

**Effects of Selected Natural Health Products on Drug Metabolism:
Implications for Pharmacovigilance**

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**This thesis submitted as a partial fulfillment of
the M.Sc. program in Cellular and Molecular Medicine**

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Acknowledgement

I would like to express my heartfelt thanks to my supervisor Dr. Brian Foster, who gave me this great opportunity, and continually supported and guided me through this graduate project especially for the guidance on my English which is my second language. I would like to sincerely thank Dr. Anthony Krantis who is also my supervisor, for his support, guidance, and sincerity through the process. I would like to thank Dr John Thor Arnason, who is my advisor committee team member, for his assistance and advice. I would also like to thank Dr William Staines for his help during the past three years. I would also like to thank Rany Tang, the former technician of CMM, for her generous donation of Goji berry.

I have very special thanks to the Elders and Healers from the Cree Nation of Mistissini, and from Whapmagoostui First Nation who kindly participated in these studies. They made this thesis possible by allowing us to use, for the purposes of this research, their knowledge relating to medicinal plants, transmitted to them by their Elders. Their trust has also enabled a useful exchange between Indigenous knowledge and Western science.

Many of my colleagues from the Foster and Arnason labs have given me great help during the past three years. I would like to send my special thank to Teresa Tam, who was my first English speaking friend and my teacher of plate reader and rat cardiomyocytes assay; Jingqin Mao, my teacher on cell culture experiment and plant extraction; Ammar Saleem, who trained and assistance me on HPLC; Carolina Cieniak, another cell culture

experiment teacher of mine. Overall, I'd like to thank all my colleagues from the Foster and Arnason labs for their company and assistance.

At last, I would like to thank my family from the bottom of my heart, without their love, encouragement and support from the other side of the Pacific, I would never have accomplished my studies.

Abstract

Seventeen Cree anti-diabetic herbal medicines and eight Traditional Chinese Medicines have been examined for their potential to cause interactions with drugs, which is considered as a major reason for adverse drug effects. Specifically, the effect of these natural health products was examined on major Phase I drug metabolism enzymes including cytochrome P450, human carboxylesterase-1 and flavin-containing monooxygenases. Several of these natural health products have the potential to cause adverse drug effect through the inhibition of major drug metabolism enzymes. The results indicated that 7 Cree medicines plant extracts inhibited CYP3A4 activity, and 3 of them have been proven to cause potent mechanism-based inactivation of CYP3A4. Seven of eight Traditional Chinese Medicines have been identified as strong CYP3A4 inhibitors; the ethanol extract of Goji has identified as a potent inhibitor for CYP2C9 and 2C19. Goji juice showed a universal inhibitory effects on most of the tested enzymes except flavin-containing monooxygenases 3.

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Abbreviations

AA	Arachidonic acid
AD	Anti-diabetic
ADME	Absorption, distribution, metabolism and excretion
AHR	Aryl hydrocarbon receptor
ACN	Acetonitrile
AMMC	3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7- methoxy-4-methylcoumarin Iodide
CAR	Androstane receptor
CEC	3-cyano-7-ethoxycoumarin
CEI	Cree Nation of Eeyou Istchee
CES	Carboxylesterase
CYP	Cytochrome P450 monooxygenase
DAD	Diode array detector
DBF	Dibenzylfluorescein
DGB	Dried Goji berry
DME	Drug metabolism enzymes
DMSO	Dimethyl sulfoxide
EET	Epoxyeicosatrienoic acid
EtOAc	Ethyl acetate
EtOH	Ethanol
FA	Formic acid
FGB	Fresh Goji berry
FMO	Flavin-containing monooxygenases
HETE	Hydroxyeicosatetraenoic acid
HGJ	100% Himalaya Goji juice
HPLC	High performance liquid chromatography
MBI	Mechanism-based inactivation
MeOH	Methanol
MFC	7-methoxy-4-(trifluoromethyl)-coumarin
MpTS	Methyl p-tolyl sulphide
MpTSO	Methyl p-tolyl sulfoxide
MpTSO ₂	Methyl p-tolyl sulfone
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NHP	Natural health products
NRC	Neonatal rat cardiomyocytes
PDAM	1-pyrenyldiazomethane
Pgp	P-glycoprotein
PXR	Pregnane X receptor
SNPs	Single-nucleotide polymorphisms
T2D	Type II diabetes
TCM	Traditional Chinese Medicines
TFA	Trifluoroacetic acid
TFK	Trifluoromethyl ketone
W	Whapmagoostui

