frac{\text{CM IC}_{50}}{\text{Extract IC}_{50}} \times \frac{\text{VAM}}{\text{VET}} \times \frac{\text{VHE}}{\text{DWH}}$$

where "CM IC_{50}" is the concentration of the chemical marker to elicit 50% of the maximal inhibition (mg/mL), "extract IC_{50}" is the dilution factor of the prepared extract required to elicit 50% of the maximal inhibition (unitless); VAM is the volume of the assay medium (mL); VET is the volume of extract tested (µL); VHE is the volume of herb stock extract (µL); DWH is the dry weight of herb used to prepare the herb stock extract (g).

4.4.5.2 Concentration/dose addition (CA) approach

The CA approach is considered the most common and default chemical mixture assessment model (Seed, 1995). The CA approach is a model-based method of relating individual chemical concentrations to a specific biological activity with the assumption that individual chemical congeners exert similar mechanism of biological effect and are additive in nature. The CA approach is defined as the sum of individual furanocoumarin relative potency factor (RPF) multiplied with the measured individual furanocoumarin concentration as shown in Equation 4-3.

Equation 4-3: The CA approach model (Safe, 1998; ATSDR, 2004)

$$\text{Dose/Concentration Equivalents} = \sum [\text{C}_\text{I}] \times \text{RPF}$$

where C_I is the concentration of the individual furanocoumarin in the chemical mixture (mg/g) and RPF is the relative potency of each chemical in comparison to 8-MOP.

Individual furanocoumarin concentrations in the extracts of *A.majus* seeds and *A.archangelica* roots were determined previously (see Chapter 2). The furanocoumarins

were separated and measured using a Phenomenex (Torrance, CA, USA) Kinetex pentafluorophenyl column (250 x 4.6 mm, 5 µm particle size) and a gradient high-performance liquid chromatography method using water and acetonitrile as solvents with the ultra-violet detector set at 310 nm. The measured concentrations for 8-MOP, 5-MOP, and ISOP was 3.21, 0.72, and 7.54 mg/g dry weight for *A.majus* seeds and 0.86, 0.47, and 0.74 mg/g dry weight for *A.archangelica* roots, respectively. The RPF value for each furanocoumarin was determined by dividing the IC_{50} of 8-MOP with individual furanocoumarin IC_{50} values.

4.4.6. Statistics and data analysis

The significance of +90ºC, -NADPH, +N_2, and +CO pretreatment for HLM and rCYP1A2 was determined by Student's (1908) paired *t*-test, two-tailed (95% confident interval). The GraphPad Prism Software version 5.04 (San Diego, CA, USA) and Microsoft Excel software version 2010 (Redmond, WA, USA) were used for plotting, statistical analysis, and IC_{50}, K_I, and k_{inact} estimation. The IC_{50}, K_I, and k_{inact} values were expressed as mean ± standard deviation (SD) of three experiments.

4.5. Results and Discussion

4.5.1. IC_{50} determination

We investigated the inhibitory potencies of 8-MOP, 5-MOP, and ISOP on CYP1A2 with HLM. Figures 4-5 to 4-7 depict the concentration-inhibition curves of the furanocoumarins. These dose-response curves were used to determine the IC_{50} of 8-MOP, 5-MOP, and ISOP inhibition on caffeine metabolism; they were 0.09 ± 0.05 µM, 0.13 ± 0.11 µM, and 0.29 ± 0.22 µM, respectively.

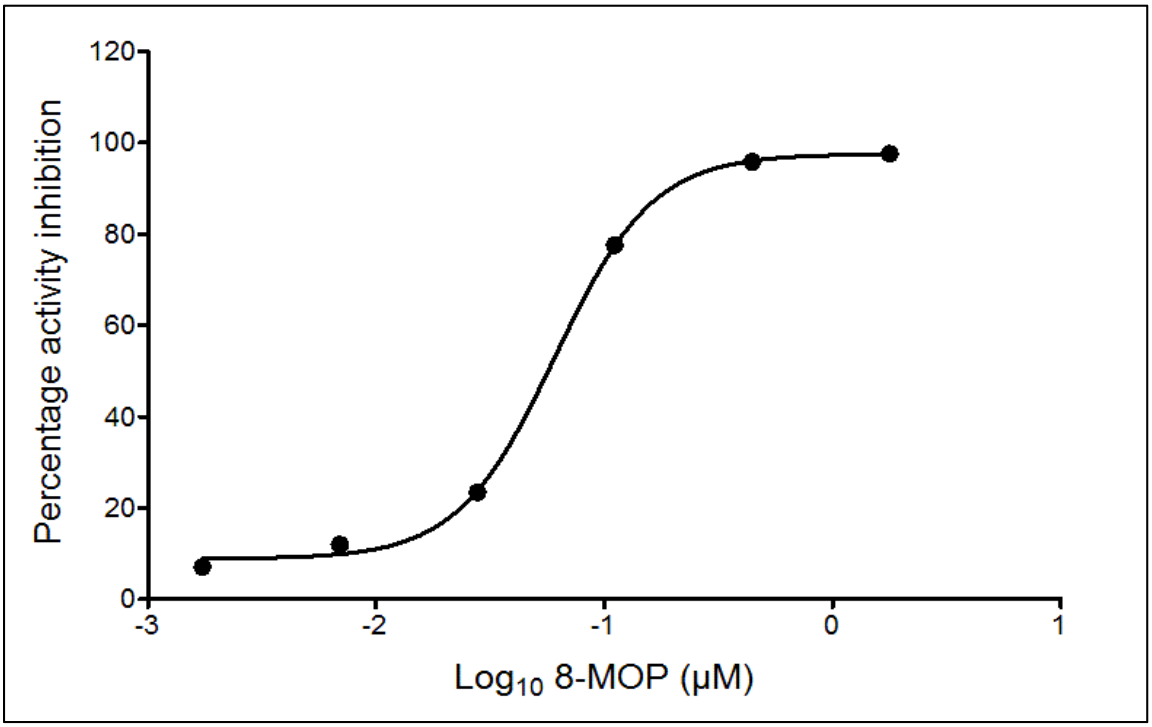

Figure 4-5: Inhibition of CYP1A2 by 8-MOP standard using HLM. Each point represents single concentration incubation.

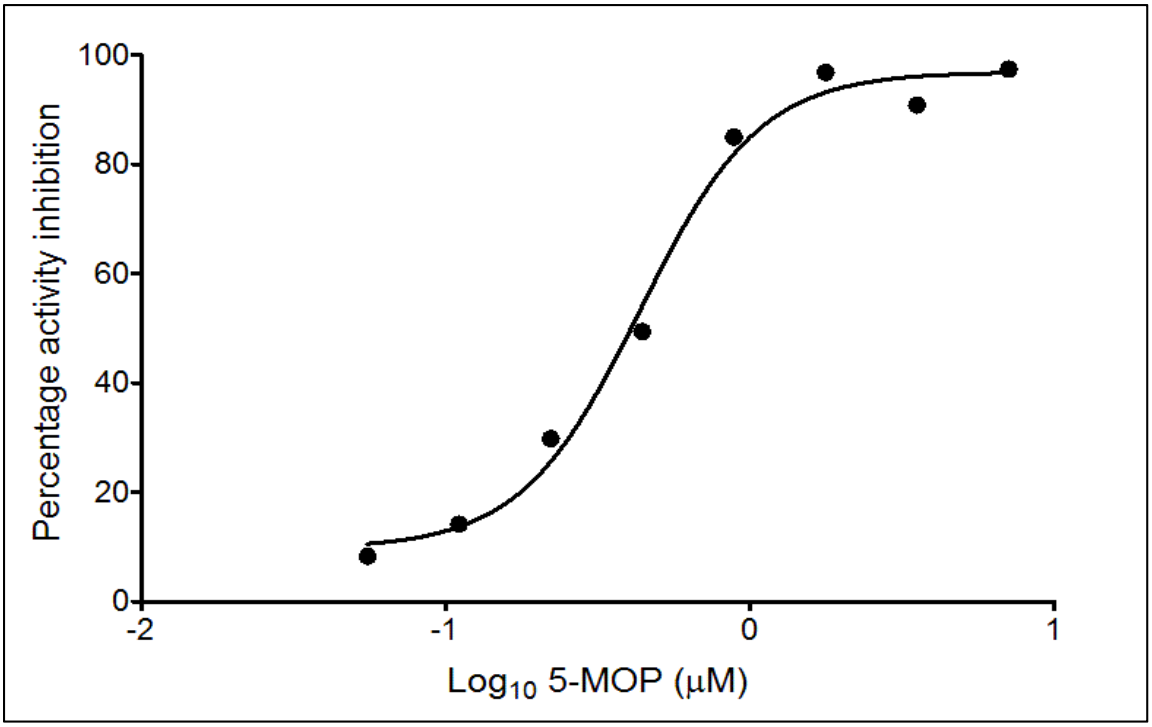

Figure 4-6: Inhibition of CYP1A2 by 5-MOP standard using HLM. Each point represents single concentration incubation.

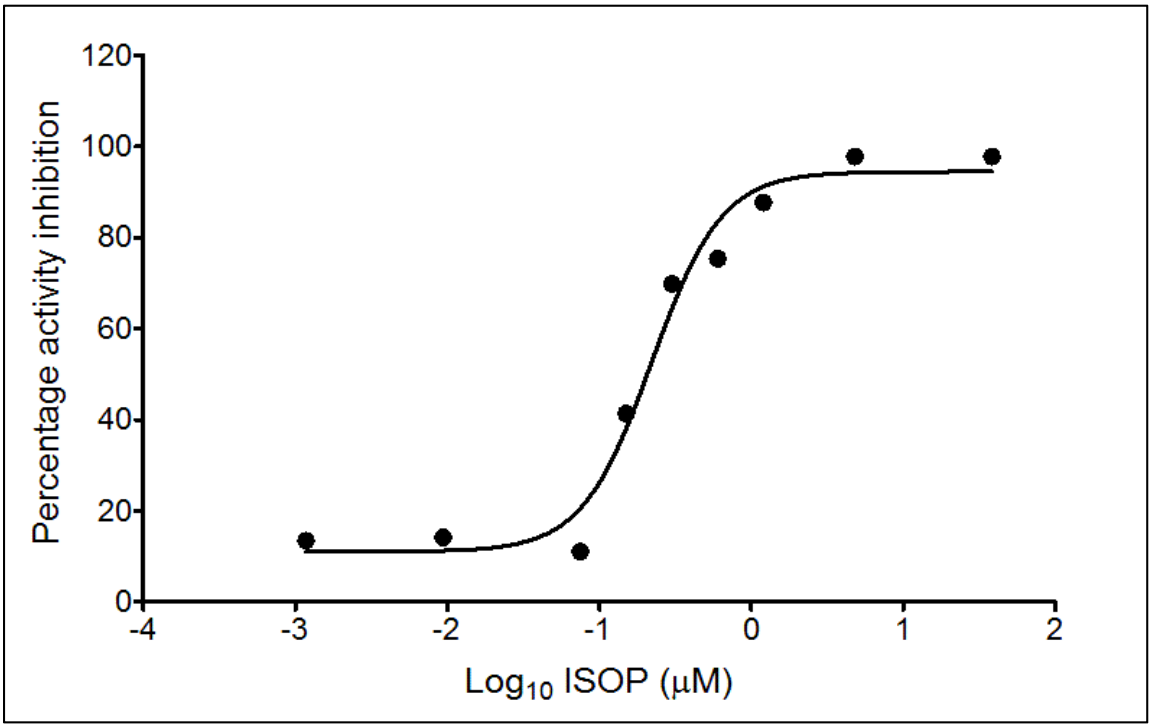

Figure 4-7: Inhibition of CYP1A2 by ISOP standard using HLM. Each point represents single concentration incubation.

4.5.2. K_I and k_{inact} determination

We also performed a preliminary study on the mechanism of caffeine metabolism inhibition using the dilution method of Obach et al. (2010). Thus, 8-MOP, 5-MOP, or ISOP was dissolved in DMSO and incubated with various preincubation periods with and without NADPH. The concentrations of 8-MOP, 5-MOP, and ISOP in the incubation were 0.7 μM, 1.77 μM, and 3.75 μM, respectively. Figures 4-8 to 4-10 show the inhibition of caffeine metabolism *in vitro* by 8-MOP, 5-MOP, and ISOP is dependent on NADPH as well as the preincubation time, the typical characteristics of time-dependent inhibition or mechanism-based inhibition.

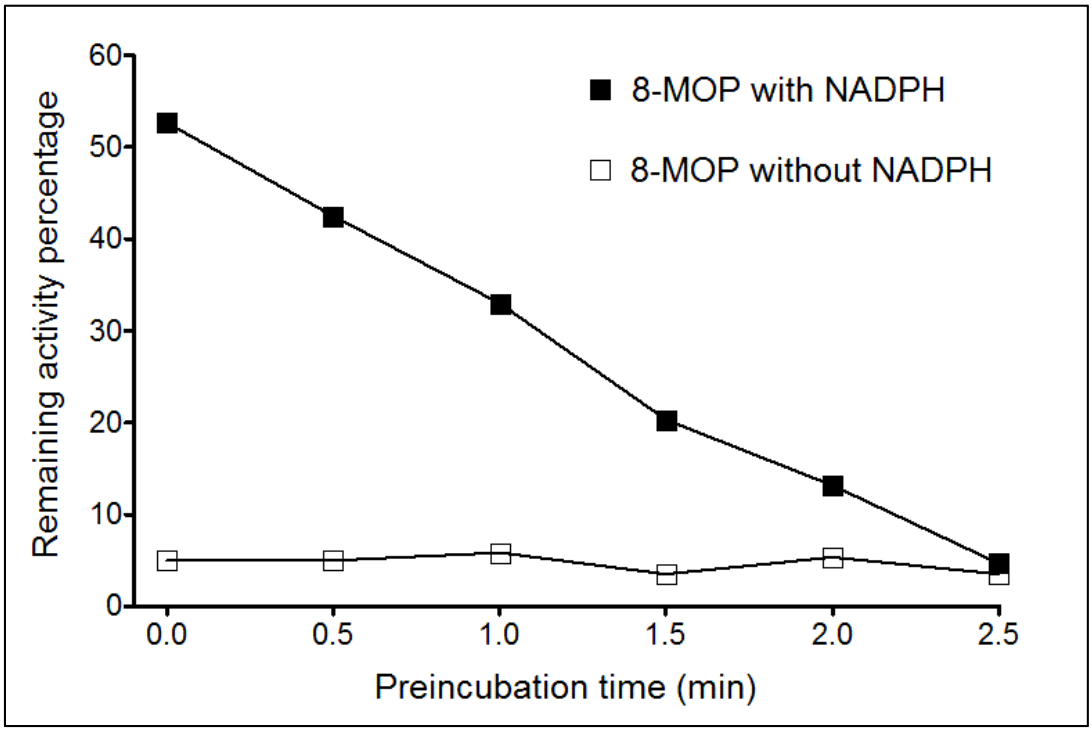

Figure 4-8: Time and NADPH-dependent of 8-MOP inactivation of CYP1A2 using HLM. Each point represents the average of three measurements.

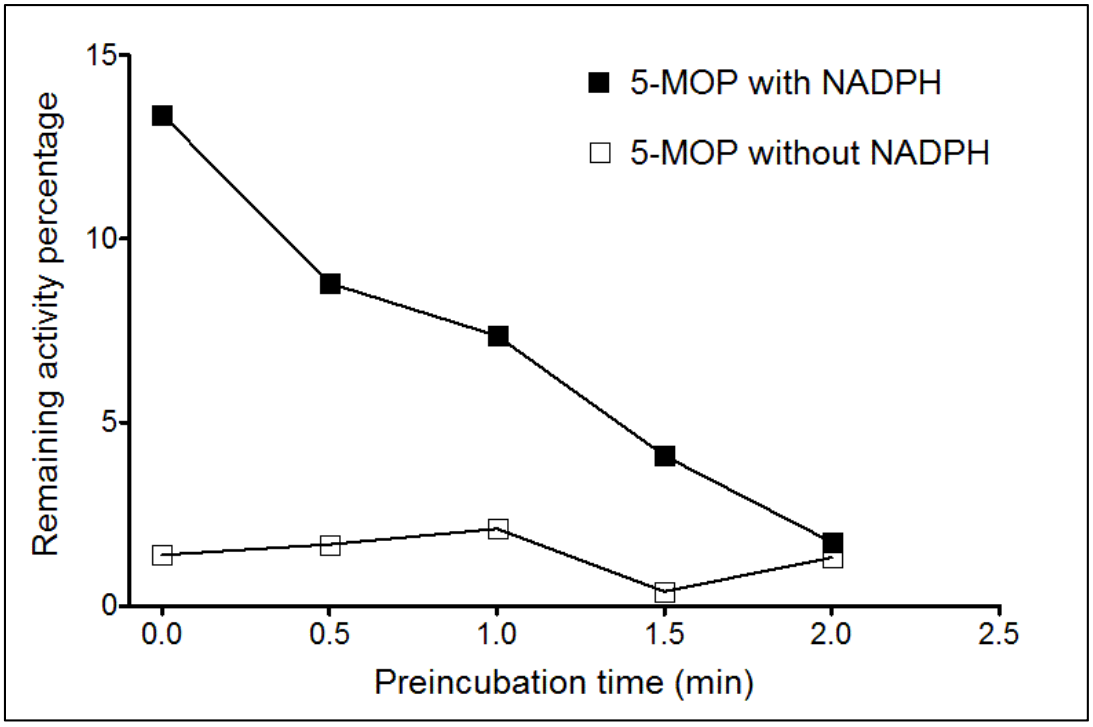

Figure 4-9: Time and NADPH-dependent of 5-MOP inactivation of CYP1A2 using HLM. Each point represents the average of three measurements.

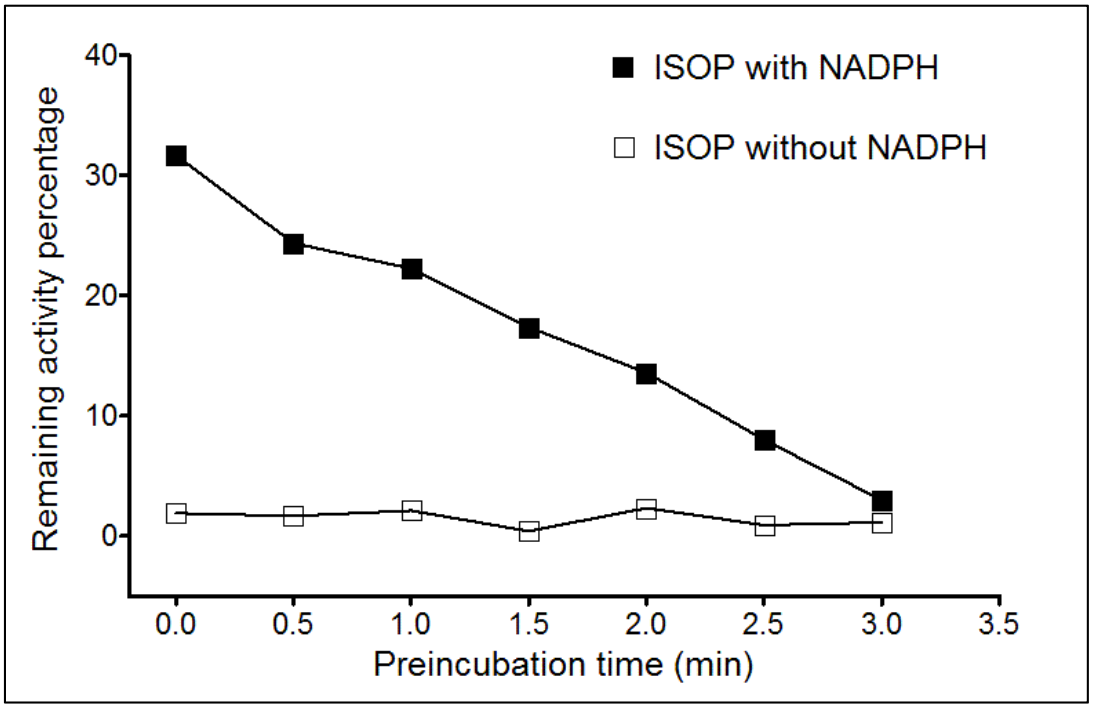

Figure 4-10: Time and NADPH-dependent of ISOP inactivation of CYP1A2 using HLM. Each point represents the average of three measurements.

Based on these preliminary results, we selected the following pre-incubation time periods for a full confirmatory study: 0.5, 1.0, 1.5, 2.0 min for 8-MOP and 5-MOP, and 0.5, 1.0, 2.0, and 3.0 min for ISOP. The full confirmatory study was conducted by preincubating HLM with different inhibitor concentrations and preincubation time periods to obtain the k_{obs} values for individual furanocoumarins. These results are shown in Figures 4-11 to 4-13.

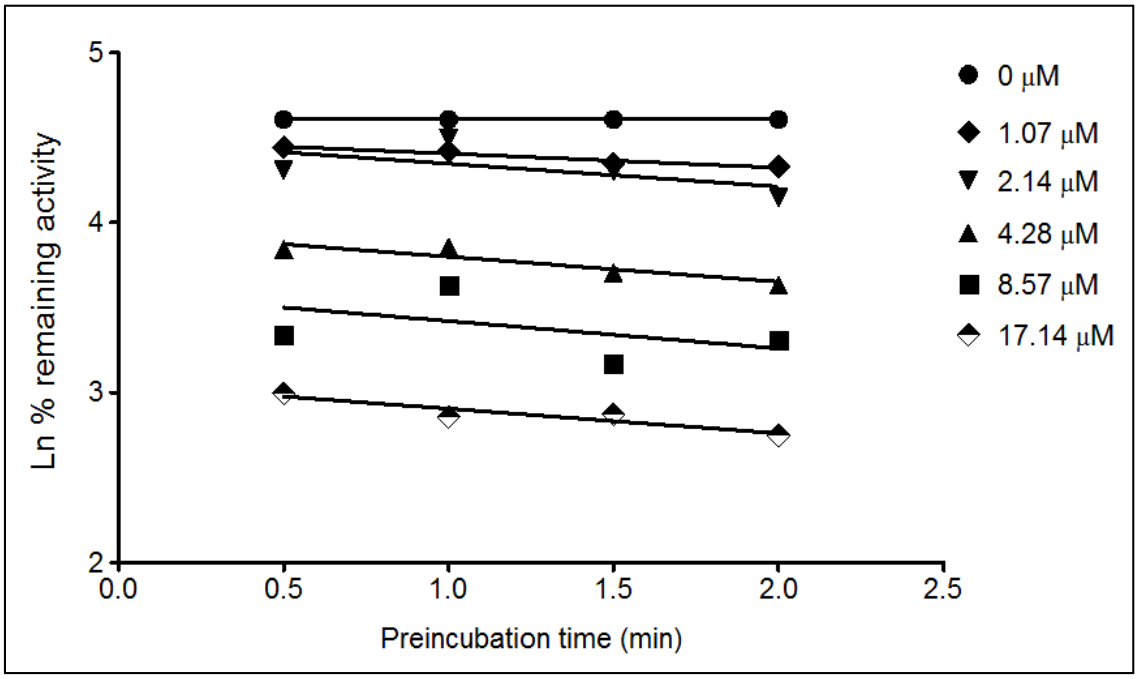

Figure 4-11: Time- and concentration-dependent of CYP1A2 inactivation by 8-MOP using HLM. Each data point represents the average of three experiments.

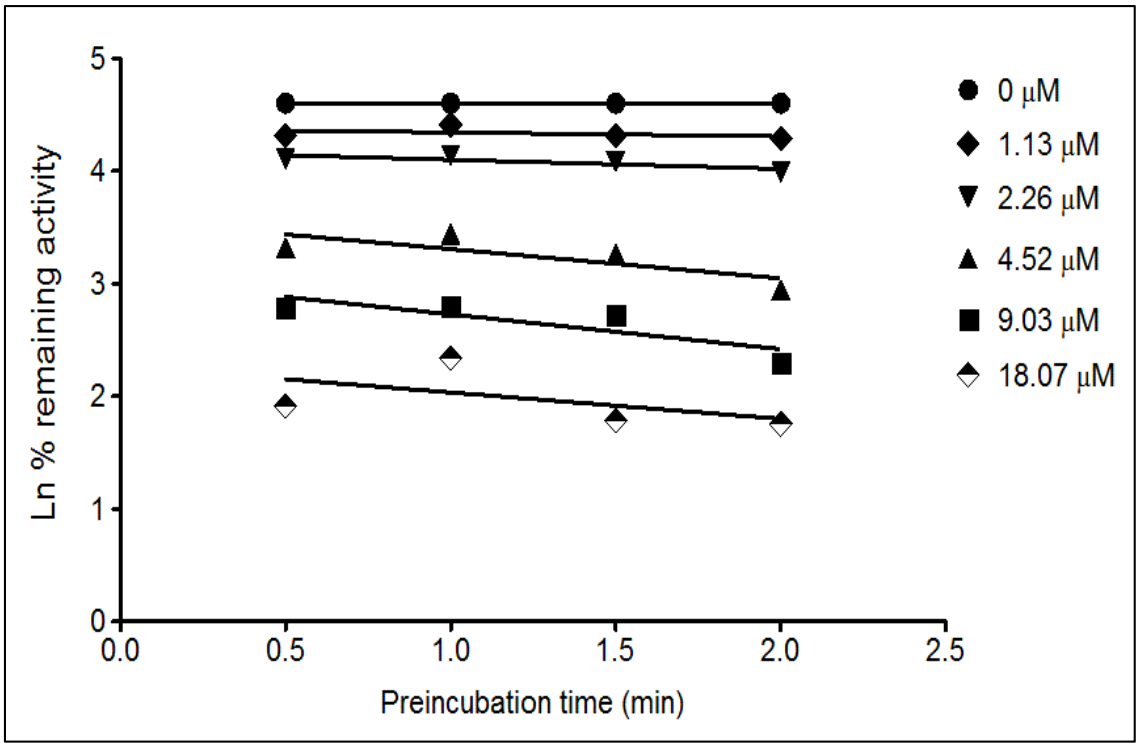

Figure 4-12: Time- and concentration-dependent of CYP1A2 inactivation by 5-MOP using HLM. Each data point represents the average of three experiments.

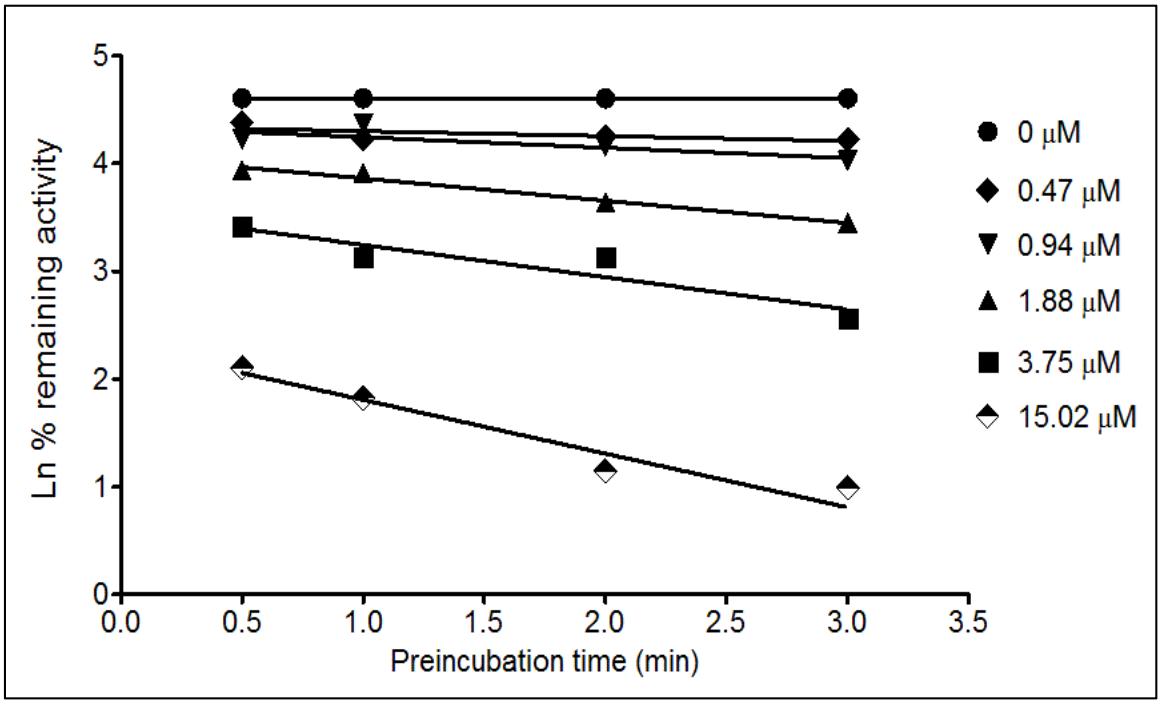

Figure 4-13: Time- and concentration-dependent of CYP1A2 inactivation by ISOP using HLM. Each data point represents the average of three experiments.

The k_{obs} *versus* concentration plots of 8-MOP, 5-MOP, and ISOP are shown in Figures 4-14, 4-15, and 4-16, respectively. The K_I values estimated for 8-MOP, 5-MOP, ISOP were 0.78 ± 0.32 μM, 3.72 ± 3.66 μM, 4.48 ± 0.56 μM, respectively. The corresponding k_{inact} values were 0.17 ± 0.01 min^{-1}, 0.35 ± 0.12 min^{-1}, 0.65 ± 0.03 min^{-1}, respectively. These results show 8-MOP, 5-MOP and ISOP are potent TDI or MBI of CYP1A2 enzyme.Table 4-1 summerizes the inactivation constants and IC_{50} values for the linear furanocoumarins.

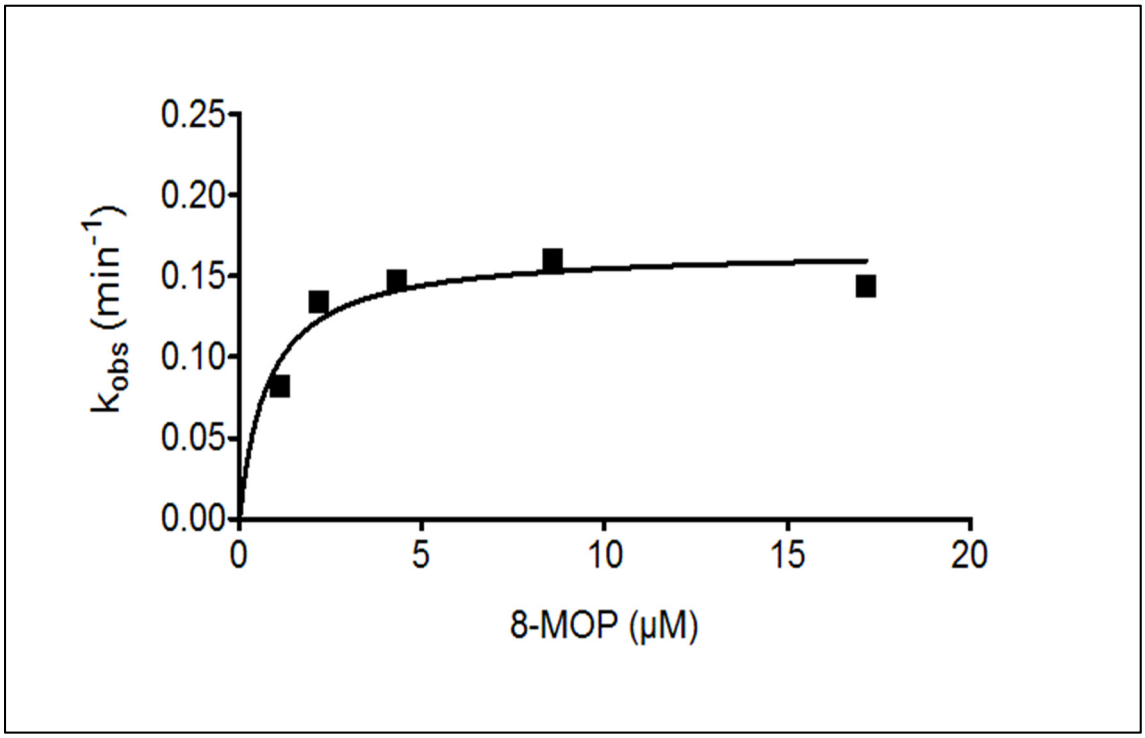

Figure 4-14: Non-linear relationship between k_{obs} (min^{-1}) and 8-MOP concentration (μM). Each data point represents the average of three experiments.

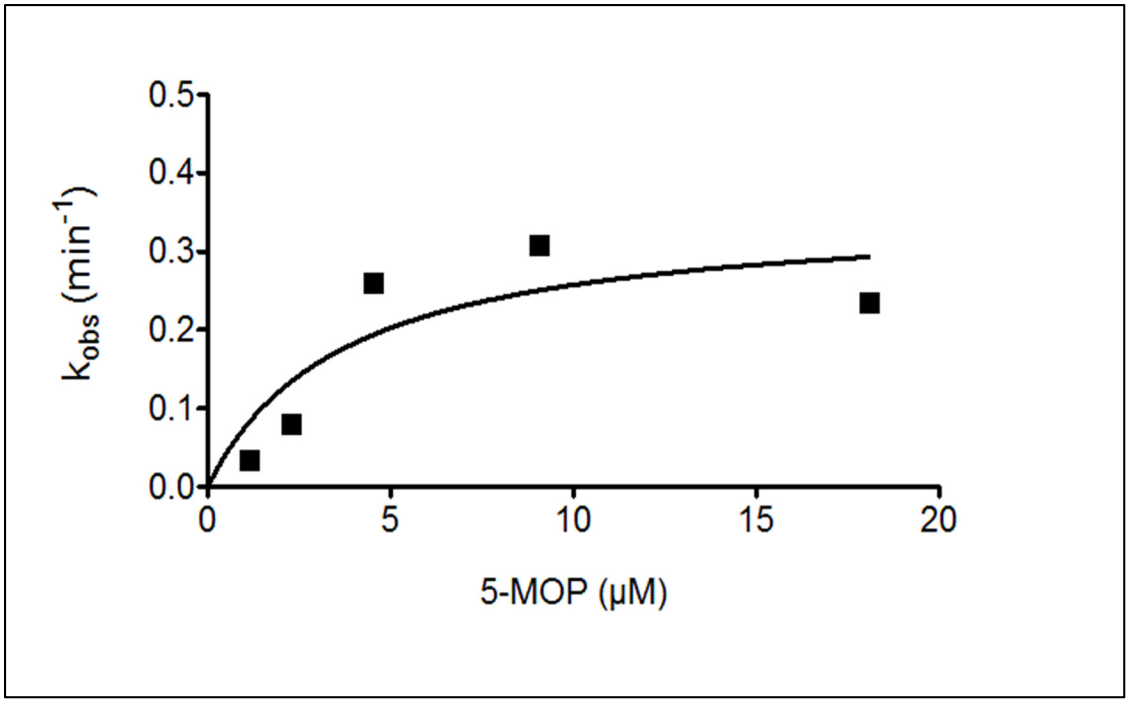

Figure 4-15: Non-linear relationship between k_{obs} (min^{-1}) and 5-MOP concentration (μM). Each data point represents the average of three experiments.

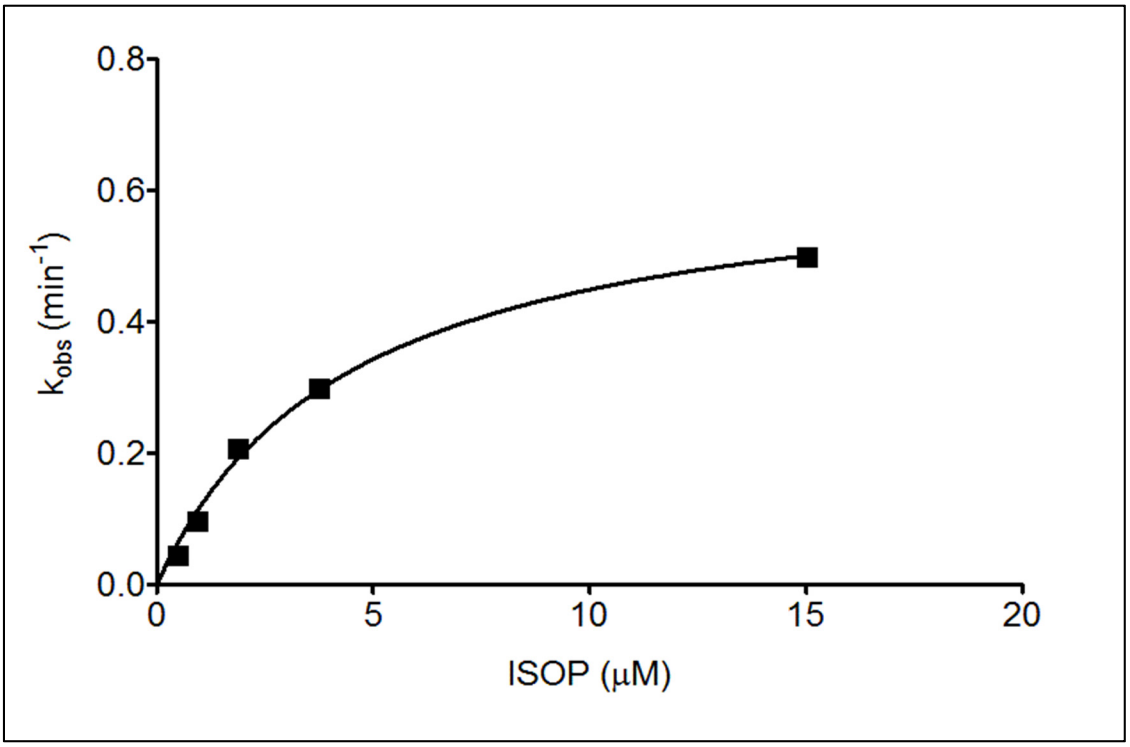

Figure 4-16: Non-linear relationship between k_{obs} (min^{-1}) and ISOP concentration (µM). Each data point represents the average of three experiments.

Table 4-1: A summary of *in vitro* inhibition kinetic constants for 8-MOP, 5-MOP, and ISOP on CYP1A2 enzyme using HLM.

Furanocoumarin	MW	*In Vitro* Inhibition Parameters [a]								
		IC_{50} (µM)			K_I (µM)			k_{inact} (min^{-1})		
8-MOP	216.19	0.09	±	0.05	0.78	±	0.32	0.17	±	0.01
5-MOP	216.19	0.13	±	0.11	3.73	±	3.66	0.35	±	0.12
ISOP	246.22	0.29	±	0.22	4.48	±	0.56	0.65	±	0.03

[a] Results are expressed as mean ± SD of three experiments.

Kang et al. (2011) have reported that ISOP is a MBI with K_I and k_{inact} values of 1.2 µM and 0.34 min^{-1}, respectively. Our results are in general agreement with their findings although we have slightly higher K_I values (Table 4-1). The authors have monitored methoxyresorufin *O*-demethylase (MROD) activity in microsomes prepared from *Saccharomyces cerevisiae* expressing human CYP1A2. In contrast, we used caffeine and HLM to characterize the inactivation constants of ISOP. The discrepancy in results is probably due to the use of different probe substrates and CYP1A2 enzyme to obtain the inactivation constants. The use of HLM closely reflectes *in vivo* human situation and

should yield, in theory, more accurate results in *in vitro-in vivo* extrapolation; which also explains the observed preferential use of HLM in drug metabolism studies among researchers (Grimm et al., 2009).

The inactivation constants in the present study are comparable to those of furafylline, a known suicidal inactivator of CYP1A2. The K_I and k_{inact} values for furafylline range from 6.9-23 μM and 0.07-0.87 min^{-1} respectively (Kunze and Trager, 1993; Tassaneeyakul et al., 1994). Our results also are consistent with the general belief that linear furanocoumarins are more potent inhibitors for CYP1A2 than angular furanocoumarins (Cai et al., 1993; Kang et al., 2011), although angelicin, an angular furanocoumarin, is a more potent inactivator for hCYP1A2 than psoralen, a linear furanocoumarin (Zhuang et al., 2013).

4.5.3. 8-MOP adduct formation with CYP1A2 isozyme

We incubated ^{14}C-8-MOP with HLM or rCYP1A2 to demonstrate the formation of 8-MOP reactive metabolite(s) which bound irreversibly to microsomal proteins. The radiolabeled assay was rapid, sensitive, and specific methods to determine binding with liver microsomes as it had minimal background interference. At the conclusion of the incubation, the microsomal proteins were denatured with TCA, and the unbound ^{14}C-8-MOP was removed with three solvent washing cycles as described earlier. In addition to methanol and acetonitrile, we also used acetone to ensure the removal of interfering ^{14}C-8-MOP metabolites. In the preliminary study, 9 solvent washes were determined sufficient to remove unbound radioactivity from the HLM. Figure 4-17 shows the radioactivity bound to HLM and rCYP1A2 are dependent on the availability of NADPH cofactor in the incubation mixture. These results clearly show ^{14}C-8-MOP is metabolized to reactive metabolite(s) which bind irreversibly, and presumably covalently, to the microsomal proteins.

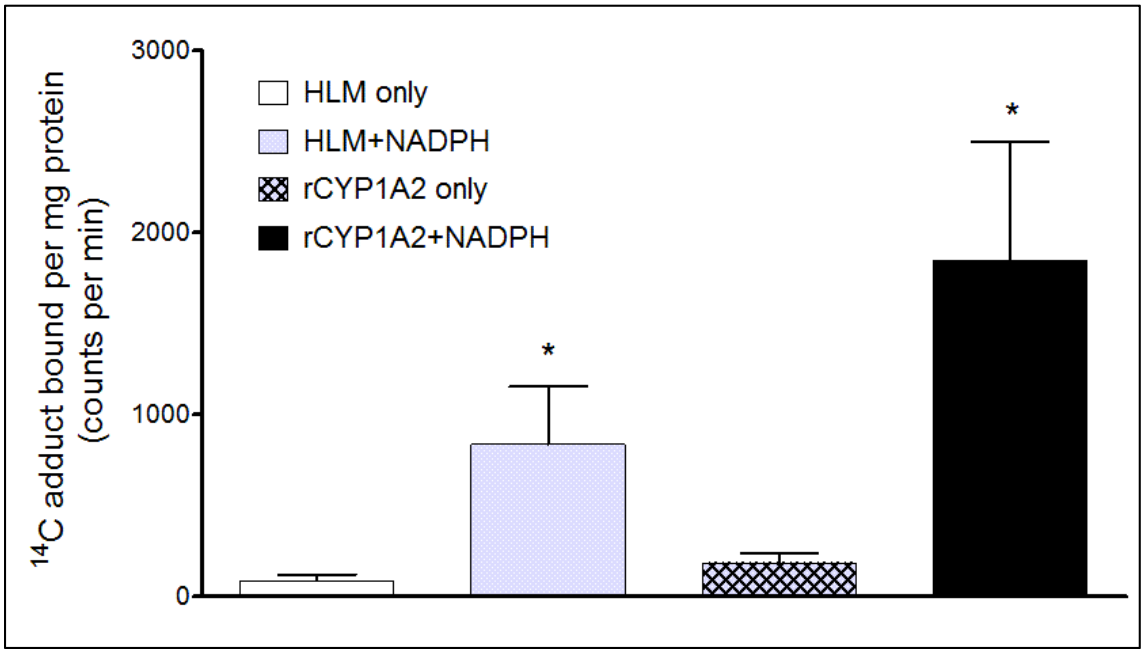

Figure 4-17: NADPH-dependent covalent-binding of ^{14}C-8-MOP with HLM and rCYP1A2 (n=3). Asterisk indicates significantly different to incubations without NADPH (P≤0.05).