1 Introduction

1.1 Background

Traditional herbal medicines have played an important role in human history, but their importance had declined in the 20th century with the growing conventional pharmaceutical industry; this trend has now changed in developed countries, use of traditional medicines as natural health products (NHP) has become a significant factor in personal health care since 1990 (WHO 2008). And according to World Health Organization (WHO), over 80% of the population across developing countries still use traditional herbal medicine (WHO 2008). The spending on complementary and alternative medicine in North America has kept increasing as people are using more acupuncture, feldenkrais, chiropractic, massage therapy and NHPs. The consumption of NHPs is considered the most common alternative or complementary healthcare (Kennedy et al. 2009).

The markets for NHPs are growing fast. According to a survey study by Health Canada in 1999; only 26% of the participants reported to take NHPs to prevent or treat an illness (Non-Prescription Drug Manufacturers Association of Canada 1999/2000). However, this number grew to 71% in 2005 (Health Canada 2005).

Traditional use has demonstrated that most of these NHPs are relatively safe with few or no serious adverse effects. However, as these products have pharmacological properties, there is the potential risk to cause NHP-drug interactions. Recent evidence is that

constituents from NHPs may affect the pharmacodynamic and pharmacokinetic profile of other therapeutic products and this may lead to therapeutic failure or other serious adverse effects which pose a threat to human health. This research project focused on the potential of two groups of NHP's of interesting in Canada today: traditional Cree botanicals and Traditional Chinese Medicine used as complementary or alternative medicines to affect human drug metabolizing enzymes.

1.2 Natural Health Products

The Natural Health Products Directorate of Health Canada defined natural health product as “any plant or plant material, a bacterium, fungus, algae, non-human plant material, or extracts or isolates of these materials” used to treat, support or prevent health conditions including vitamins and minerals, herbal remedies, homeopathic medicines, traditional medicines such as traditional Chinese medicines (TCM) and other products such as probiotics, amino acids and essential fatty acids (Health Canada 2005). They are not only commercialized in the traditional form such as creams, teas, tinctures, ointments or raw materials, but are also available in other forms such as capsules, tablets, or caplets through modern pharmaceuticals preparation processes (Health Canada 2005).

NHP manufacture in Canada is under the regulatory control by Health Canada. The uses, recommended doses, ingredients and adverse effect of manufactured NHPs are reviewed and approved as the basic recommendation for the consumers (Health Canada 2005).

However, NHPs such as practitioner formulated products, raw plant material or traditional medicinal material used by aboriginal communities are not under the

regulatory control of Health Canada (Moss et al. 2007).

NHPs differ from other therapeutic products in that they are usually not single-active ingredient products but highly complex mixtures of constituents where the safety profiles of many of these products is still not clear. NHPs, especially herbal products, may contain a large number of natural constituents from multiple classes of phytochemicals such as alkaloids, anthraquinones, carotenoids, flavonoids, monoterpenes, organosulfur compounds, phenolics-polypyrroles, tocopherols-tocotrienols, triterpenes, polyphenols and furanocoumarins. These constituents not only provide therapeutic effects (Table 1), they may also be responsible for some adverse effects. In a survey, about 12% of the NHPs consuming population experienced unexpected side effects or reactions (Health Canada 2005).

NHPs are routinely used together with conventional drugs. Kuo and colleagues (2004) reported that 46% of the participants were concomitantly using NHPs with conventional medicine. Some constituents of NHPs may affect both pharmacokinetics and pharmacodynamics of drug (Table 2) which is considered as a major source of adverse effect. As a result of increased NHP consumption, there is an urgent need to identify potential NHP-drug interactions.

Incorrect identification of the source materials (species, organs, etc), environmental growth conditions (climate, pollution, etc), harvesting (season, climate conditions, etc), storage conditions (temperature, humidity, exposure to light, etc), and manufacturing

Table 1. Major chemical constituents in plants (Leo, 1990).