We also investigated the possibility of signal quenching due to the presence of HLM in the liquid scintillation counter using a Harvey Biological Instruments oxidizer (Tappan, NY, USA). The oxidizer combustion temperature was set at 900ºC. About 200.0 µL of the incubation mixture was added to 0.5 g of sucrose and burned in the oxidizer. The $^{14}CO_2$ generated was trapped in a scintillation cocktail solution which was counted in the liquid scintillation counter. The biological oxidizer was found to yield lower counts compared to adding the incubation mixture directly to the scintillation cocktail perhaps due to the loss of $^{14}CO_2$ during combustion. Nevertheless, these results indicate that the incubation mixture does not cause significant signal quenching in the liquid scintillation counter when added directly to the scintillation cocktail (Figure 4-18).

The formation of ^{14}C-adducts with HLM was significantly reduced with boiled microsomes, removal of NADPH cofactor, reduction of O_2, and the addition of CO gas before incubation (Table 4-2). These results indicate that adduct formation is mediated by CYP450 enzyme, a finding which is consistent with the report that 8-MOP binds strongly to the apoprotein moiety of the liver microsomes of mice (Mays et al., 1990), rats (Fouin-Fortunet et al., 1986), and humans (Tinel et al., 1987).

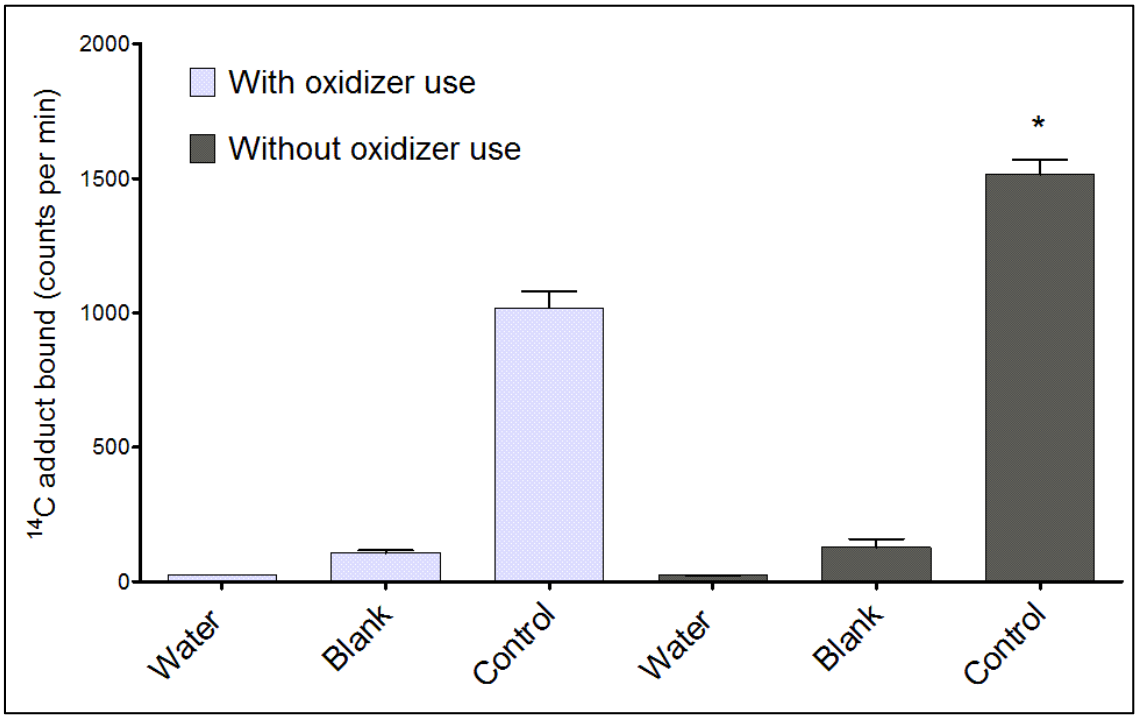

Figure 4-18: Measurement of radiolabelled adducts with HLM (1.0 mg protein) with and without using a biological oxidizer (*n*=2). Asterisk indicates significantly different to oxidizer use (*P*≤0.05).

Table 4-2: Inhibition of ^{14}C-8-MOP metabolite binding with HLM under different incubation conditions.

Reaction Mixture	*n*	^{14}C-8-MOP Metabolite Bound [a b] (pmol per mg protein)			Average Inhibition %			*P* value
Boiled Microsomes (+90ºC) [c]	3	6.30	±	2.99	89.35	±	5.06	≤ 0.01
Without NADPH (-NADPH) [c]	3	7.55	±	3.11	87.24	±	5.26	≤ 0.01
Oxygen Reduction (+N_2) [d]	3	47.17	±	10.20	44.24	±	12.05	0.02
With Carbon Monoxide (+CO) [e]	2	15.44	±	1.02	58.38	±	2.76	0.02

[a] Results are expressed as mean ± SD.

[b] Incubation time is 10 min.

[c] Complete system (without pretreatment) is 59.1 pmol per mg protein.

[d] Complete system (without pretreatment) is 84.6 pmol per mg protein.

[e] Complete system (without pretreatment) is 37.1 pmol per mg protein.

The formation of ^{14}C adducts with HLM indicates that ^{14}C-8-MOP molecule remains intact and is in agreement with previous studies reporting the opening of the molecule's furan ring during biotransformation (Sahali-Sahly et al., 1996). These results also agree

with previous reports that the main pathway of 8-MOP metabolism in dog is epoxidation of the furan ring (Kolis et al., 1979) and that *O*-hydroxylation is only a minor metabolic pathway in humans (Ehrsson et al., 1978). The formation of ^{14}C adducts provides additional evidence that 8-MOP is a MBI. In view of the similarities in molecular structures and metabolic pathways (John et al., 1992; Schmid et al., 1980), it is likely that 5-MOP is also a MBI which binds irreversibly to HLM.

4.5.4. Integrated dose/concentration for a mixture of furanocoumarins

We also examined if 8-MOP would be a good chemical marker (CM) to represent the mixtures of furanocoumarin bioactive in *A.majus* and *A.archangelica* extracts. An integrated dose of the furanocoumarin bioactive was estimated using the following models: (a) the concentration addition (CA) model which assumed additivity of individual furanocoumarin concentrations after being adjusted for their inhibition potencies, and (b) the whole mixture (WM) model which, unlike the CA approach, does not assume additivity of the chemicals in the mixture but to measure the inhibitory potency of the furanocoumarin mixture as a single entity.

Using the CA model (Equation 4-3), the total dose/concentration of furanocoumarin bioactive in *A.majus* and *A.archangelica* extracts were estimated to be 5.86 and 1.42 mg 8-MOP equivalent/g of dry herb, respectively. It is appropriate to use the CA model to calculate an integrated dose of furanocoumarin bioactive in the herbal extract because 8-MOP, 5-MOP, and ISOP meet the requirements of model application. For example, the furanocoumarin chemicals are isomers or congeners. The dose-response curves of 8-MOP, 5-MOP, and ISOP are parallel with slopes of 2.0, 2.2, and 1.9, respectively. The IC_{50} of caffeine metabolism inhibition for 8-MOP, 5-MOP, and ISOP were 0.09 ± 0.05 μM, 0.13 ± 0.11 μM, and 0.29 ± 0.22 μM, respectively (Figures 4-5 to 4-7). Thus, the estimated RPE were 1.00, 0.69 and 0.31 for 8-MOP, 5-MOP and ISOP respectively.

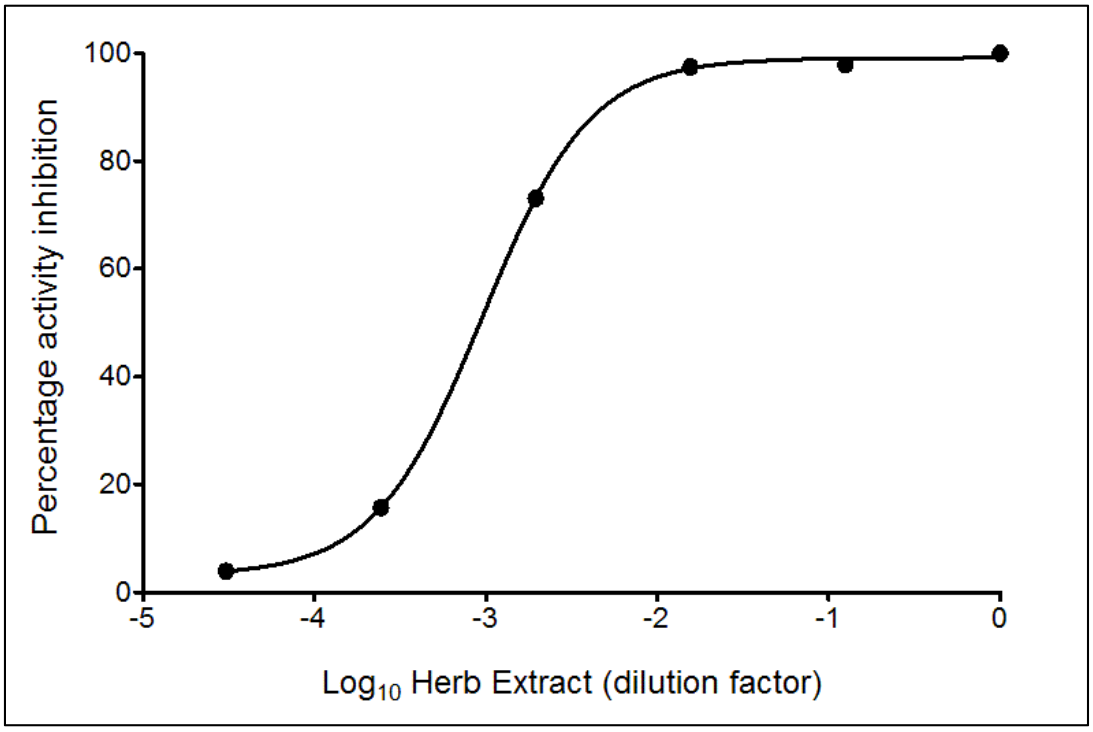

Figure 4-19: Inhibition of CYP1A2 by *A.majus* seed extract using HLM. Each point represents single incubation of the diluted extract.

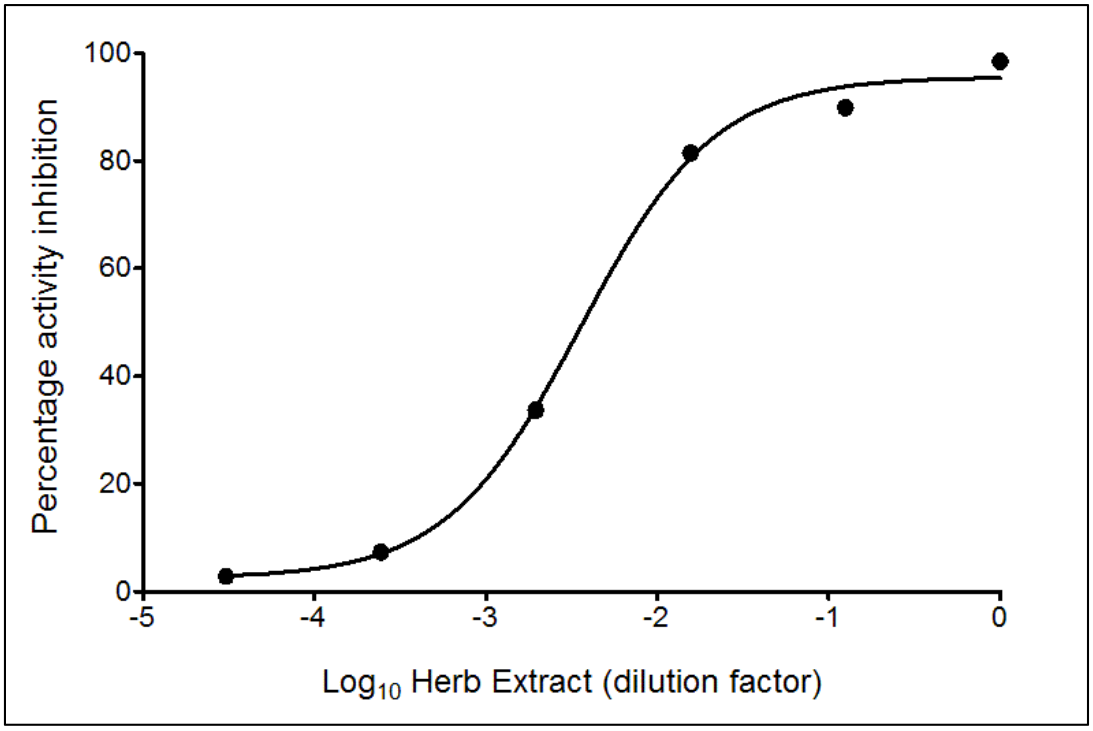

Figure 4-20: Inhibition of CYP1A2 by *A.archangelica* root extract using HLM. Each point represents single incubation of the diluted extract.

The IC_{50} of caffeine metabolism inhibition by *A.majus* and *A.archangelica* extracts were 0.001292 and 0.003868 dilution factor (unitless), respectively (Figures 4-19 and 4-20). The VAM, VET, and VHE values were 0.2 mL, 2.0 µL, and 2000.0 µL respectively and the DWH values used for *A.majus* and *A.archangelica* herbal extracts were 0.4 and 0.6 g dry weight respectively. Using the WM approach, the integrated dose/concentration of the furanocoumarin bioactive in *A.majus* and *A.archangelica* extracts were estimated to be 7.5 and 1.8 mg 8-MOP equivalent/g dry herb, respectively. The integrated doses calculated by the WM approach (Equation 4-2) are comparable to those calculated by the CA approach (Equation 4-1).

Because 8-MOP, 5-MOP, and ISOP are the major furanocoumarins in the *A.majus* and *A.archangelica* herbs (see Chapter 2), it is not surprising that both CA and WM approaches would yield similar integrated doses for the herbal extracts. The success of the CA approach in predicting integrated doses suggests that the inhibitory potencies of 8-MOP, 5-MOP, and ISOP contribute additively to caffeine metabolism inhibition. Results of the study also confirm a previous report that the furanocoumarins of (R)-6′,7′-dihydroxybergamottin, bergamottin, GF-I-1, GF-I-4, and GF-I-6, contribute additively to the inhibitory effects of grapefruit juice on CYP3A-mediated testosterone 6β-hydroxylase activity (Guo et al. 2000). In the present study, the WM approach generally predicts a higher integrated dose/concentration than the CA approach. An explanation for the difference in results most probably is due to the inclusion of other minor furanocoumarin bioactive(s), or CYP1A2 inhibitor(s), in the WM approach but not in the CA approach.

4.6. Conclusions

Linear furanocoumarins such as 8-MOP, 5-MOP, and ISOP are potent MBI of CYP1A2 activity. The abundance of 8-MOP, 5-MOP, and ISOP in foods and herbs from the *Apiaceae* and *Rutaceae* plant families suggest the importance of studying the reduction of CYP1A2 activity in humans by these plant products. As expected, the furanocoumarin bioactive in *A.majus* and *A.archangelica* extracts inhibited CYP1A2 activity additively. Both the CA and WM approaches are capable of predicting an integrated dose/concentration for the furanocoumarin mixture in the *A.majus* or *A.archangelica* extract. Thus, either CA or WM model may be used to calculate an

integrated dose for the bioactive chemicals in functional foods, health supplements, and nutraceuticals.

4.7. References

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Chapter 5.

In Vitro-In Vivo Extrapolation of Herb-Caffeine Interaction in Humans

5.1. Abstract

Background: New drug candidates (NDC) must undergo extensive tests and scrutiny on different issues related to drug safety and efficacy before they are sold in the market. Drug-drug interaction (DDI) is one of the issues of concern. As a result, many *in vitro-in vivo* extrapolation (*IVIVE*) models have been developed to predict DDI before initiating clinical trial studies. Herb-drug interaction (HDI) has also become an increasingly important research area in recent years due to the increasing popularity of natural health products. HDI is difficult to predict as a botanical drug usually contains a complex mixture of phytochemicals which might interact with the prescription drug. Additionally, there is no pharmacological tool to study HDI since almost all current *IVIVE* DDI models deal with the interaction between one "perpetrator inhibitor drug" and one "victim substrate drug".

Objectives: To modify the *IVIVE* DDI models of Mayhew et al. (2000) and Wang et al. (2004) for the prediction of *in vivo* interaction between caffeine and a furanocoumarin-containing herb, and to confirm model prediction by comparing the predictive results with experimental data. In the present study, caffeine is the victim drug and the herbal extracts, which contain 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and/or isopimpinellin (ISOP), are the inhibitors of the interaction.

Methods: The Mayhew et al. (2000) and Wang et al. (2004) DDI models were modified and used to predict *in vivo* herb-caffeine interaction. These *IVIVE* models were implemented using the same set of inhibition constants (see Chapter 4) but different integrated dose/concentration of the furanocoumarin inhibitor mixture in the liver. In the present study, four different *in vivo* hepatic inlet inhibitor concentration ($[I]_H$) surrogates were calculated from plasma C_{max} of each furanocoumarin. In the Mayhew et al. (2000) model, the $[I]_H$ was predicted using the concentration addition (CA) model for chemical mixtures. In the Wang et al. (2004) model, the $[I]_H$ was calculated by adding individual

furanocoumarin $[I]_H$ together. Once the $[I]_H$ was determined, the *IVIVE* models were used to predict an area-under-curve ratio (AUCR) (*i.e.*, area-under-curve (AUC) with herbal pre-treatment divided by AUC with no treatment) of the interaction. The predicted AUCRs were compared with actual AUCRs obtained from our previous human pharmacokinetic (PK) studies (see Chapter 3).

Results: Both Mayhew et al. (2000) and Wang et al. (2004) DDI models, after being modified for HDI, were able to predict the experimental AUCR of 9 herbal products reasonably well. The prediction appeared to depend on the C_{max} value as well as the model used. The *IVIVE* model of Wang et al. (2004) is more predictive of the experimental data probably because it uses the more accurate C_{max} of individual furanocoumarins to calculate the $[I]_H$ value. The Mayhew et al. (2000) model is less predictive of the experimental data probably because it uses the less accurate hybrid or composite C_{max} value to calculate the $[I]_H$ value

Conclusions: Results of this study indicate that the DDI models of Mayhew et al. (2000) and Wang et al. (2004), after modification, could be used to predict HDI in humans. The model-predicted AUCR and experimental AUCR are very close for some C_{max} values. These results render further support to my earlier findings that herbal extracts containing 8-MOP, 5-MOP, and ISOP are capable of inhibiting CYP1A2 enzyme *in vivo*.

5.2. Introduction

Regulatory bodies such as Health Canada, the United States Food Drug Administration (USFDA), and European Medicines Agency (EMEA) require the investigation of DDI prior to NDC approval (Health Canada, 1998; USFDA, 2012; EMEA, 2010). Based on the 2012 guidelines of USFDA, new drug applicants are required to follow a step-wise protocol which includes the investigation of key metabolizing enzymes inhibition, most notably phase-I cytochrome P-450 (CYP450) enzymes, in an effort to reduce unnecessary clinical trials and post-marketed drug withdrawals. Several DDI prediction models have been developed by researchers and adopted by regulatory bodies. Until recently, DDI prediction is based mainly on reversible CYP450 enzyme inhibition mechanisms (competitive or non-competitive) and has been carried out routinely as part

of the preclinical studies (Fahmi et al., 2008). If irreversible inhibition were to occur, this would result in underestimation of the magnitude of DDI (Einolf, 2007). Co-administration of a pharmaceutical drug and an herbal product with bioactive constituents that interfered with drug metabolite(s) formation, might significantly alter the pharmacokinetics of the victim drug. The outcome of such alterations, in many cases, may result in serious clinical consequence and even deaths (Ebbesen et al., 2001).

In the last decade, predictive DDI models have been developed with increased accuracy in prediction not only for reversible DDI but also for irreversible or mechanism-based DDI. For example, Mayhew et al. (2000) reported a predictive DDI model which involves an irreversibly bound inhibitor. Fahmi et al. (2008) proposed a combination model to sum up the inhibitory effects of reversible and irreversible enzyme inhibition along with enzyme induction. Despite these improvements, the DDI predictive model usually addresses only the interaction between one victim drug and one inhibitor, which is not always the case in HDI as herbal products usually consist of a complex mixture of bioactive chemicals which may interact with the victim drug.

To implement a predictive DDI model involving time-dependent inhibitors (TDI) or the mechanism-based inhibitors (MBI) requires information on *in vitro* inactivation kinetics of the victim drug as well as *in vivo* $[I]_H$ of the inhibitor. In the case of an herbal extract, the challenge is to estimate the$[I]_H$ or an integrated dose of the bioactive chemicals of the herb in the liver. Wang et al. (2004) had modified the model of Mayhew et al. (2000) to account for a multitude of inhibitors, which probably can be applied to a mixture of bioactive inhibitors in an herbal extract. The DDI model of Wang et al. (2004) requires detailed PK information on each of the inhibitors in an herbal extract. These can be time-consuming and expensive at the early drug development stage. In this study, we propose to use the concentration addition (CA) model (Safe, 1998; ATSDR, 2004) to calculate an integrated dose/concentration for the furanocoumarin mixture in the liver. The integrated dose is then used to calculate the AUCR with the Mayhew et al. (2000) model.

The “victim drug” in this study is caffeine. A popular drug with adverse health effects upon abusive consumption (Dews, 1982). Caffeine is also an ideal chemical probe to measure *in vivo* CYP1A2 activity (Doehmer et al., 1992; Miners et al., 1996). The

pharmacokinetics of caffeine in humans has been studied in great detail (Kot and Daniel, 2008). The “perpetrators or inhibitors” in the present study are the linear furanocoumarins which are chemical isomers and congeners found in *Apiaceae, Leguminosae*, *Moraceae,* and *Rutaceae* plant families (Diawara and Trumble, 1997). Previous *in vitro* liver microsomal studies have shown that the main metabolic pathway of 8-MOP and 5-MOP is the oxidative ring-opening of the furan structure to form an epoxide, or an intermediate electrophilic reactive metabolite, which binds covalently to human CYP proteins (Fouin-Fortunet et al., 1986; Tinel et al., 1987; Mays et al., 1990; John et al., 1992). Other linear furanocoumarins such as ISOP (Kang et al., 2011) and psoralen (Zhuang et al., 2013) also have been shown to be TDI or MBI in human liver microsomes (HLM) or recombinant human CYP1A2 expressed in yeast.

Our previous studies have shown that detectable levels of 8-MOP, 5-MOP, and/or ISOP are found in the following herbal extracts: *Ammi majus* L. seeds, *Angelica archangelica* L. roots, *Angelica pubescens* Maxim roots, *Apium graveolens* L. seeds, *Apium graveolens* L. flakes, *Cnidium monnieri* (L.) Cusson fruits, *Petroselinum crispum* (Mill.) Fuss leaves, *Pimpinella aniseum* L. seeds, and *Ruta graveolens* L. leaves (see Chapter 2). We also have shown 5 of the furanocoumarin-containing herbs significantly reduced the oral clearance of caffeine in human volunteers (see Chapter 3). The aforementioned linear furanocoumarins were found to be potent TDI or MBI of CYP1A2 isozyme (see Chapter 4).

The objectives of this study were: (a) to modify the predictive DDI models of Mayhew et al. (2000) and Wang et al. (2004) for *in vivo* herb-caffeine interaction using *in vitro* caffeine metabolism data in HLM and *in vivo* C_{max} of furanocoumarins in humans, and (b) to determine the accuracy of Mayhew et al. (2000) and Wang et al. (2004) DDI predictive models by comparing predicted caffeine AUCR with experimental AUCR from Chapter 3.

5.3. Methods

5.3.1. Using DDI models to predict herb-caffeine interaction

In the present study, caffeine is the victim drug and the furanocoumarin bioactive in the herbs are the perpetrators or inhibitors. As both caffeine and furanocoumarin inhibitors are metabolized by the same CYP1A2 enzyme, metabolic inhibition may occur in humans after co-administration. Indeed, the furanocoumarins have been shown in Chapter 4 to be MBI of CYP1A2. We have used two different DDI models to predict the inhibition of caffeine metabolism by furanocoumarin-containing herbs.

5.3.1.1 Using Mayhew et al. (2000) model to Predict Caffeine-Herb Interaction

Mayhew et al. (2000) first proposed a DDI model involving the destruction of CYP450 enzyme by the reactive metabolite(s) of the inhibitor or the mechanism-based DDI model as follows:

Equation 5-1: The *IVIVE* DDI prediction model of Mayhew et al. (2000)

$$\mathrm{AUCR} = \frac{\mathrm{AUC_I}}{\mathrm{AUC}} = \frac{1}{1 + \left(\frac{\mathrm{f_m}}{\frac{[\mathrm{I}]_\mathrm{H} \times \mathrm{k_{inact}}}{\mathrm{k_{deg}} \times ([\mathrm{I}]_\mathrm{H} + \mathrm{K_I})}} \right) + (1 - \mathrm{f_m})}$$

where AUC is the area-under-curve of caffeine with no herbal extract pre-treatment, AUC_I is the area-under-curve of caffeine with herbal extract pre-treatment, k_{deg} is the first-order *in vivo* degradation rate constant for P-450 enzyme, K_I represents the equilibrium dissociation constant for the inactivator, k_{inact} is the maximum rate of enzyme inactivation at saturating concentrations of inhibitor, f_m is the fraction of metabolic pathway, and $[I]_H$ represents the *in vivo* hypothetical intra-hepatic concentration(s) of the integrated dose/concentration of furanocoumarin inhibitors. Thus the model of Mayhew et al. (2004) required the determination of K_I, k_{inact}, k_{deg} and $[I]_H$ for the chemical marker (CM) furanocoumarin of 8-MOP only.

The theoretical background of mechanism-based model has been described in great details elsewhere (Mayhew et al., 2000). The model is able to predict quantitatively the inhibition of drugs if the following conditions are met: (a) the well-stirred liver conditions are met, (b) the *in vitro* inhibition constants are applicable to *in vivo* interaction, and (c) the $[I]_H$ value is lower than the K_I inhibition constant value.

An integrated dose/concentration of the furanocoumarin mixture in the liver, $[I]_H$ was predicted using the CA model (Safe, 1998; ATSDR, 2004) with 8-MOP as the CM (see Equation 4-3). The CA model is applicable to the present study because: (a) the furanocoumarin inhibitors are chemical isomers and congeners that exhibit similar biological mechanisms, (b) the dose-inhibition curves of individual furanocoumarins are parallel to one another as the slopes of the dose-inhibition curves of 8-MOP, 5-MOP, and ISOP are 2.0, 1.6, and 1.9 respectively, and (c) the inhibitory potencies of individual furanocoumarins are additive in nature (see Chapter 4).

5.3.1.2 Using Wang et al. (2004) model to predict caffeine-herb interaction

Instead of calculating a single $[I]_H$ with the CA model (Safe, 1998; ATSDR, 2004) (section 5.3.1.1), individual furanocoumarin $[I]_H$ were calculated separately before adding them together to yield the AUCR. Thus, Wang et al. (2004) model required to determine the K_I, k_{inact}, k_{deg} and $[I]_H$ of individual furanocoumarin inhibitors as follows:

Equation 5-2: The *IVIVE* DDI prediction model of Wang et al. (2004)

$$\mathrm{AUCR} = \frac{\mathrm{AUC_I}}{\mathrm{AUC}} = \frac{1}{1 + \left(\sum \frac{f_m}{\frac{[I]_H \times k_{inact}}{k_{deg} \times K_I}} \right) + (1 - f_m)}$$

Since caffeine undergoes negligible first-pass metabolism (Kalow and Tang, 1993), the DDI model of Wang et al. (2004) was modified for the present study by excluding the intestinal metabolism term from the original equation.

Figure 5-1 summarizes the experimental procedure and validation steps of the herb-caffeine interaction studies. Model-predicted AUCR was compared with experimental AUCR in the PK studies of Chapter 3 in order to validate the *IVIVE* models for herb-caffeine interaction prediction.

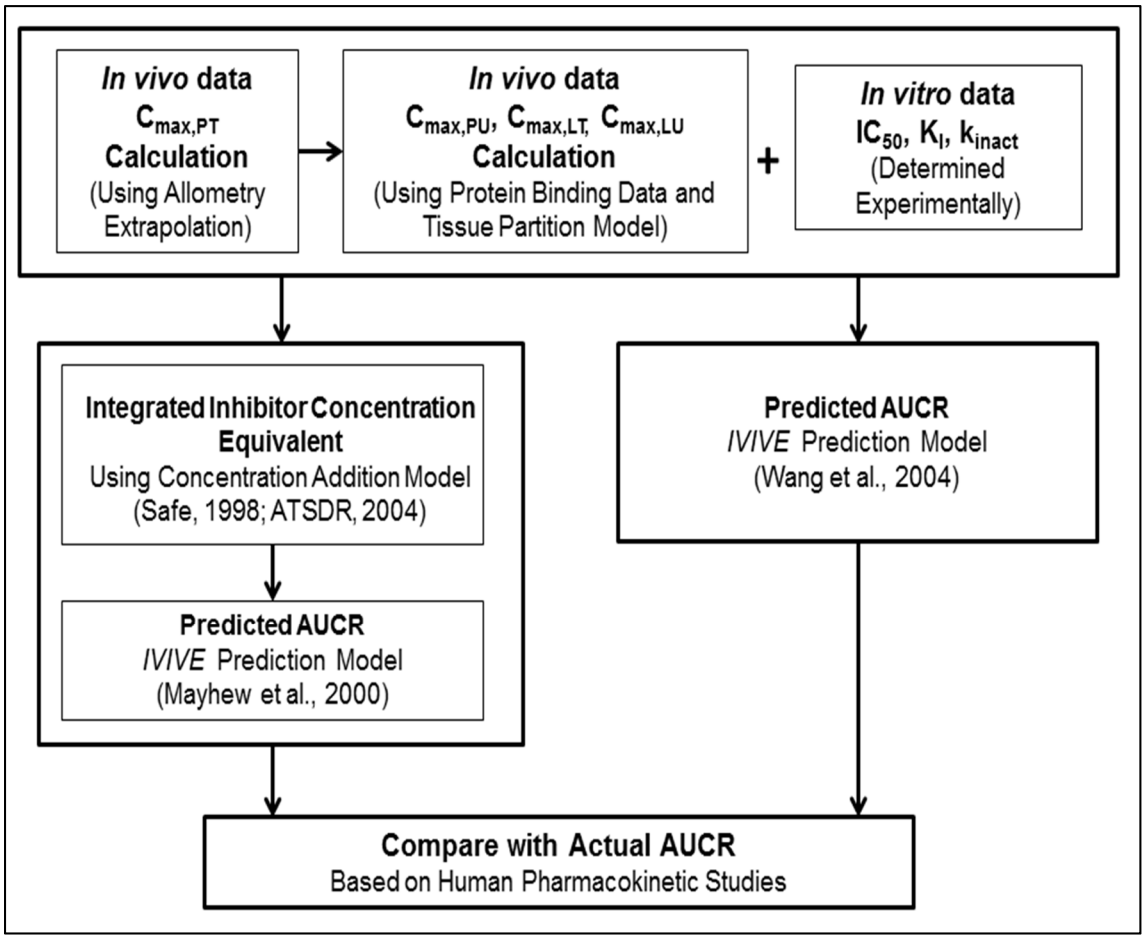

Figure 5-1: Flow-chart summary of experimental procedures and validation steps in the use of drug-drug interaction models to predict herb-drug interaction.

5.3.2. *IVIVE* DDI model input parameters

The predictive models of Mayhew et al. (2000) and Wang et al. (2004) used a combination of *in vitro* caffeine metabolism inhibition parameters (see Chapter 4) and *in vivo* furanocoumarin [I_H] concentrations to predict quantitative impairment of *in vivo* caffeine clearance due to co-administration of caffeine and furanocoumarin-containing herbs. As shown in equations 5-1 and 5-2, *in vivo* caffeine clearance impairment does not depend on the concentration of caffeine but is dependent on the concentrations of

furanocoumarin inhibitors at the site of metabolism, the liver. As the intrahepatic concentrations of furanocoumarin inhibitors could not be determined by direct experiment, four different furanocoumarin dose surrogates *i.e.* maximum total plasma concentration ($C_{max,PT}$), maximum unbound plasma concentration ($C_{max,PU}$), maximum total liver concentration level ($C_{max,LT}$), and maximum unbound liver concentration ($C_{max,LU}$) were used to calculate the $[I]_H$ for the predictive models.

5.3.2.1 Furanocoumarin C_{max} extrapolation based on dose-C_{max} relationship

Table 5-1 lists the body weights (BW) of the volunteers and the doses of individual furanocoumarins administered as described in Chapter 3. These furanocoumarin doses were used as the administered doses in predicting the $C_{max,PT}$ of 5-MOP and 8-MOP in the plasma of humans based on allometric extrapolation (Gabrielsson and Weiner, 2006). The $C_{max,PT}$ of ISOP was the average of the 5-MOP and 8-MOP values. The following was a detailed description of predicting $C_{max,PT,}$ $C_{max,PU}$, $C_{max,LT}$, and $C_{max,LU}$ concentration levels in humans:

(a) The $C_{max,PT}$ of 8-MOP and 5-MOP were obtained by allometric extrapolation from the experimental data of Schfifer-Korting and Korting (1982) and Stolk et al. (1981) studies, respectively. Figure 5-2 shows the administered dose *versus* serum $C_{max,PT}$ plot of 8-MOP (Schfifer-Korting and Korting, 1982). Figure 5-3 shows the administered dose *versus* serum $C_{max,PT}$ plot of 5-MOP (Stolk et al. 1981). Despite our best effort, we were unable to find any animal or human data for proper allometric extrapolation of ISOP. Instead, we assumed the $C_{max,PT}$ values of ISOP to be the average of 8-MOP and 5-MOP extrapolated values.

Table 5-1: A summary of BW and individual furanocoumarin doses for human volunteers in the caffeine PK studies.

Botanical Name	*n*	Human Furanocoumarin Oral Dose											
		BW [a] kg			8-MOP Dose [a] µg/kg BW			5-MOP Dose [a] µg/kg BW			ISOP Dose [a] µg/kg BW		
A.majus seeds	4	77.5	±	17.1	260.89	±	73.37	58.19	±	16.36	611.91	±	172.1
A.archangelica roots	5	73.2	±	17.6	42.2	±	11.2	25.5	±	6.8	39.3	±	10.4
C.monnieri fruits	5	75.8	±	7.4	28.2	±	3.0	71.3	±	7.5	18.6	±	2.0
R.graveolens leaves	4	72.0	±	6.9	56.3	±	5.7	22.4	±	2.3	12.3	±	1.3
A.pubescens roots	4	75.0	±	8.2	4.1	±	0.5	5.2	±	0.6		n.d. [b]	
A.graveolens seeds	4	79.3	±	5.8	2.7	±	0.2	2.1	±	0.1	29.9	±	2.1
A.graveolens flakes	2	81.5	±	3.5	1.5	±	0.1	29.9	±	1.3	1.2	±	0.1
P.crispum leaves	2	81.5	±	3.5		n.d. [b]		4.2	±	0.2		n.d. [b]	
P.aniseum seeds	4	75.8	±	8.5	2.1	±	0.3		n.d. [b]			n.d. [b]	

[a] Expressed as mean ± standard deviation (SD).

[b] n.d. = not detected.

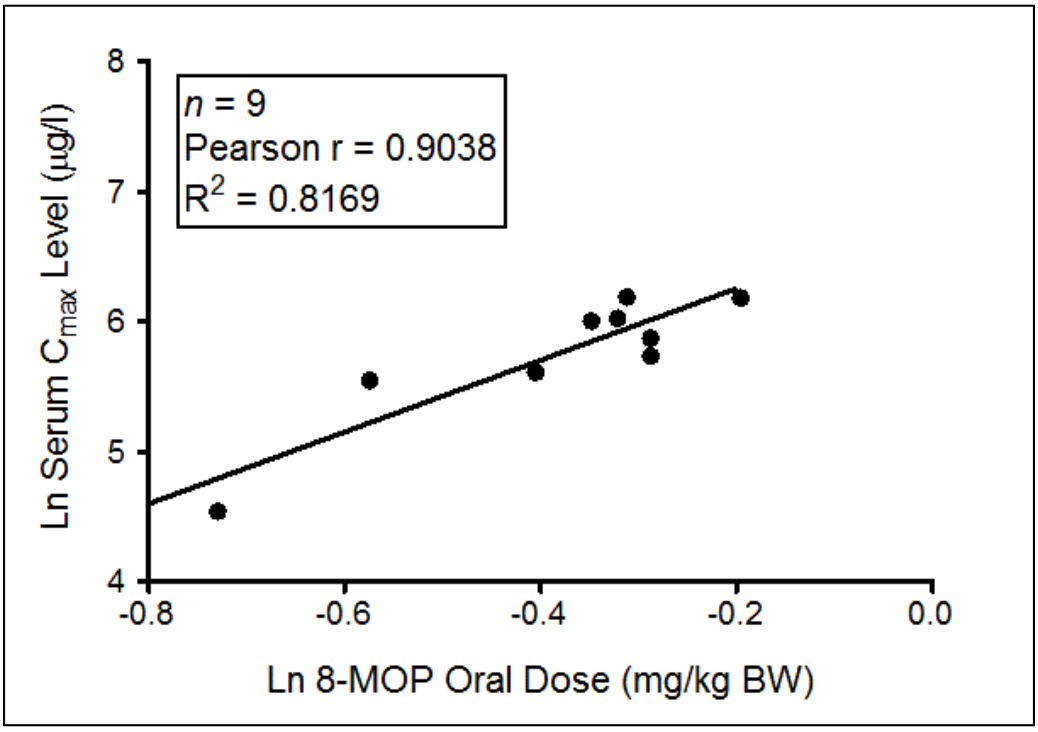

Figure 5-2: Ln-Ln plot of 8-MOP dose *versus* $C_{max,PT}$ data from Schfifer-Korting and Korting (1982). The numerical data are presented in Appendix S.

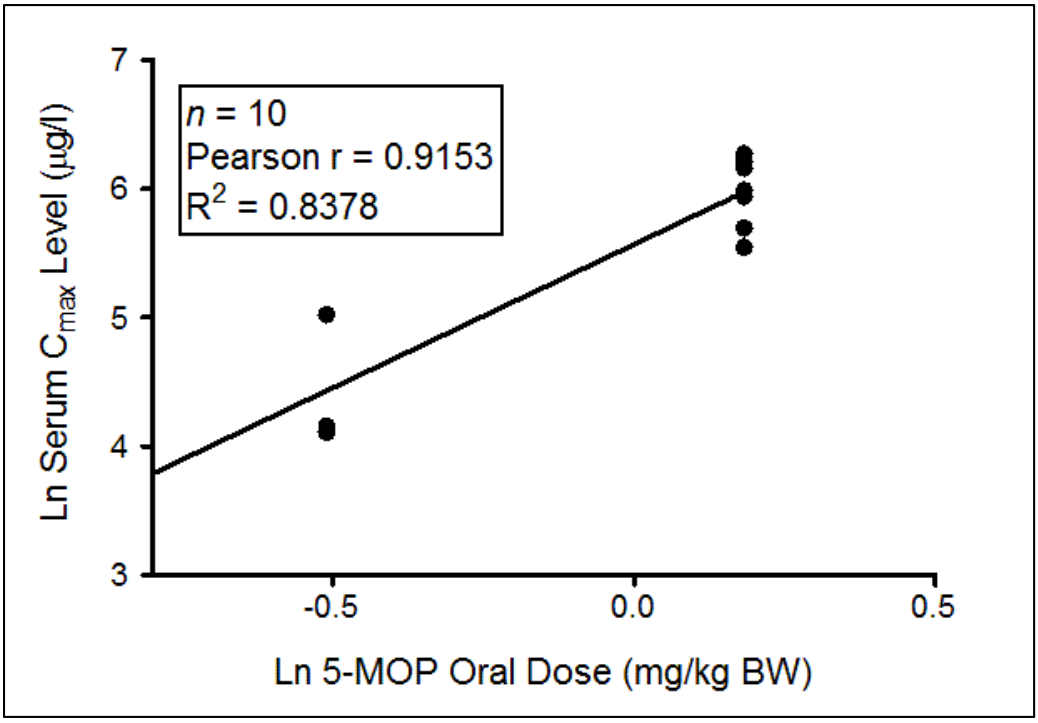

Figure 5-3: Ln-Ln plot of 5-MOP dose *versus* $C_{max,PT}$ data from Stolk et al. (1981). The numerical data are presented in Appendix T.

(b) $C_{max,PU}$ was derived from $C_{max,PT}$ by multiplying the later with the unbound fractions (f_{up}) of 8-MOP or 5-MOP in the plasma; they were 0.1703 and 0.0357 respectively (Table 5-2). No information was found for ISOP. Therefore, an average of 8-MOP and 5-MOP values (0.1030) was used.

Table 5-2: A summary of 8-MOP and 5-MOP plasma protein binding studies

Furanocoumarin	Percent Bound	Percent Unbound	References
8-MOP	77.50	22.50	Artuc et al., 1979
	80.00	20.00	Veronese et al., 1978
	91.40	8.60	Pibouin et al., 1987
Average	82.97	17.03	
5-MOP	99.00	1.00	Artuc et al., 1979
	95.00	5.00	Muret et al., 1993a
	95.30	4.70	Makki et al., 1991
Average	96.43	3.57	
ISOP [a]	89.70	10.30	

[a] Based on the average of 8-MOP and 5-MOP protein-binding percentage

(c) The $C_{max,LT}$ was derived by multiplying $C_{max,PT}$ with the liver:plasma partition coefficient ($P_{t:p}$) which was calculated as follows:

Equation 5-3: Non-adipose tissue:plasma partition model of Poulin and Theil (2002)

$$P_{t:p} = \frac{P_{o/w} \times \left(V_{nt} + 0.3 \times V_{pht}\right) + \left(V_{wt} + 0.7 \times V_{pht}\right)}{P_{o/w} \times \left(V_{np} + 0.3 \times V_{php}\right) + \left(V_{wp} + 0.7 \times V_{php}\right)} \times \frac{f_{up}}{f_{ut}}$$

where $P_{o/w}$ is the *n*-octanol/water partition for non-ionized inhibitor, V_{nt} is the fraction weight of neutral lipids in liver tissue, V_{pht} is the fraction weight of phospholipids in liver tissue, V_{wt} is the fraction weight of water in liver tissue, V_{np} is the fraction weight of neutral lipids in plasma, V_{wp} is the fraction weight of water in plasma, V_{php} is the fraction weight of phospholipids in plasma, f_{up} is the fraction unbound in plasma, and f_{ut} is the fraction unbound in liver tissue.

The $P_{o/w}$ of 8-MOP, 5-MOP, and ISOP were 120.2, 128.8, and 169.8 respectively; they were obtained from the Advanced Chemistry Development I-lab databases (ACD,

2015). The values of V_{nt}, V_{pht}, V_{wt}, V_{np}, V_{wp}, and V_{php} were 0.035, 0.025, 0.751, 0.004, 0.945, and 0.002 respectively; they were also obtained from Poulin and Theil (2000).

(d) The $C_{max,LU}$ was derived by multiplying the $C_{max,LT}$ by the calculated unbound fraction (f_{ut}) in tissue. The f_{ut} was determined using the following equation:

Equation 5-4: Unbound tissue fraction model of Poulin and Theil (2000)

$$f_{ut} = \frac{1}{1 + ((\frac{1 - f_{up}}{f_{up}}) \times 0.5)}$$

5.3.2.2 *In vitro* caffeine metabolism inhibition data

Previous *in vitro* studies (see Chapter 4) showed that the IC_{50} of 8-MOP, 5-MOP, and ISOP were 0.09 μM, 0.13 μM, and 0.29 μM, respectively; the corresponding K_I values were 0.78 μM, 3.73 μM, and 4.48 μM, and k_{inact} values were 0.17 min^{-1}, 0.35 min^{-1}, and 0.65 min^{-1} respectively. The k_{deg} value of CYP1A2, 0.0003 min^{-1}, was obtained from Faber and Fuhr (2004). The f_m value for caffeine in humans, 0.95, was taken from Kalow and Tang (1993). Thus, only the C_{max}-derived $[I]_H$ remained to be determined in the *IVIVE* models.