Class	Compounds	Example Sources	Example Some Effects and Uses
Alkaloids	Nicotine, cocaine	Tobacco	Interferes with neurotransmission, block enzyme action.
Coumarins	Hydrangine	Carrots, parsnip	Cross-links DNA, blocks cell division.
Flavonoids	Anthocyanin, catechin	Almost all plants	Inhibit enzymes, anti- and pro-oxidants, estrogenic.
Monoterpenes	Menthol linalool	Mint and relatives	Interferes with neurotransmission, blocks ion transport, anaesthetic.
Phenolic acids	Caffeic acid, chlorogenic acid	All plants	Causes oxidative damage.
Sterols	Spinasterol	Spinach	Interferes with animal hormone action.
Tannins	Gallotannin	Oak, hemlock trees	Binds to proteins and enzymes, block digestion, antioxidant.
Tetraterpenoids	Carotene	Carrots	Antioxidant.
Triterpenes	Digitogenin	Foxglove	Stimulates heart muscle, alter ion transport.

Table 2. Drugs that interact with NHPs (Dhananjay et al. 2006; Zhou et al. 2007).

NHPs	Drug	Interaction outcome	Possible mechanism
Garlic	Acetaminophen	Increased sulfation	Induction of enzyme
	Chlorpropamide	Hypoglycemic response	Additive effect
	Saquinavir	Decreased blood drug level, lower drug effect	Induction of CYP3A4 and Pg-P
	Warfarin	Increased internationally normalized ratio and clotting time	Additive effect
Ginkgo	Aspirin	Spontaneous hyphema	Additive effect
	Trazodone	Coma	Unknown
	Warfarin	Bleeding	Additive effect
Ginseng	Phenelzine	Serotonin syndrome	Additive effect
	Warfarin	Decreasing internationally normalized ratio	Antagonistic effect and/or enzyme induction
Black/long pepper	Theophylline	Increased blood drug level	Inhibition of enzymes
	Propranolol	Increased blood drug level	Inhibition of enzymes
St John's wort	Digoxin	Decreased blood drug level	P-glycoprotein induction
	Fexofenadine	Decreased the maximum plasma concentration, increasing oral clearance	P-glycoprotein inhibition
	Loperamide	Acute delirium episode	Monoamine oxidase inhibition
	Omeprazole	Induced metabolism	Induction of CYP2C9
	Warfarin	Decreased blood drug level	Enzyme induction

processes may change the composition of NHPs which can alter their pharmacokinetic properties and hence their efficiency and toxicity (Health Canada 2005). Although traditional medicinal products have few reported adverse effects if used as directed, this may be due in large part to careful administration (Table 3). Herbal practitioners are highly skilled, but as more consumers are self-medicating and occasionally use more than one product there is an increasing risk of serious adverse effects from misuse.

Most herbal medicine systems such as TCM have well defined traditional usage and strict rules to guide the use of medical plants. These rules are mainly based on long-term practice rather than scientific evaluation, but based on observation which helped herbal practitioners to use these herbal medicines effectively and safely. Combination, or blending, of different NHPs is a common practice to enhance the therapeutic efficacy. This requires specific knowledge and experience as improper combinations may lead to adverse effects. For example, Gan Sui (*Euphorbia kansui*) and liquorice (*radix liquiritiae*) should be used together carefully. A 1:1 mixing ratio showed the highest toxicity, whereas ratios of 1:2 or 2:1 had lower toxicity. A 1:4 or 4:1 mixing ratio exhibited the lowest toxicity which was even lower than exclusive use of Gan Sui (Yanping et al. 2007).

1.3 Drug Disposition

1.3.1 Disposition processes

Absorption, distribution, metabolism and excretion (ADME) are the four systemic processes that affect the safety and efficacy of all xenobiotics, of which therapeutic products are only one part. It is accepted that these processes are controlled by both

Table 3. Toxicities and usages of some NHPs (Dasgupta and Bernard 2007).

Common name	Species Name	Toxicity	Intended Use
Ephedra	<i>Ephedra equisetina</i>	Cardiovascular	Weight loss
Chan Su	Venenum bufonis*	Cardiovascular	Tonic for heart
Kava-kava	<i>Piper methysticum</i>	Hepatotoxic	Sleeping aid, anti-anxiety
Comfrey	<i>Symphytum officinale</i>	Hepatotoxic	Repairing of bone and muscle, prevention of kidney stones
Germander	<i>Teucrium sp.</i>	Hepatotoxic	Weight loss, general tonic
Chaparral	<i>Larrea tridentata</i>	Hepatotoxic, nephrotoxic, carcinogenic.	General cleansing tonic, blood thinner, arthritis remedies, and weight loss.
Borage	<i>Borago officinalis</i>	Hepatotoxic, hepatocarcinogenic	Source of essential fatty acids, rheumatoid arthritis, hypertension
Calamas	<i>Acorus calamus</i>	Carcinogenic	Psychoactive, not promoted in United States
Senna	<i>Senna alexandrina</i>	Carcinogenic, hepatotoxic	Laxative
Licorice	<i>Glycyrrhiza glabra</i>	Pseudoadosteronism	Treatment of peptic ulcer, flavoring agent