5.3.3. Data and statistical analysis

Data plotting and extrapolation were performed using GraphPad Prism Software version 5.04 (San Diego, CA, USA). Statistical analysis was performed using Microsoft Excel software version 2010 (Redmond, WA, USA). Model-predicted AUCR were reported as mean ± SD. Herb-caffeine interaction occurred when the mean AUCR was equal or greater than 2.0. No interaction occurred when the mean AUCR was less than 2.0 (Einolf, 2007). The geometric mean-fold error (GMFE) (Equation 5-5) was also used to assess the accuracy of model prediction by equal weighting under-predictions and over-predictions. The model that predicted perfectly would give a GMFE value of 1; GMFE value of 2-fold or less is considered to be accurate.

Equation 5-5: The geometric mean-fold error (GMFE) (Obach et al., 1997)

$$\mathrm{GMFE} = 10^{\frac{\Sigma \left|\log\frac{\text{Predicted HDI}}{\text{Actual HDI}}\right|}{n}}$$

where *n* is the number of predictions for each herb.

5.4. Results and Discussion

Tables 5-3 to 5-10 show the experimental and model-predicted AUCR of caffeine in humans as a result of pre-treatment with an herbal extract. The variability of the experimental AUCR is large but consistent with the large variation of 8-MOP (Herfst and De Wolff, 1982) and 5-MOP (Ehrsson et al., 1994) concentrations observed in the serum. Relatively high experimental AUCR were observed in volunteers pre-treated by *A. majus, A. archangelica, A.pubescens, C.monnieri,* or *R.graveolens* (Table 5-3 to 5-10). These results are consistent with the relatively high levels of furanocoumarins found in the herbal extracts (Table 2-5). Moreover, the presence of osthole, a CYP1A2 inhibitor (Yang et al., 2012), in *A.pubescens* may contribute to the high AUCR of this herbal extract. Similarly, the relatively high AUCR of *C.monnieri* may be due to osthole in addition to high levels of 8-MOP, 5-MOP, and ISOP (Table 2-5). Despite only low levels of furanocoumarins have been found in *P.crispum* (Table 2-5)*;* mean AUCR in the volunteers is noticeable (Table 5-3 to 5-10). This may be due to the presence of flavones such as apigenin in the extract (Meyer et al., 2006), which is a CYP1A2 inhibitor (Peterson et al., 2006).

Wang et al. (2004) *IVIVE* model predicted a ≥ 2-fold increase in mean caffeine AUCR after pre-treating the volunteers with *A.majus* seeds or *C.monnieri* fruits extract regardless of C_{max} value used to derive the $[I]_H$ (Tables 5-3 to 5-6). However, the interaction due to *A.archangelica* roots pretreatment was not predicted successfully using $C_{max,PU}$ values. The AUCR derived from $C_{max,PU}$ and $C_{max,LU}$ appeared to have fewer false positive results in the remaining 6 herbs on the list (Tables 5-4 and 5-6). The average GMFE for $C_{max,PU}$ and $C_{max,LU}$ were 1.7 and 2.0 for (Tables 5-4 and 5-6) indicating $C_{max,PU}$ and $C_{max,LU}$ yield more accurate AUCR results than $C_{max,PT}$ and $C_{max,LT}$ which had average GMFE value of 2.6 and 3.5 respectively (Tables 5-3 and 5-5). These results are consistent with the

larger number of false positive results in $C_{max,PT}$ and $C_{max,LT}$ (Tables 5-3 and 5-5). The actual AUCR *versus* predicted AUCR correlation plot confirms $C_{max,PU}$ and $C_{max,LU}$ are better dose surrogates to predict caffeine AUCR than $C_{max,PT}$ and $C_{max,LT}$ (Figure 5-4).

In contrast, when the *IVIVE* model of Mayhew et al. (2000) was used to predict the AUCR of caffeine in humans pre-treated by herbal extracts, only $C_{max,LT}$ predicted the experimental AUCR closely. An exception was *R.graveolens* leaves which overpredicted the experimental AUCR (Table 5-9). The average GMFE were similar for $C_{max,LU}$, $C_{max,PT}$ and $C_{max,PU}$ at 1.7 (Tables 5-7, 5-8, and 5-10). However, $C_{max,PU,}$ $C_{max,LU}$, and $C_{max,PT}$ generally underpredicted the experimental AUCR with the exception of *A.majus* seeds which was always overpredicted regardless of the DDI model used. Thus, $C_{max,LT}$ yielded the most accurate prediction for caffeine metabolism inhibition with average GMFE of 1.8 (Table 5-9). The actual AUCR *versus* predicted AUCR correlation plot also confirms $C_{max,LT}$ is a better dose surrogate to predict caffeine AUCR than $C_{max,LU}$, $C_{max,PT}$ and $C_{max,PU}$ (Figure 5-5).

Despite no agreement is reached on the most accurate $[I]_H$ surrogate for DDI predictions, previous studies have reported accurate DDI prediction by using either total C_{max} or unbound C_{max} in the liver and plasma to estimate $[I]_H$ (Grimm et al., 2009). For example, Ito et al. (2004; 2005) have reported that $C_{max,LT}$ yields the most accurate prediction for reversible-based inhibition DDI. This is consistent with the suggestion of Brown et al. (2006) that incorporating protein binding into the predictive model did not improve reversible-based DDI prediction. In contrast, Obach et al. (2006) and Fahmi et al. (2009) have concluded that $C_{max,LU}$ is most accurate in reversible-based DDI prediction. Blanchard et al. (2004) have reported $C_{max,PU}$ provides the most accurate DDI predictions based on reversible inhibition. For irreversible-based inhibition predictions, Obach et al. (2007) and Fahmi et al. (2009) have suggested $C_{max,PU}$ is the most accurate dose surrogate for DDI prediction. In contrast, both Ito et al. (2003) and Shardlow et al. (2011) reported $C_{max,LU}$ provided the most accurate DDI predictions.

Table 5-3: A comparison of model-predicted $C_{max,PT}$-based AUCR and experimental AUCR in humans due to herbal extract pretreatment. *IVIVE* model was modified from Wang et al. (2004).

Botanical Name	Actual AUCR			Predicted AUCR				
				Wang et al. (2004) Model				
	Mean	±	SD	Mean	±	SD	Predictive [a]	GMFE [b]
A.majus seeds	4.7	±	1.0	19.9	±	0.0	+	4.3
A.archangelica roots	2.3	±	0.6	6.3	±	2.4	+	2.7
C.monnieri fruits	2.6	±	1.2	11.9	±	1.1	+	5.0
R.graveolens leaves	1.7	±	0.5	2.7	±	0.4	—	1.6
A.pubescens roots	1.9	±	0.8	1.0	±	0.0	+	1.7
A.graveolens seeds	1.3	±	0.7	3.4	±	0.3	—	2.8
A.graveolens leaves	1.2	±	0.3	2.8	±	0.2	—	2.4
P.crispum leaves	1.6	±	0.9	1.0	±	0.0	+	1.6
P.aniseum seeds	1.1	±	0.2	1.0	±	0.0	+	1.2
							Average	2.6

[a] Based on the two-fold rule; (+) predictive and (—) not predictive.

[b] Geometric mean-fold error; (≤2) indicate accurate prediction.

Table 5-4: A comparison of model-predicted $C_{max,PU}$-based AUCR and experimental AUCR in humans due to herbal extract pretreatment. *IVIVE* model was modified from Wang et al. (2004).

Botanical Name	Actual AUCR			Predicted AUCR				
				Wang et al. (2004) Model				
	Mean	±	SD	Mean	±	SD	Predictive [a]	GMFE [b]
A.majus seeds	4.7	±	1.0	19.3	±	0.3	+	4.1
A.archangelica roots	2.3	±	0.6	1.7	±	0.5	—	1.4
C.monnieri fruits	2.6	±	1.2	3.3	±	0.6	+	1.6
R.graveolens leaves	1.7	±	0.5	1.2	±	0.0	+	1.4
A.pubescens roots	1.9	±	0.8	1.0	±	0.0	+	1.8
A.graveolens seeds	1.3	±	0.7	1.3	±	0.0	+	1.4
A.graveolens leaves	1.2	±	0.3	1.1	±	0.0	+	1.2
P.crispum leaves	1.6	±	0.9	1.1	±	0.1	+	1.5
P.aniseum seeds	1.1	±	0.2	1.0	±	0.0	+	1.2
							Average	1.7

[a] Based on the two-fold rule; (+) predictive and (—) not predictive.

[b] Geometric mean-fold error; (≤2) indicate accurate prediction.

Table 5-5: A comparison of model-predicted $C_{max,LT}$-based AUCR and experimental AUCR in humans due to herbal extract pretreatment. *IVIVE* model was modified from Wang et al. (2004).

Botanical Name	Actual AUCR			Predicted AUCR				
				Wang et al. (2004) Model				
	Mean	±	SD	Mean	±	SD	Predictive [a]	GMFE [b]
A.majus seeds	4.7	±	1.0	20.0	±	0.0	+	4.3
A.archangelica roots	2.3	±	0.6	10.2	±	2.9	+	4.4
C.monnieri fruits	2.6	±	1.2	15.8	±	0.8	+	6.7
R.graveolens leaves	1.7	±	0.5	4.5	±	0.8	—	2.7
A.pubescens roots	1.9	±	0.8	1.1	±	0.0	+	1.6
A.graveolens seeds	1.3	±	0.7	6.3	±	0.6	—	5.2
A.graveolens leaves	1.2	±	0.3	4.6	±	0.3	—	3.8
P.crispum leaves	1.6	±	0.9	1.1	±	0.0	+	1.6
P.aniseum seeds	1.1	±	0.2	1.0	±	0.0	+	1.2
							Average	3.5

[a] Based on the two-fold rule; (+) predictive and (—) not predictive.

[b] Geometric mean-fold error; (≤2) indicate accurate prediction.

Table 5-6: A comparison of model-predicted $C_{max,LU}$-based AUCR and experimental AUCR in humans due to herbal extract pretreatment. *IVIVE* model was modified from Wang et al. (2004).

Botanical Name	Actual AUCR			Predicted AUCR				
				Wang et al. (2004) Model				
	Mean	±	SD	Mean	±	SD	Predictive [a]	GMFE [b]
A.majus seeds	4.7	±	1.0	19.8	±	0.1	+	4.3
A.archangelica roots	2.3	±	0.6	3.9	±	1.6	+	1.6
C.monnieri fruits	2.6	±	1.2	8.6	±	1.2	+	3.6
R.graveolens leaves	1.7	±	0.5	1.7	±	0.2	+	1.3
A.pubescens roots	1.9	±	0.8	1.0	±	0.0	+	1.8
A.graveolens seeds	1.3	±	0.7	2.3	±	0.2	—	1.9
A.graveolens leaves	1.2	±	0.3	1.3	±	0.0	+	1.2
P.crispum leaves	1.6	±	0.9	1.0	±	0.0	+	1.6
P.aniseum seeds	1.1	±	0.2	1.0	±	0.0	+	1.2
							Average	2.0

[a] Based on the two-fold rule; (+) predictive and (—) not predictive.

[b] Geometric mean-fold error; (≤2) indicate accurate prediction.

Table 5-7: A comparison of model-predicted $C_{max,PT}$-based AUCR and experimental AUCR in humans due to herbal extract pretreatment. *IVIVE* model was modified from Mayhew et al. (2000).

Botanical Name	Actual AUCR			Predicted AUCR				
				Mayhew et al. (2000) Model				
	Mean	±	SD	Mean	±	SD	Predictive [a]	GMFE [b]
A.majus seeds	4.7	±	1.0	17.9	±	0.9	+	3.9
A.archangelica roots	2.3	±	0.6	2.0	±	0.6	+	1.2
C.monnieri fruits	2.6	±	1.2	1.8	±	0.2	—	1.5
R.graveolens leaves	1.7	±	0.5	2.0	±	0.3	—	1.4
A.pubescens roots	1.9	±	0.8	1.0	±	0.0	+	1.8
A.graveolens seeds	1.3	±	0.7	1.1	±	0.0	+	1.4
A.graveolens leaves	1.2	±	0.3	1.2	±	0.0	+	1.2
P.crispum leaves	1.6	±	0.9	1.0	±	0.0	+	1.6
P.aniseum seeds	1.1	±	0.2	1.0	±	0.0	+	1.2
							Average	1.7

[a] Based on the two-fold rule; (+) predictive and (—) not predictive.

[b] Geometric mean-fold error; (≤2) indicate accurate prediction.

Table 5-8: A comparison of model-predicted $C_{max,PU}$-based AUCR and experimental AUCR in humans due to herbal extract pretreatment. *IVIVE* model was modified from Mayhew et al. (2000).

Botanical Name	Actual AUCR			Predicted AUCR				
				Mayhew et al. (2000) Model				
	Mean	±	SD	Mean	±	SD	Predictive [a]	GMFE [b]
A.majus seeds	4.7	±	1.0	11.1	±	2.9	+	2.3
A.archangelica roots	2.3	±	0.6	1.1	±	0.1	—	2.0
C.monnieri fruits	2.6	±	1.2	1.1	±	0.0	—	2.2
R.graveolens leaves	1.7	±	0.5	1.2	±	0.1	+	1.4
A.pubescens roots	1.9	±	0.8	1.0	±	0.0	+	1.8
A.graveolens seeds	1.3	±	0.7	1.0	±	0.0	+	1.4
A.graveolens leaves	1.2	±	0.3	1.0	±	0.0	+	1.2
P.crispum leaves	1.6	±	0.9	1.0	±	0.0	+	1.6
P.aniseum seeds	1.1	±	0.2	1.0	±	0.0	+	1.2
							Average	1.7

[a] Based on the two-fold rule; (+) predictive and (—) not predictive.

[b] Geometric mean-fold error; (≤2) indicate accurate prediction.

Table 5-9: A comparison of model-predicted $C_{max,LT}$-based AUCR and experimental AUCR in humans due to herbal extract pretreatment. *IVIVE* model was modified from Mayhew et al. (2000).

Botanical Name	Actual AUCR			Predicted AUCR				
				Mayhew et al. (2000) Model				
	Mean	±	SD	Mean	±	SD	Predictive [a]	GMFE [b]
A.majus seeds	4.7	±	1.0	19.1	±	0.4	+	4.1
A.archangelica roots	2.3	±	0.6	2.7	±	1.0	+	1.4
C.monnieri fruits	2.6	±	1.2	2.8	±	0.4	+	1.5
R.graveolens leaves	1.7	±	0.5	3.1	±	0.6	—	1.9
A.pubescens roots	1.9	±	0.8	1.0	±	0.0	+	1.8
A.graveolens seeds	1.3	±	0.7	1.2	±	0.0	+	1.4
A.graveolens leaves	1.2	±	0.3	1.5	±	0.0	+	1.2
P.crispum leaves	1.6	±	0.9	1.0	±	0.0	+	1.6
P.aniseum seeds	1.1	±	0.2	1.0	±	0.0	+	1.2
							Average	1.8

[a] Based on the two-fold rule; (+) predictive and (—) not predictive.

[b] Geometric mean-fold error; (≤2) indicate accurate prediction.

Table 5-10: A comparison of model-predicted $C_{max,LU}$-based AUCR and experimental AUCR in humans due to herbal extract pretreatment. *IVIVE* model was modified from Mayhew et al. (2000).

Botanical Name	Actual AUCR			Predicted AUCR				
				Mayhew et al. (2000) Model				
	Mean	±	SD	Mean	±	SD	Predictive [a]	GMFE [b]
A.majus seeds	4.7	±	1.0	16.6	±	1.4	+	3.6
A.archangelica roots	2.3	±	0.6	1.4	±	0.3	—	1.6
C.monnieri fruits	2.6	±	1.2	1.4	±	0.1	—	1.7
R.graveolens leaves	1.7	±	0.5	1.6	±	0.2	+	1.3
A.pubescens roots	1.9	±	0.8	1.0	±	0.0	+	1.8
A.graveolens seeds	1.3	±	0.7	1.0	±	0.0	+	1.4
A.graveolens leaves	1.2	±	0.3	1.0	±	0.0	+	1.2
P.crispum leaves	1.6	±	0.9	1.0	±	0.0	+	1.6
P.aniseum seeds	1.1	±	0.2	1.0	±	0.0	+	1.2
							Average	1.7

[a] Based on the two-fold rule; (+) predictive and (—) not predictive.

[b] Geometric mean-fold error; (≤2) indicate accurate prediction.

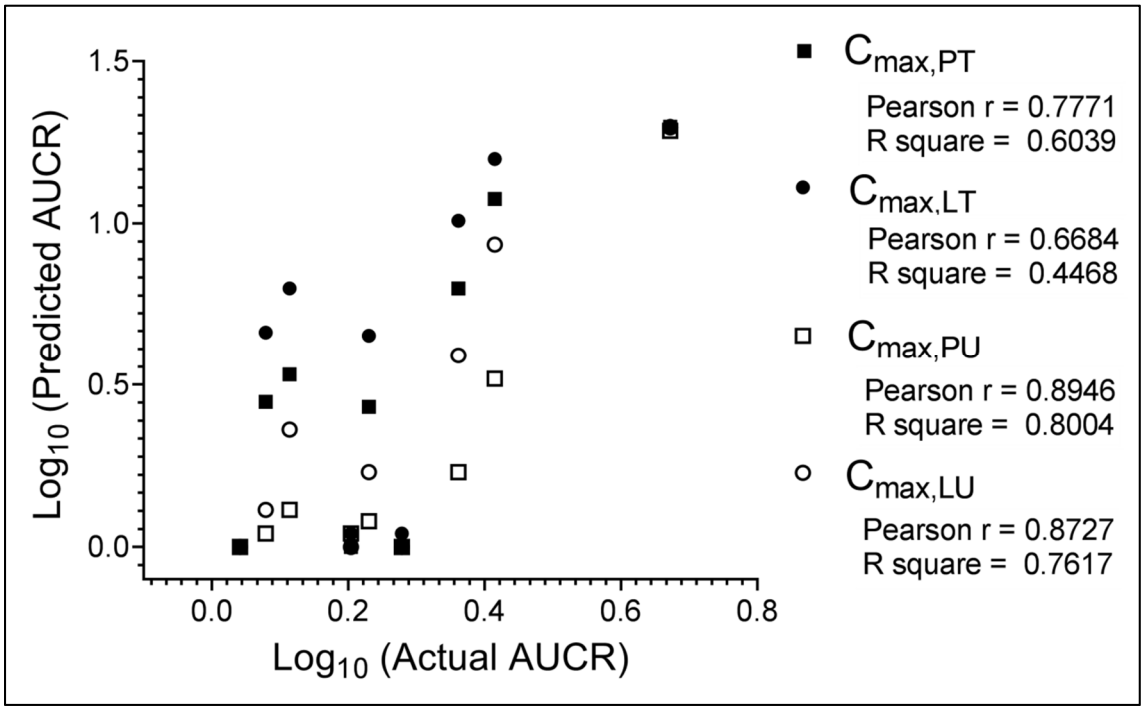

Figure 5-4: Correlation of Wang et al. (2004) model-predicted AUCR and actual AUCR using different $[I]_H$ surrogate values.

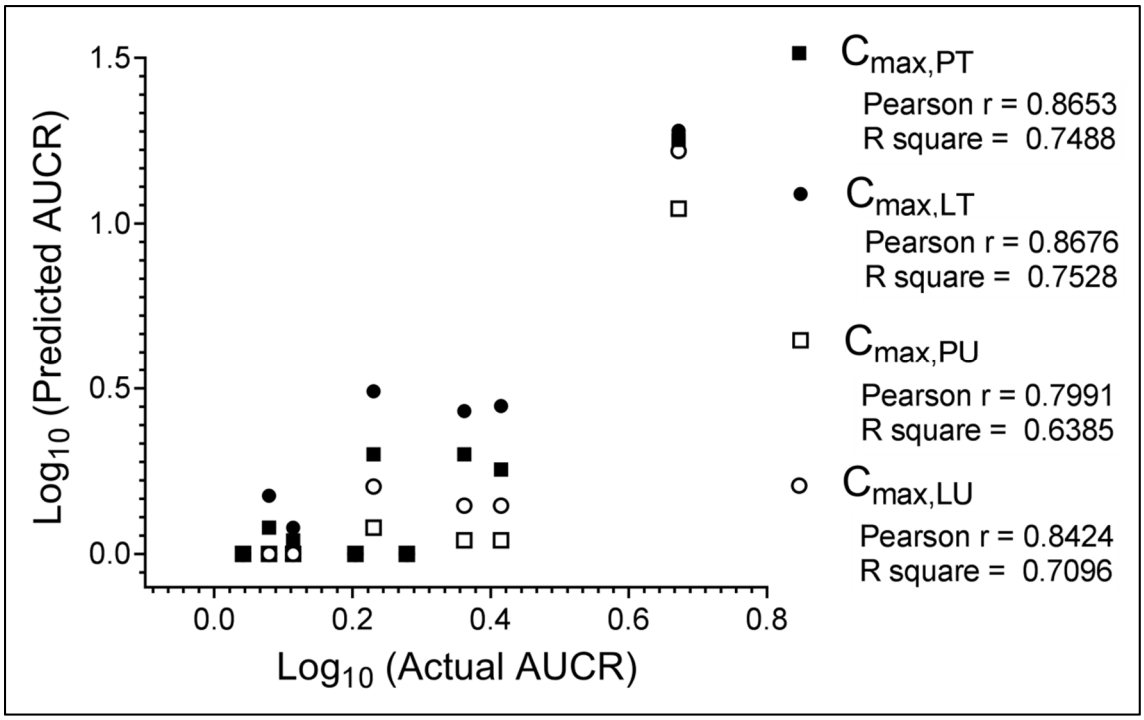

Figure 5-5: Correlation of Mayhew et al. (2000) model-predicted AUCR and actual AUCR using different $[I]_H$ surrogate values.

Although both Wang et al. (2004) and Mayhew et al. (2000) models are able to predict the empirical AUCR of caffeine, Wang et al. (2004) model is more predictive than Mayhew et al. (2000) model because the later is implemented with a single, composite $[I]_H$ value which is less accurate than a combination of individual $[I]_H$ values in Wang et al. (2004) model. The predictability of the *IVIVE* model is also affected by the following kinetic parameters: (a) the k_{deg} value of hCYP1A2. The 0.00030 min^{-1} k_{deg} used in the present study is based on the 38 h $t_{1/2}$ of a tobacco smoking cessation study (Faber and Fuhr 2004). Mayhew et al. (2000) has reported a k_{deg} value of 0.00083 min^{-1} which is derived from rats. If this k_{deg} was used in the present study, both predictive models would underestimate the AUCR of caffeine, (b) *in vitro* kinetic inhibition parameters such as IC_{50}, K_I and k_{inact} are derived using pooled HLM from multiple donors. This also appears to improve the accuracy of our predictions, and (c) caffeine as the victim drug has simplified model prediction by eliminating the need to account for parallel metabolic pathways by other CYP450 isoforms and urinary excretion of unchanged caffeine. As a result, the uncertainty involved in the AUCR calculation is greatly reduced.

The following are some of the uncertainties or limitations of the present study: (a) the data used to establish the 8-MOP dose-response curves for allometric extrapolation are based on psoriasis patients and these raised concern that plasma 8-MOP levels in healthy and psoriasis subjects might be different. However, Shephard et al. (1999) has shown that similar free 8-MOP fractions are found in the plasma of psoriasis and healthy volunteers. Also, Muret et al. (1993b) have shown that the fractions of free 5-MOP in the serum of healthy and psoriasis volunteers do not differ significantly. Together, these results suggest that systemic 8-MOP and 5-MOP levels do not differ significantly in healthy and psoriasis subjects, (b) in the present study, the PK values of individual furanocoumarins in an herbal extract and pure furanocoumarins are assumed to be similar. This assumption might be incorrect since PK interaction between individual furanocoumarins in an herbal extract may occur, and (c) previous studies have shown that both 8-MOP (Mays et al., 1987; Apseloff et al., 1990) and ISOP (Baumgart et al., 2005) induce hCYP1A2 enzyme activity in rats after multiple dosing. In contrast, Tantcheva-Poór et al. (2001) dosed humans with 0.6 mg of 8-MOP/kg/day and found no significant change in caffeine clearance even after a week of treatment. In the present study, the inductive effect of 8-MOP and ISOP is ignored as only a single dose of herbal extract is administered

3 h before the caffeine PK studies, and it is unlikely hCYP1A2 can be induced with a short pretreatment period.

5.5 Conclusions

Using *IVIVE* models to predict HDI is an ongoing research program in our laboratory. Our goal is to develop a reliable and simple prediction tool for HDI. Results of this study suggest that linear furanocoumarins such as 8-MOP, 5-MOP and ISOP are responsible for the inhibition of caffeine metabolism in humans after consuming herbal extracts containing furanocoumarin derivatives. The described modeling approaches in this study may also be applicable to health supplements and functional food.

5.6 References

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Chapter 6

Conclusions and Future Research

In this thesis, we confirm the results of earlier studies that linear furanocoumarins of 8-MOP, 5-MOP, and ISOP are present in plant products found in human diets and herbal medicine. Although previous studies have reported 8-MOP, 5-MOP, and ISOP as inactivators for cytochrome (CYP) 1A2, this is the first study which provides the inactivation constants for the aforementioned furanocoumarins using human liver microsomes (HLM). Results of the studies show that despite the complexity of an herbal extract, individual furanocoumarin bioactive in the extract still obeys the same pharmacokinetic rules as a pure chemical. Thus, we are able to demonstrate caffeine clearance reduction by furanocoumarin-containing herbs with pharmacokinetic studies in humans. We also have successfully modified *in vitro-in vivo* extrapolation (*IVIVE*) models of drug-drug interactions to predict the reduction of caffeine clearance in humans and conclude that the aforementioned linear furanocoumarins are indeed responsible for the *in vivo* inhibitory effects of the herbal extracts.

The most significant outcome of this thesis is the application of different drug-drug interaction (DDI) models for herb-drug and food-drug interaction. The modified DDI procedure can now be used to help developing new botanical drugs. The *IVIVE* procedure may be used as a screening tool to avoid carrying out unnecessary and expensive clinical trial studies in the future. The framework as described in the present study is especially useful to screen for herb-drug and food-drug interactions in patients who take prescription drugs and botanical drugs concurrently. For example, patients who are on theophylline and tizanidine should avoid consuming furanocoumarin-containing herbs and vegetables during the course of their treatment. The study also advocates for proper regulation and labelling of herbs and natural health products containing bioactive ingredients which may pose a health risk to sensitive individuals.

The following are some suggestions for future research studies: we suggest using the chemical mixture assessment models recommended by the United States Environmental Protection Agency (USEPA) to study the risks of phytochemical mixtures

on prescription drugs. Upon the successful use of such models, a phytochemical toxic equivalency factor (PTEF) database may be established. The PTEF database would help providing an accurate and consistent approach for phytochemical-related pharmacokinetic and risk assessment studies. Future studies should also compare the pharmacokinetics of 8-MOP, 5-MOP, and ISOP as a pure chemical and as a component of a furanocoumarin mixture. This would further define the role of individual furanocoumarins in the mixture more precisely and improve the accuracy of predicting interaction between furanocoumarin-containing plant products and prescription drugs.

Appendices

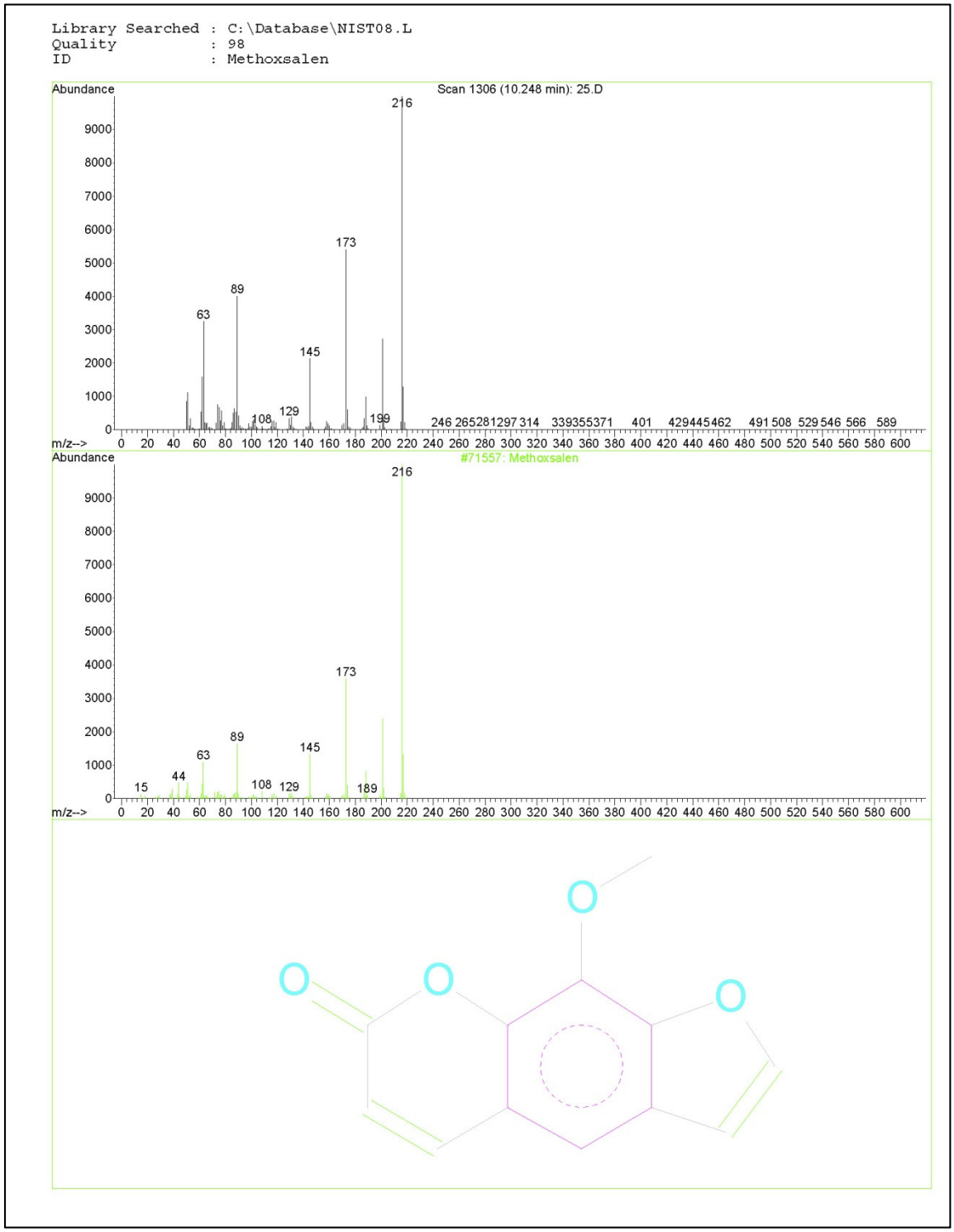
Appendix A.

List of Herb Suppliers and Origins

Botanical Name	Plant Part	Supplier Brand	Supplier Country	Origin Country
Ammi majus L.	Seeds	Everwilde	USA	USA
Ammi visnaga L.	Seeds	(Local Shop)	Jordan	Not available
Angelica archangelica L.	Roots	Mountain Rose	USA	Bulgaria
Angelica dahurica (Hoffm.) Maxim	Roots	Spring Wind	USA	China
Angelica pubescens Maxim	Roots	Spring Wind	USA	China
Angelica sinensis (Oliv.) Diels	Roots	Mountain Rose	USA	China
Anethum graveolens L.	Leaves	Mountain Rose	USA	Egypt
Anethum graveolens L.	Seeds	Mountain Rose	USA	USA
Anthriscus cerefolium Hoffm.	Leaves	Silk Road	Canada	Netherlands
Apium graveolens L.	Seeds	Mountain Rose	USA	Egypt
Apium graveolens L.	Flakes	A1 Spice World	USA	India
Carum carvi L.	Seeds	A1 Spice World	USA	Canada
Citri reticulatae Blanco	Peels	Spring Wind	USA	China
Cnidium monnieri (L.) Cusson	Seeds	Health and Wellness	Canada	China
Coriandrum sativum L.	Seeds	A1 Spice World	USA	Canada
Cuminum cyminum L.	Seeds	A1 Spice World	USA	India
Foeniculum vulgare Mill.	Seeds	Silk Road	Canada	India
Levisticum officinale W.D.J.Koch	Roots	Mountain Rose	USA	Bulgaria
Levisticum officinale W.D.J.Koch	Leaves	Silk Road	Canada	Canada
Ligusticum chuanxiong S.H.Qiu, Y.Q.Zeng, K.Y.Pan, Y.C.Tang and J.M.Xu	Roots	Spring Wind	USA	China
Ligusticum porteri J.M.Coult. and Rose	Roots	Mountain Rose	USA	USA
Ligusticum sinense Oliv.	Roots	Star West	USA	China
Ocimum basilicum L.	Leaves	A1 Spice World	USA	Egypt
Pastinaca sativa L.	Roots	Forest Rx	USA	Ecuador
Petroselinum crispum (Mill.) Fuss	Leaves	A1 Spice World	USA	UK
Petroselinum crispum (Mill.) Fuss	Roots	Mountain Rose	USA	Croatia
Pimpinella aniseum L.	Seeds	Mountain Rose	USA	Egypt
Psoralea corylifolia L.	Seeds	Spring Wind	USA	China
Ruta graveolens L.	Leaves	Mountain Rose	USA	USA

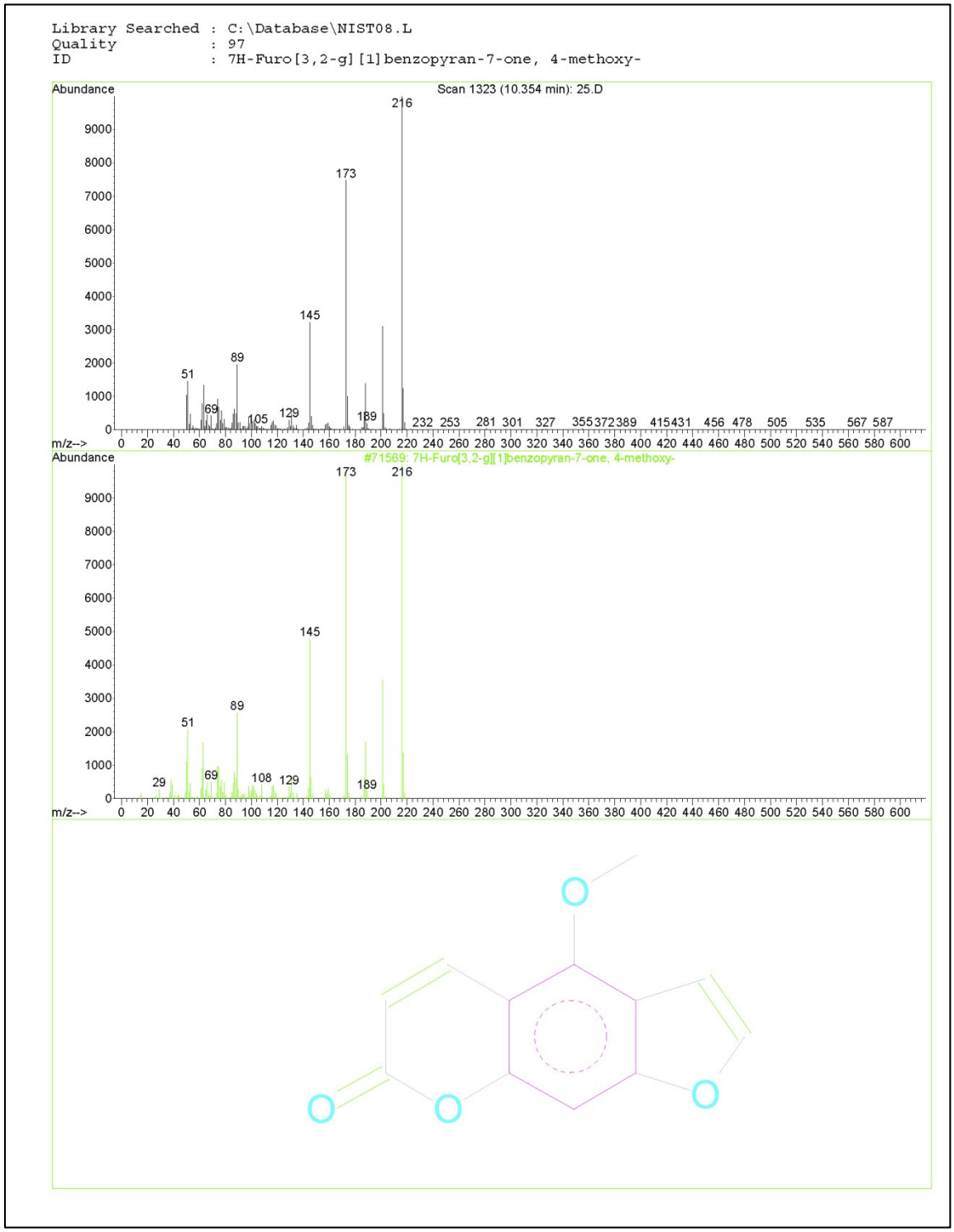
Appendix B.

Mass Spectra for 8-methoxypsoralen



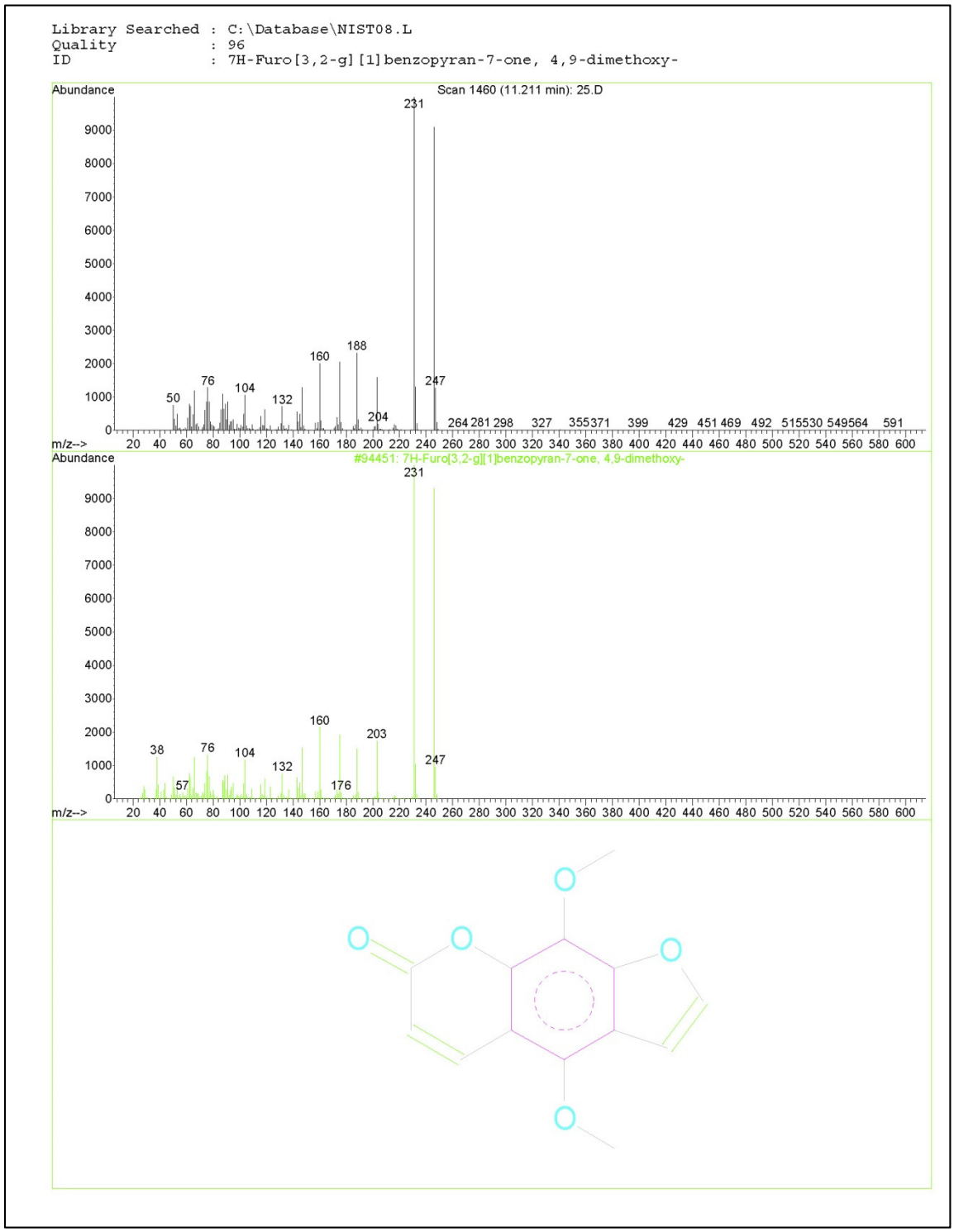
Appendix C.

Mass Spectra for 5-methoxypsoralen



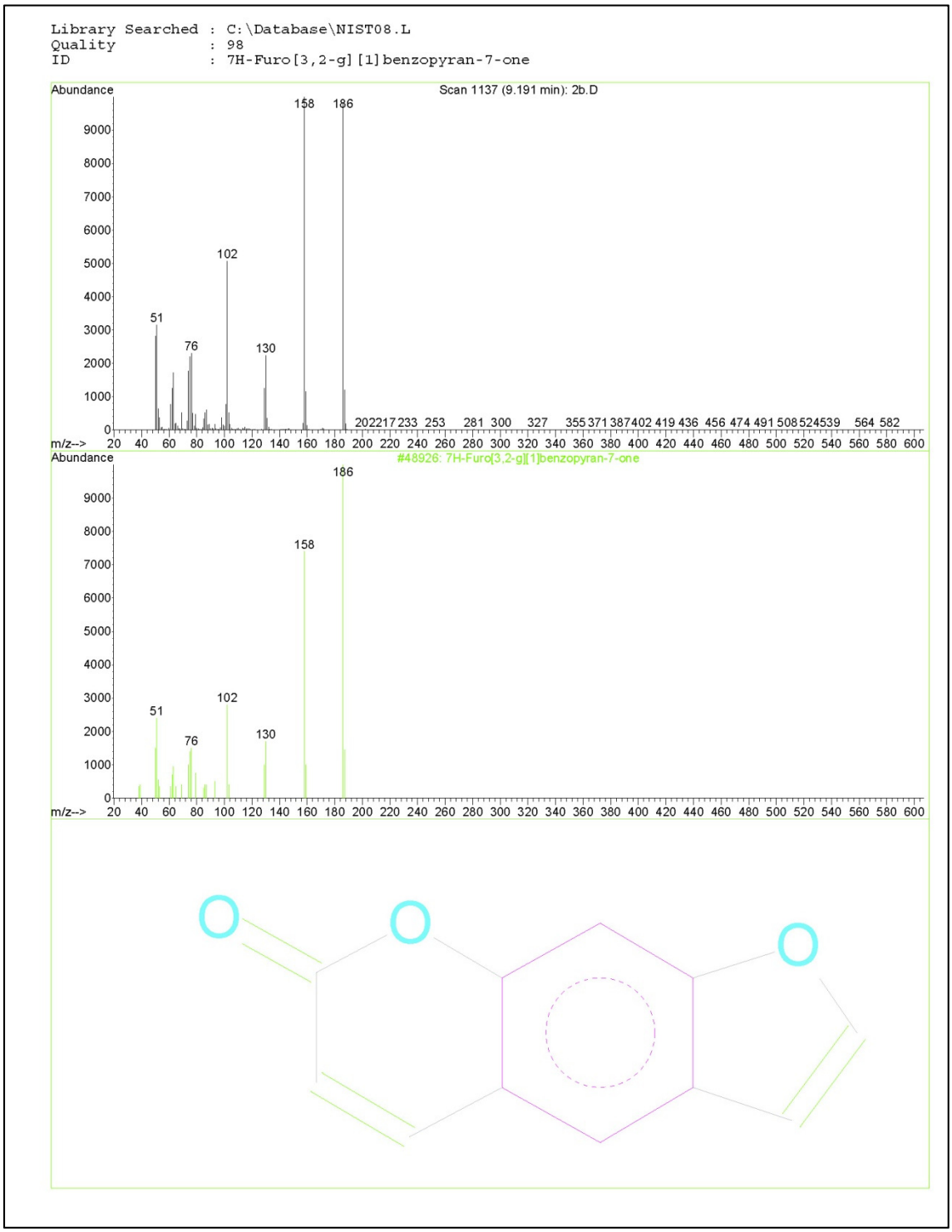
Appendix D.

Mass Spectra for Isopimpinellin



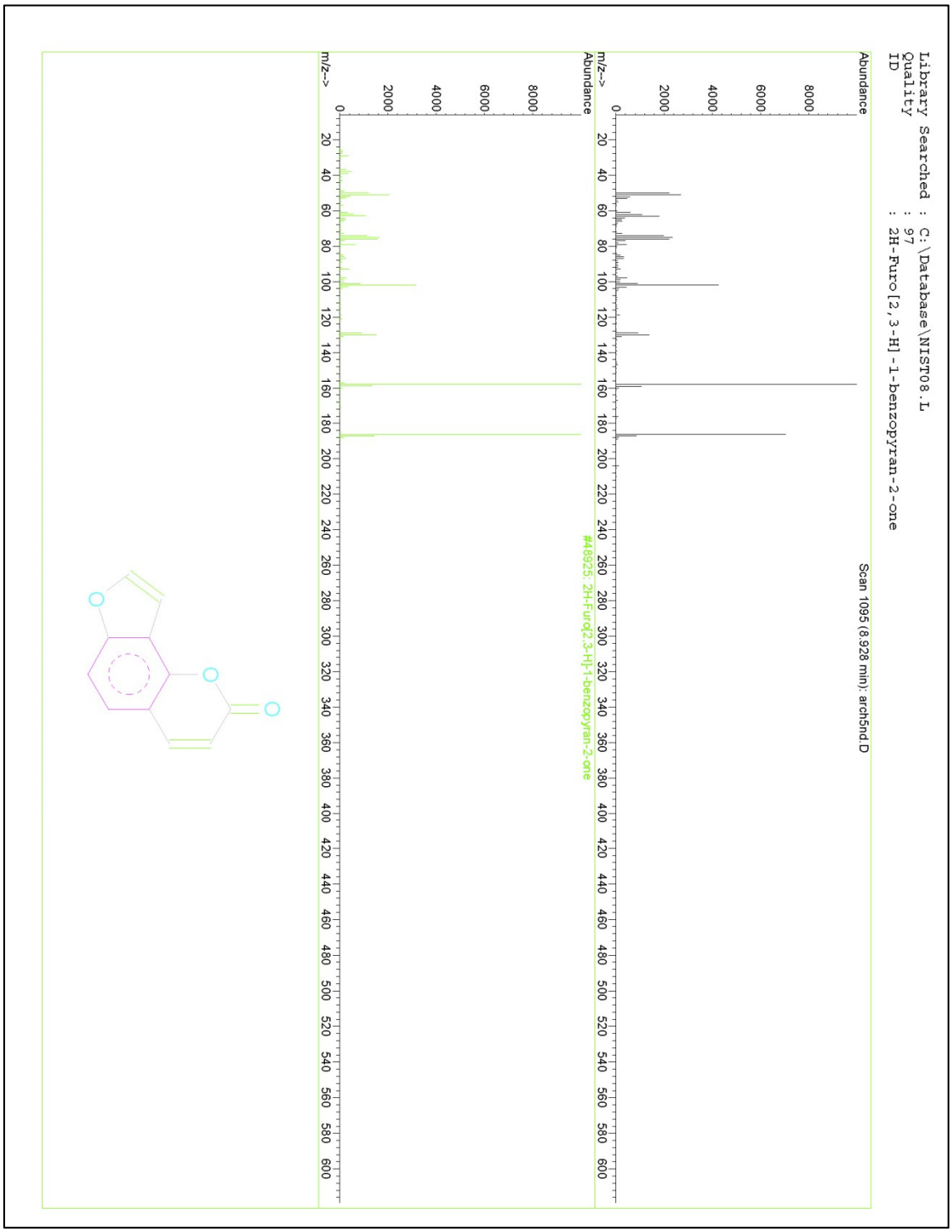
Appendix E.

Mass Spectra for Psoralen



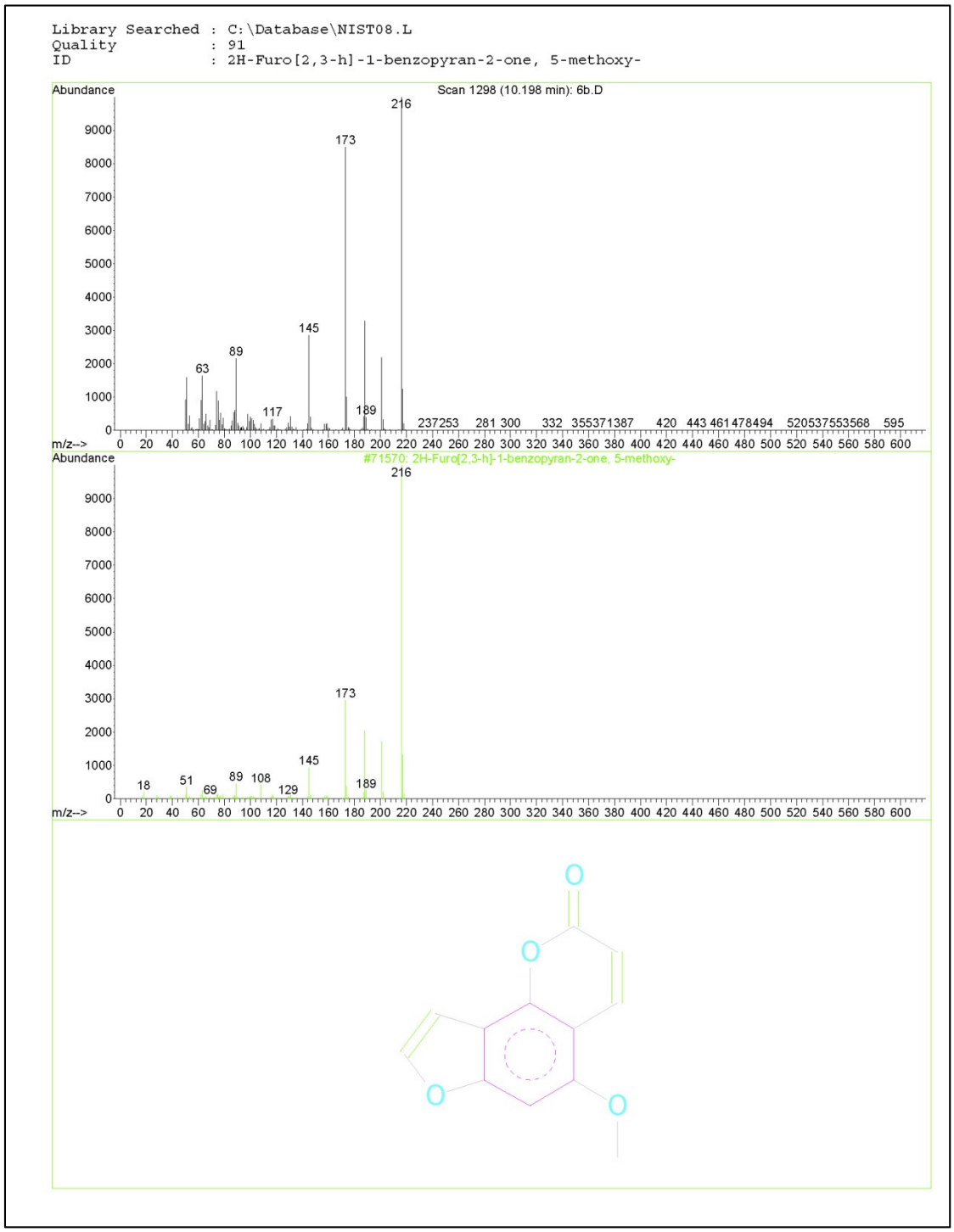
Appendix F.

Mass Spectra for Angelicin



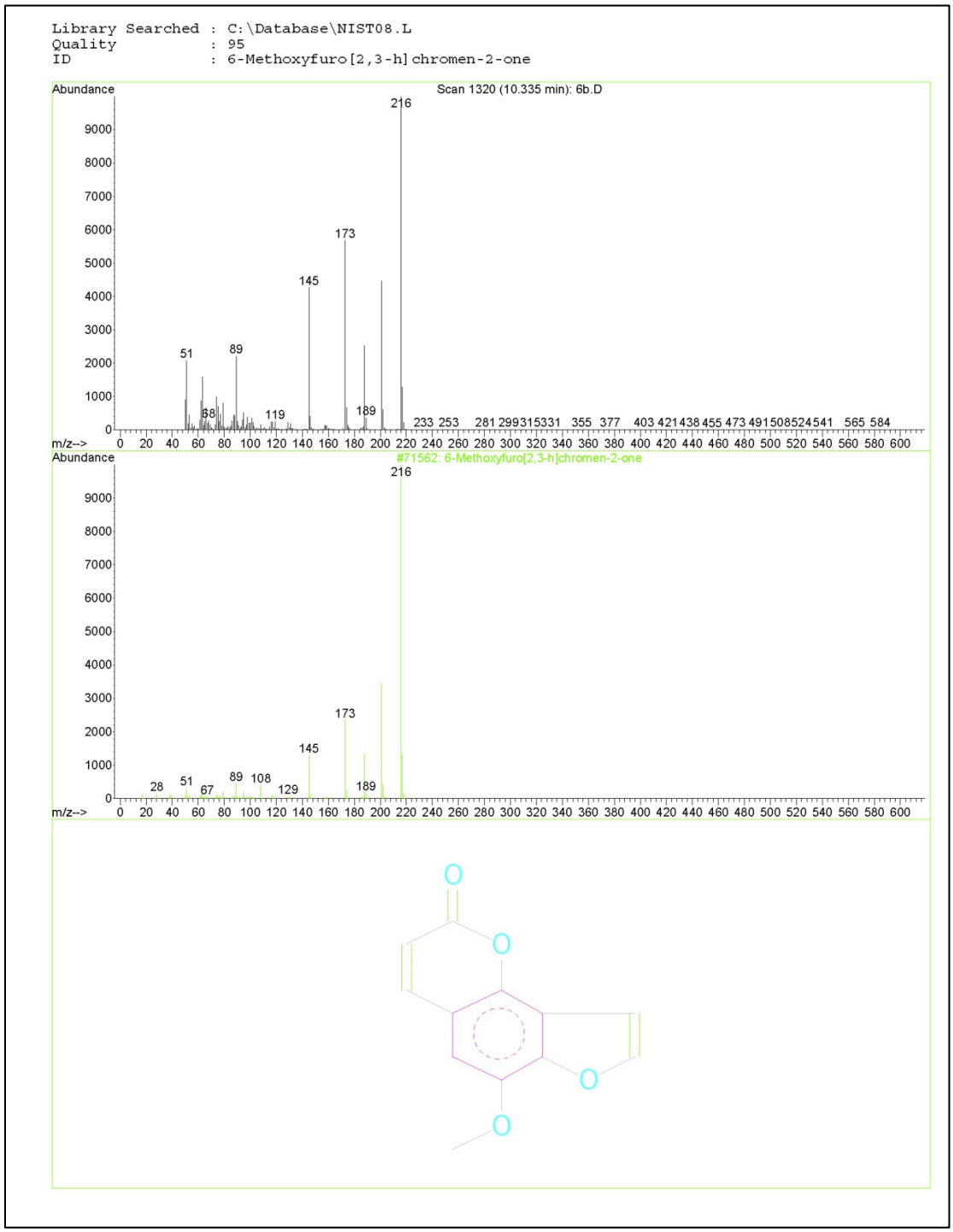
Appendix G.

Mass Spectra for Isobergapten



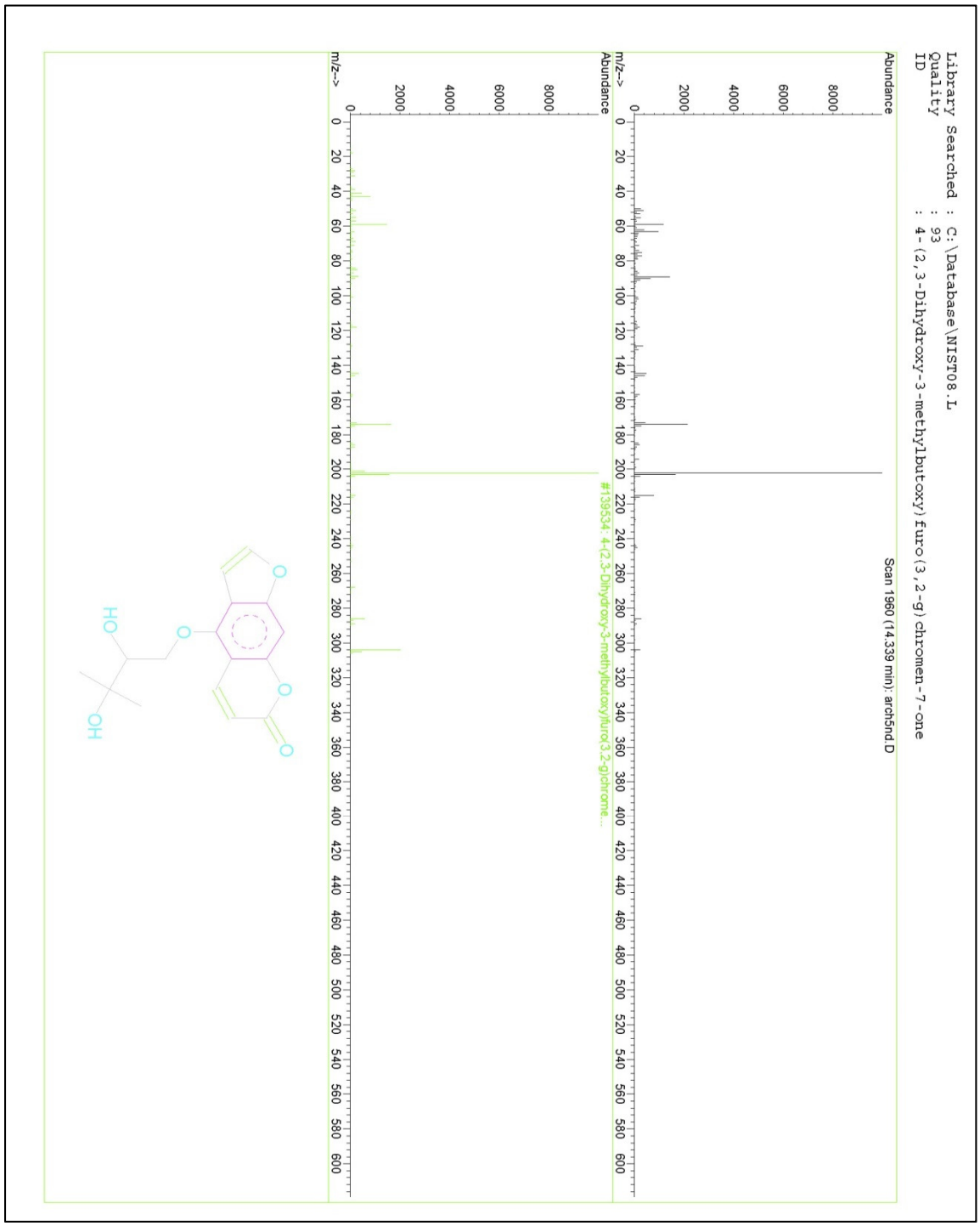
Appendix H.

Mass Spectra for Sphondin



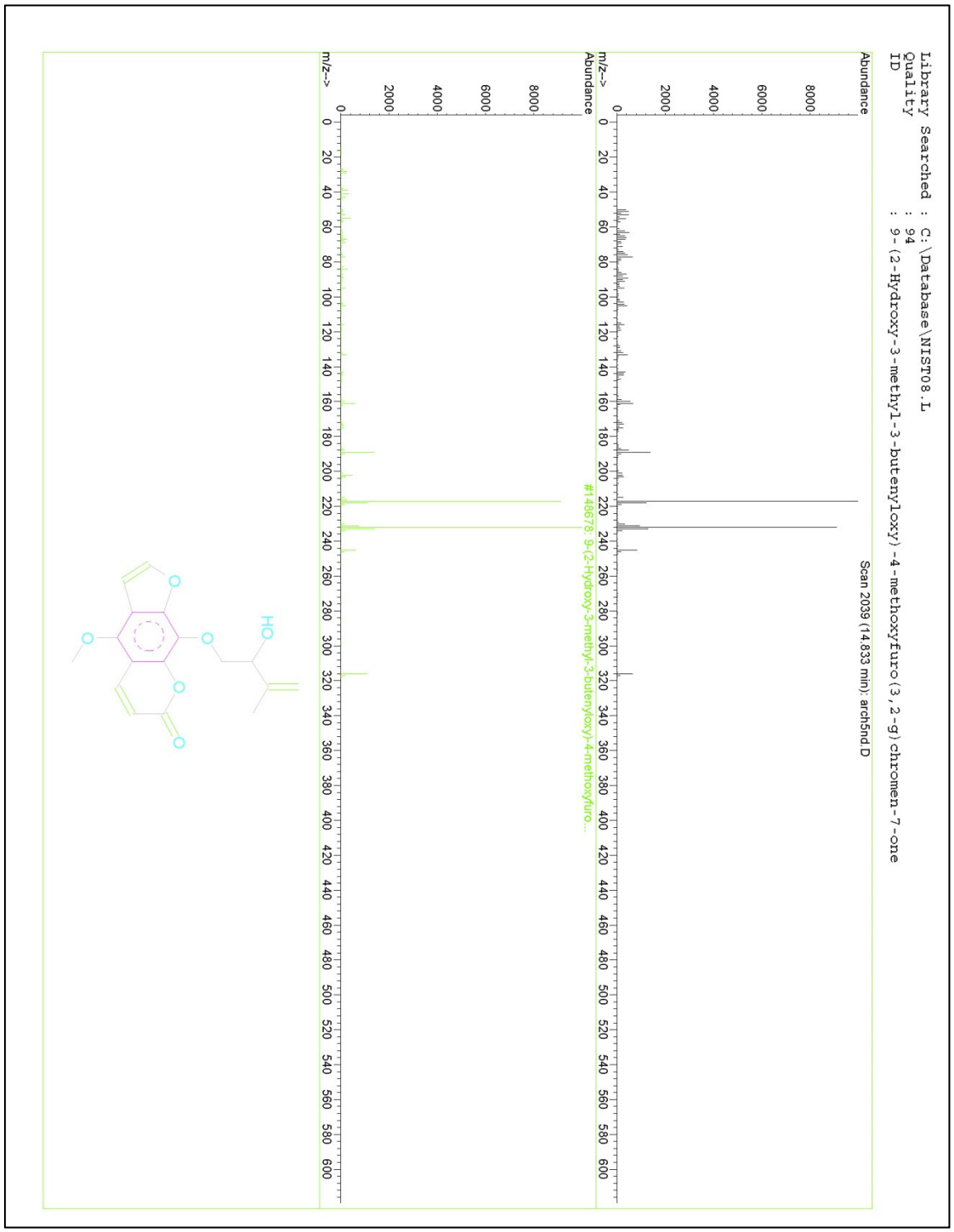
Appendix I.

Mass Spectra for Oxypeucedanin Hydrate



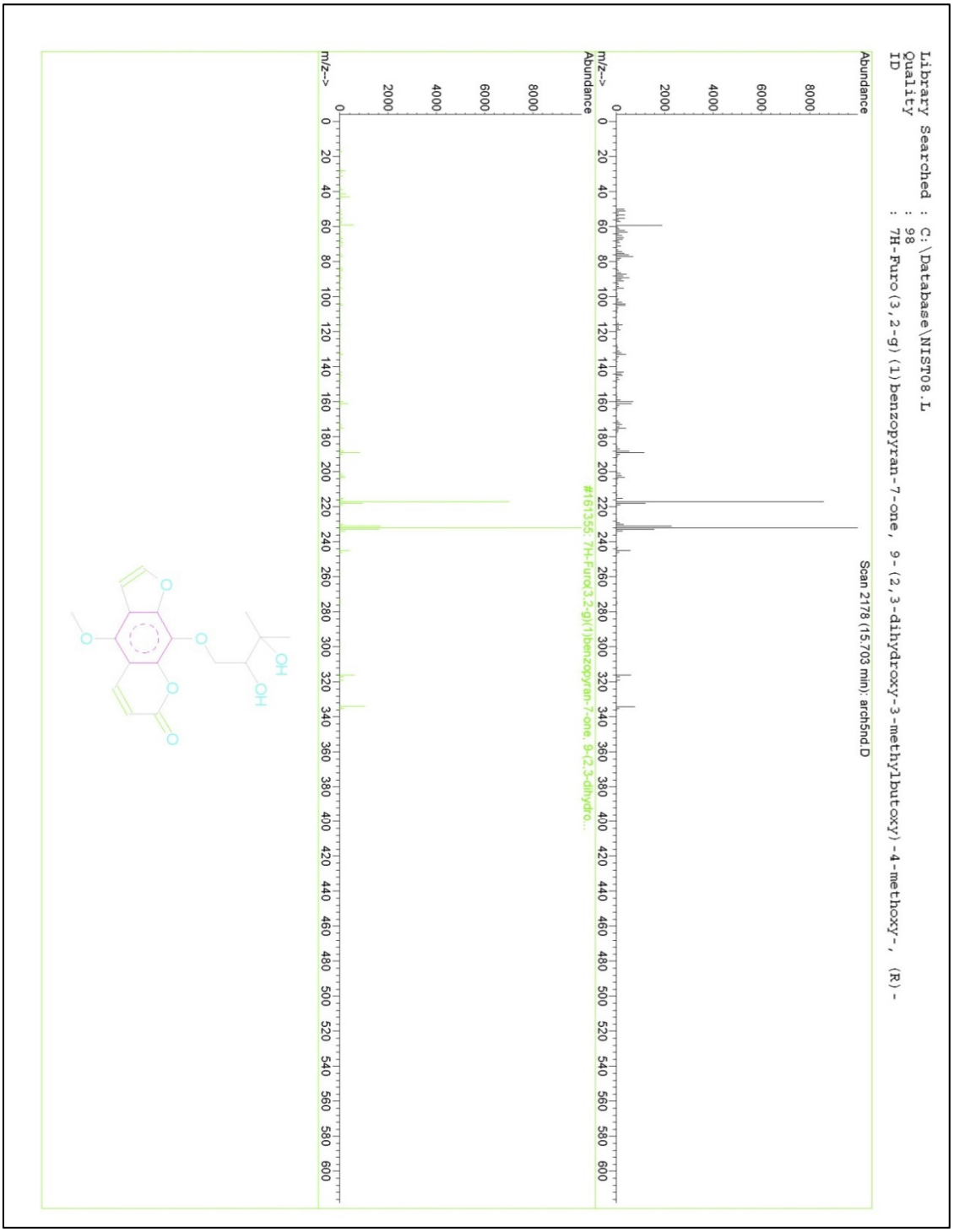
Appendix J.

Mass Spectra for Neobyakangelicol



Appendix K.

Mass Spectra for Byakangelicin



Appendix L.

Advertisement Flyer for Volunteer Participation

Simon Fraser University

*** Volunteers Wanted for a Research Study ***

Researchers at the Department of Biological Sciences are searching for volunteers to participate in a pharmacokinetic study of foods. The goal of this study is to study potential interactions between caffeine and furanocoumarin-containing foods in humans. As a volunteer for this study, you will be asked to refrain from caffeine and furanocoumarin containing foods and drinks during the study. You will be asked to consume caffeine tablets and an extract of food. You are required to give multiple saliva and urine samples during the study. No clinic visits needed as this study will be done at the convenience of your own home. The total duration of the study is twelve days.

Eligibility Criteria: You must be between the ages of 20-35 years, non-smoking, no recreational drug used in the last three months, no heavy alcohol consumption in the last three months, no major heart or liver medical history, non-pregnant nor breast-feeding.

Benefits: No benefits to you. However, your voluntarily participation may benefit the medical community to better understand caffeine and furanocoumarin(s) interactions in humans.

Privacy: All information related to your identification and contact information will be confidential and restricted to the main researcher only.

Risks: No risks expected. The amount of caffeine is equivalent to a cup of coffee and the amount of furanocoumarin-containing food is similar to the average food consumption levels.

This study will be conducted with the approval of Simon Fraser University's Human Ethics Office (Approval No. 2012s0565).

If you wish to participate and/or need more information, please contact:

Mr. Zeyad Alehaideb

Cell phone: [redacted]

Email: [redacted]

Mail: [redacted]

Appendix M.

Consent Form for Participated Volunteers

CONSENT FORM [2012s0565]

INFORMED CONSENT BY SUBJECTS TO PARTICIPATE IN THE FOLLOWING EXPERIMENT:

PREDICTING IN VIVO INTERACTIONS BETWEEN CAFFEINE AND FURANOCOUMARINS-CONTAINING HERBS OR FOODS BASED ON IN VITRO DATA FROM HUMAN LIVER MICROSOMES.

You are being asked to participate in a research study. In order to decide whether or not you should agree to be part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent.

This consent form gives detailed information about the research study that the investigator will discuss with you. Once you understand the study, you will be asked to sign this form if you wish to participate. You will have a copy to keep as a record.

The proposed research study will be to collect timed saliva and urine samples from you after ingesting caffeine tablets (200 mg) alone and caffeine tablets (200 mg) with an herb (or vegetable) together (total caffeine consumption by you is 400 mg for the whole study).

PURPOSE OF THE RESEARCH STUDY:

To determine if a single meal of furanocoumarin-containing herb (or vegetable) would cause inhibition of caffeine metabolism after co-administration.

DESCRIPTION OF THE RESEARCH PROCEDURES:

You have been selected to participate in this study to find out if common furanocoumarin-containing vegetables (or herbs) are capable of modulating caffeine metabolism in humans. ***You should be a non-smoker between the ages of 20-35 years but not pregnant or breast-feeding. You will be asked to refrain from ingesting caffeine, caffeinated drinks and furanocoumarin-containing foods for 3 days before and after participating in the first pharmacokinetic study (without co-treatment with an herb) and until the end of the second pharmacokinetic study (with co-treatment of an herb).*** You will be provided with a study kit consisting of caffeine tablets (400 mg), an herbal extract, and several coded containers for saliva and urine sample collection. You will conduct the following studies in the privacy of your homes:

First pharmacokinetic study: Time course of caffeine and metabolite concentrations in the saliva of humans without herb/food extract co-treatment. On the day of the experiment, you will ingest 200 mg caffeine tablets (equivalent to the amount of caffeine in a cup of coffee or in a can of energy drink). A saliva sample (about 3 ml) will be collected in a coded, siliconized glass tubes just before dosing. Serial saliva samples also will be collected at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5,

6, 7, 8 and 12 hr post-dosing. A 30 ml urine sample will be collected at 4-8 hr post caffeine administration since the half-life of caffeine clearance in the human is about 4-4.5 hr.

Second pharmacokinetic study: Time course of caffeine and metabolite concentrations in the saliva of humans co-treated with an herb/food extract. After a 3-day wash-out period, you will ingest 4.5 g (or 9 g) of a dehydrated herb (or food) in the form of an aqueous extract 3 hr before ingesting the caffeine tablets. You will be given one of the following herbs or vegetables: parsnip, celery, dill, parsley, angelica, false bishop's weed, common rue, lovage, khella, dong quai, and baizhi. A saliva sample (about 3 ml) will be collected in a coded, siliconized glass tubes just before dosing. Serial saliva samples also will be collected immediately after dosing with an herb extract at 0.5, 1, 1.5, 2.5, 3.0 hr and after dosing with 200 mg caffeine at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8 and 12 hr. A 30 ml urine samples will be collected before dosing and at 4-8 hr post-caffeine ingestion.

At the conclusion of the study, the saliva and urine samples from both pharmacokinetic studies will be submitted to the principle investigator for chemical analysis. Information learned from study of this study will be used in a PhD thesis, and may be shared with the medical communities to better understand the nature of caffeine/herbs (foods) interaction.

PRIVACY:

Your research records are confidential. Your identity and all personal and confidential information about you will NOT be divulged to anybody. All private information related to you will be kept in a locked cabinet at Simon Fraser University, which is accessed by the principle investigator only. A coded number will be given to the saliva and urine specimen, and used by the investigators for reference only. The only information needed to compare your saliva and urine samples with those taken from other participants will be your age, sex, race, and whether you are sensitive to caffeine which might affect the interpretation of results. Participating scientists will not have access to your identity. All private information about you will be destroyed permanently in year 2015.

BENEFITS:

You will not benefit directly from this study. **No information or results obtained by this study will be made available to you.** However, there is the potential to benefit other people in the future if the study leads to the development of an effective method for predicting caffeine/herb interaction using *in vitro* data.

RISKS:

There will be no risk to your health because the amount of caffeine ingested is equivalent that in a cup of coffee. Moreover, the herbs (or foods) selected for the study are found in our daily diets. Please note that caffeine overdose only occurs when large amount of caffeine (more than the recommended dose by Health Canada) is ingested. Caffeine overdose may result in adverse health effects including nausea, vomiting, irritability, nervousness, anxiety, panic attacks,

dehydration, and sleep disorders in sensitive individuals (Health Canada, 2012). By signing this consent form you give to Simon Fraser University your saliva and urine samples for the advancement of science and will relinquish all rights and privileges obtained from analysis and experimental work on your samples or information obtained.

RIGHT TO REFUSE OR WITHDRAW:

The choice to enter or not to enter this study is yours. You are in a position to make a decision if you understand what the principle investigator has explained and what you have read about the research study. ***You also have the right to withdraw at any time without prejudice.*** As long as the principle investigator can still identify the sample, the subject can ask to have it withdrawn. The only way a subject cannot have a collected sample withdrawn is if once the sample is collected it is made absolutely anonymous. This means there can be no link anywhere to the subject's name, record number, etc. Following the procedure, you give up all rights to retract consent to use of the saliva and urine samples and information obtained.

This study has been reviewed by the Research Ethics Board at Simon Fraser University, which is responsible for making sure that research with participants is appropriate and that the rights and welfare of the participants are protected. If you have any questions or need more information about the conduct of this study, contact Dr. [redacted] Professor, Biological Sciences, at [redacted] or phone [redacted]. If you have questions about your rights as a research subject, contact Dr. [redacted], the Director, Office of Research Ethics at [redacted]

I have read this consent form and the research study has been explained to my satisfaction. I also certify that I have received a copy of this consent form.

PERSON OBTAINING CONSENT	PARTICIPANT SIGNATURE
PRINTED NAME OF PARTICIPANT	DATE
INVESTIGATOR'S SIGNATURE	DATE

Appendix N.

Supplementary Notice Letter for Study Volunteers

*****Note*****

Please refrain from consuming common herb extracts and drinks during the experiment. The following are known to interact and interfere with this experiment:

- Hibiscus.
- Chamomile.
- Ginger.
- Peppermint.
- Black Lemon.
- Cumin.
- Tamarind.
- Tea (including decaffeinated tea).
- Coffee (including decaffeinated coffee).
- Yerbe Mate.
- Medications that contain caffeine.
- Grape fruit juice.
- Lemon juice.
- Foods and drinks that contain chocolate.

Please also avoid pop drinks that contain caffeine and/or contain any ingredient mentioned above.

Please check the ingredient label and/or contact *Zeyad* at [redacted] for more information.

Thank you for volunteering

Appendix O.

Supplementary Form for Caffeine Study (Phase I)

***** CAFFEINE SALIVA STUDY *****

PLEASE READ ALL NOTES AND STEPS CAREFULLY PRIOR THE STUDY

- **Before the study, avoid caffeine-containing products for at least 12 hours. Common caffeine-containing products include coffee, black/green tea, chocolate, medication-containing caffeine, caffeinated drinks, and pop.**
- **Start the study early in the morning on an empty stomach before breakfast.**
- **The kit includes two caffeine tablets, 14 vials labelled (PRE-DOSE, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 12, and 24).**
- **Remember to avoid consuming caffeine-containing products during the study.**
- **If missing/broken/mislabelled vials or labels, or you need more information/clarification, please don't hesitate to call [redacted] at phone [redacted]**
- **Please note that an extra vial is provided in case needed i.e. broken vial.**

Form Update: Oct 23, 2013

STUDY STEPS

1- **Avoid any caffeine-containing foods and drinks 12 hours prior the study.**
2- **Avoid any drink or food (except water) three hours before consuming the caffeine tablets.**
3- **Avoid any drink or food (except water) 30 minutes after consuming the caffeine tablets.**
4- **Remember to avoid caffeine-containing foods and drinks during the remaining time of the study.**
5- **For (Vial PRE-DOSE):** Just before taking the caffeine tablets, collect minimum 1ml (or 1/10 of the vial volume) of your saliva. Record the time, screw the vial cap tightly, and store in the refrigerator.
6- **For (Caffeine Dose):** After collecting your (PRE-DOSE) saliva sample, swallow the 2 caffeine tablets together and record the (Caffeine Dose) time immediately and accurately. ***Do not collect saliva sample for (Caffeine Dose), just write the dose time only. Drink a glass of water to ensure you have consumed the tablets fully. Do not chew the tablets.***
7- **For (Vial 0.5):** After 0.5 hour (30 minutes) from taking the caffeine tablets, collect minimum 1ml (or 1/10 of the vial) of your saliva in (Vial 0.5). ***Sampling should be done fast (not more than 2 minutes).*** Screw the vial cap tightly, record the time accurately at (Vial 0.5) below, and refrigerate immediately.
8- **For (Vials 1 – 24):** Collect your saliva samples similar to (Vial 0.5). Remember to screw the vial cap tightly, record the time accurately, and refrigerate immediately. ***Note: The vial label number represents the time of your saliva sample after the (Caffeine Dose) time (in hours).***
9- Please remember to sample saliva **on time as much as possible** to provide more accurate study results.
10- **After the study, please make sure to place all vials and forms in the kit bag.**
11- Once completed, please inform me at phone [redacted] for forms/vials collection.

Volunteer Name:
Study Start Date:

** *Please Record the Time Immediately and Accurately* **

*** *Remember No Caffeinated Food or Drink 12 Hours Before and During the Experiment* ***

**** *Remember to keep the samples in the fridge* ****

Vial I.D.	Vial PRE-DOSE Saliva	Caffeine Tablets Dose (0 hour) No Saliva Sample is Needed – Record Time only	Vial 0.5 (0.5 hour) Saliva	Vial 1 (1 hour) Saliva	Vial 1.5 (1.5 hours) Saliva	Vial 2 (2 hours) Saliva	Vial 2.5 (2.5 hours) Saliva
Date							
Time	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM

Vial I.D.	Vial 3 (3 hours) Saliva	Vial 4 (4 hours) Saliva	Vial 5 (5 hours) Saliva	Vial 6 (6 hours) Saliva	Vial 7 (7 hours) Saliva	Vial 8 (8 hours) Saliva	Vial 12 (12 hours) Saliva	Vial 24 (24 hours) Saliva
Date								
Time	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM

***** *Call/TXT ([redacted]) to arrange a pickup the soonest* *****

THANK YOU FOR VOLUNTEERING

Appendix P.

Supplementary Form for Caffeine Study (Phase II)

START THIS STUDY THREE HOURS AFTER HERB TEA DOSE

(OR ONE HOUR AFTER 120 MIN SALIVA SAMPLE)

Caffeine Saliva Study

8- **Start this study three hours after you drink the (Herb Tea).**

9- For (Vial PRE-DOSE): Just before taking the caffeine tablets, collect minimum 1ml (or 1/10 of the vial volume) of your saliva. Record the time, screw the vial cap tightly, and store in the refrigerator.

10- For (Caffeine Dose): After collecting your (PRE-DOSE) saliva sample, swallow the 2 caffeine tablets together (Do not chew) and record the (Caffeine Dose) time immediately and accurately. *Do not collect saliva sample for (Caffeine Dose), just write the dose time only. Drink a glass of water to ensure you have consumed the tablets fully.*

11- For (Vial 0.5): After 0.5 hour (30 minutes) from taking the caffeine tablets, collect minimum 1ml (or 1/10 of the vial) of your saliva in (Vial 0.5). *Sampling should be done fast (not more than one minute).* Screw the vial cap tightly, record the time accurately at (Vial 0.5) below, and refrigerate immediately.

12- For (Vials 1 – 12): Collect your saliva samples similar to (Vial 0.5). Remember to screw the vial cap tightly, record the time accurately, and refrigerate immediately. *Note: The vial label number represents the time of your saliva sample after the (Caffeine Dose) time (in hours).*

13- Please remember to sample saliva on time as much as possible to provide more accurate study results.

14- After the study, please make sure to place all vials and forms in the kit bag.

15- Once completed, please inform me at phone [redacted] for forms/vials collection.

Volunteer Name:
Study Date:

Please Record the Time Immediately and Accurately For Both Studies

Study 2: CAFFEINE TABLETS

******* *Drink Plenty of Water After You Swallow Both Caffeine Tablets Together* *******

******This study should be done three hours after consuming the herb tea******

Vial I.D.	Vial PRE-DOSE Saliva	Caffeine Tablets Dose (0 hour) No Saliva Sample is Needed – Record Time only	Vial 0.5 (0.5 hour) Saliva	Vial 1 (1 hour) Saliva	Vial 1.5 (1.5 hours) Saliva	Vial 2 (2 hours) Saliva	Vial 2.5 (2.5 hours) Saliva	Vial 3 (3 hours) Saliva
Date								
Time	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM

Vial I.D.	Vial 4 (4 hours) Saliva	Vial 6 (6 hours) Saliva	Vial 8 (8 hours) Saliva	Vial 12 (12 hours) Saliva	Vial 24 (24 hours) Saliva	Vial 36 (36 hours) Saliva	Vial 48 (48 hours) Saliva
Date							
Time	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM

THANK YOU FOR VOLUNTEERING

Appendix Q.

Reproducibility Results of Human Plasma Caffeine Measurement

Volunteer	C_{max}				$AUC_{0\text{-last}}$			
	μg/mL	Mean	SD	CV	μg/mL*h	Mean	SD	CV
1	4.37 4.17 3.90	4.15	0.23	5.62	20.21 19.01 18.28	19.17	0.97	5.08
2	5.86 5.80 5.62	5.76	0.13	2.17	41.87 40.57 40.51	40.98	0.77	1.88
3	5.91 6.22 6.15	6.09	0.16	2.67	46.88 44.75 39.49	43.71	3.80	8.70
4	5.50 4.61 3.66	4.59	0.92	20.05	59.05 59.67 58.57	59.10	0.55	0.93
5	1.31 1.20 1.13	1.21	0.09	7.48	7.91 8.16 7.24	7.77	0.48	6.12
6	4.30 4.10 3.91	4.10	0.20	4.75	29.98 32.99 31.34	31.44	1.51	4.79
7	7.90 7.22 7.42	7.51	0.35	4.65	50.62 48.19 47.56	48.79	1.62	3.31
8	6.57 6.19 7.23	6.66	0.53	7.90	43.89 46.72 50.93	47.18	3.54	7.51
9	5.58 5.54 5.57	5.56	0.02	0.37	35.96 35.69 37.11	36.25	0.75	2.08
10	8.37 8.45 9.48	8.77	0.62	7.06	41.88 38.29 43.04	41.07	2.48	6.03
11	2.93 3.95 3.05	3.31	0.56	16.84	33.97 33.91 38.21	35.36	2.47	6.97
12	8.91 8.71 10.44	9.35	0.95	10.12	46.09 54.87 50.37	50.44	4.39	8.70
13	6.89 8.45 9.61	8.32	1.36	16.41	48.50 52.26 52.60	51.12	2.28	4.45
14	4.39 3.93 3.36	3.89	0.52	13.25	28.64 27.72 27.98	28.11	0.47	1.69
Mean		5.66	0.47	8.52		38.61	1.86	4.88

Appendix R.

Baseline Human Plasma Caffeine Pharmacokinetic Parameters

Volunteer	T_{max}	C_{max}	$AUC_{0\text{-}Last}$	$AUC_{0\text{-}Inf}$	CL
No.	h	µg/mL	µg/mL*h	µg/mL*h	mL/min
1	0.5	4.1	19.2	20.3	164.2
2	0.5	5.8	41.2	48.2	69.2
3	2.0	6.8	36.2	44.3	75.2
4	1.0	3.7	25.5	37.9	87.9
5	1.5	4.0	28.1	36.3	91.7
6	0.5	7.9	43.1	43.8	76.0
7	1.0	7.3	51.3	54.3	61.4
8	1.0	7.2	55.5	58.8	56.7
9	0.5	5.8	28.8	29.2	114.1
10	1.0	7.5	44.4	56.2	59.4
11	0.5	9.3	58.6	66.7	50.0
12	0.5	5.3	42.9	46.4	71.9
13	0.7	7.5	60.0	75.7	44.0
14	0.5	6.4	42.0	44.4	75.1
15	0.5	8.3	64.9	72.9	45.7
16	0.8	4.4	29.3	30.9	107.9
17	0.5	9.0	97.5	106.9	31.2
18	1.5	5.6	62.6	71.8	46.4
19	1.0	4.6	49.4	54.2	61.4

Mean	0.8	6.3	46.3	52.6	73.1
Median	0.7	6.4	43.1	48.2	69.2
Geometric Mean	0.8	6.1	43.2	49.1	67.9
SD	0.4	1.7	18.2	20.2	30.8
SEM	0.1	0.4	4.1	4.5	6.9
5^{th} Percentile	0.5	4.0	24.9	28.3	42.8
95^{th} Percentile	1.6	9.1	68.1	78.8	119.1

Note: Caffeine pharmacokinetic parameters for 200mg (oral dose).

Appendix S.

Relationship between 8-MOP dose and $C_{max,PT}$ in humans

Subject	Amount	BW	Dose/BW	$C_{max,PT}$
	mg	kg	mg/kg	µg/l
1	40.0	83.0	0.482	94.0
2	40.0	71.0	0.563	257.0
3	60.0	90.0	0.667	273.0
4	60.0	85.0	0.706	406.0
5	50.0	69.0	0.725	414.0
6	60.0	82.0	0.732	488.0
7	60.0	80.0	0.750	355.0
8	60.0	80.0	0.750	310.0
9	60.0	73.0	0.822	484.0

The data were adopted from Schfifer-Korting and Korting (1981).

Appendix T.

Relationship between 5-MOP dose and $C_{max,PT}$ in humans

Subject	Dose/BW	$C_{max,PT}$
	mg/kg	µg/l
1	0.600	64.0
2	0.600	152.0
3	0.600	61.0
4	1.200	400.0
5	1.200	257.0
6	1.200	380.0
7	1.200	500.0
8	1.200	530.0
9	1.200	475.0
10	1.200	298.0

The data were adopted from Stolk et al. (1981).