*Amphibian toxin

environmental and intrinsic factors. The intrinsic factors include genetics, gender, age, physiology, ambulatory status, health status and co-morbidities. Extrinsic factors are variable environmental factors such as sunlight, temperature, diet, nutrition, smoking, alcohol use (or abstinence), socio-economic factors that affect compliance, period of use, concomitant therapy including licit and illicit products, and acute or repeated use (William and Howard 2003; Foster et al. 2005).

Therapeutic products are given to the patient by several routes such as orally, intravenously, intramuscularly, intrathecally, subcutaneously, sublingually, rectally, vaginally, inhalation and cutaneously (Corina et al. 2005). The route of administration will affect the rate and extent in which active and other constituents are absorbed or distributed throughout systemic circulation. This may also affect the duration of activity. For the 4 major routes, the decreasing order of time to effect is intravenous > intramuscular > subcutaneous > oral. However, the order is opposite for the duration of effect where the decreasing order is oral > subcutaneous > intramuscular > intravenous (Corina et al. 2005).

Importantly, disposition can be affected by genetics. In the initial sequencing of the human genome, more than 60,000 of single-nucleotide polymorphisms (SNPs) in the coding region of genes have been identified (Sachidanandam et al. 2001). Estimates now range up to 17.3 million SNPs with 7.5 million mapped (NCBI data base 2009). In addition to SNPs, polymorphisms can result from deletion/insertion, structural variation or copy number, re-arrangements, inversions in the drug-metabolizing, transporter,

receptor, and ion channel genes. In general, there are 4 genotype or phenotypes with some populations skewed to one side of the bell curve depending on the functionality of the alleles (Ingelman-Sundburg 2004):

- poor or slow (lack functional alleles),
- intermediate (heterozygous with 1 functional allele),
- extensive or fast (homozygous with 2 normal alleles), and
- ultra-rapid (multiple copies, $n \geq 2$).

Distribution to different tissues and organs may vary by the size, blood flow of the organ or tissue, the binding specification of drug to some component in organ, serum and tissue. Tissue membrane permeability is also an important factor; generally, lipid soluble drugs are easier to cross the cell membrane or bio-barrier such as the blood-brain, placental, blood-retinal, blood-ocular, and vaginal barriers (José et al. 1997). Protein binding is also a very important factor that affects drug disposition (Corina et al. 2005), for example, many drugs can bind to serum albumin, and this may increase the solubility of drug in blood and make the transportation more efficient. However, the bound form of drug is not pharmacologically available.

1.3.2 Drug metabolism

Drug metabolism is the biochemical modification of a drug through specialized enzymatic systems. Drug metabolism often converts lipophilic chemical compounds into more readily excreted polar products. The rate of metabolism is an important determinant of the duration and intensity of the pharmacological action of drugs. The smooth

endoplasmic reticulum of the liver cell is the principal site of drug metabolism. The drug metabolites are mainly excreted from urine; however, there are also other routes for drug extraction such as bile (Corina et al. 2005).

Individuals who are poor metabolizers are low in enzyme activity, this usually leads to slow metabolism which may result in increased plasma drug concentration and can cause overdose. It has been confirmed in clinical studies that many adverse effects of drugs are evident in poor and intermediate metabolizers. For the ultra-rapid metabolizers, overexpression of functional enzymes increases drug metabolism rate faster than normal which will decrease the plasma drug concentration to levels which may lead to therapeutic failure (Ingelman-Sundberg 2004).

Xenobiotic metabolism including drugs has two phases: Phase I; metabolic modification (oxidation, reduction, hydrolysis, cyclization, and decyclization reactions), and Phase II; conjugation. Each type of biotransformation requires a specific enzyme system. Enzymes involved in drug metabolism are the so-called Drug Metabolism Enzymes (DME). The activity of the metabolite may be increased or decreased, or have a new effect relative to the parent substance.

The major Phase I enzymes include the cytochrome P450 (CYP) monooxygenase family, flavin-containing monooxygenases, alcohol dehydrogenase, aldehyde dehydrogenase, monoamine oxidase, NADPH-cytochrome P450 reductase, reduced cytochrome P450, esterases, amidases, and epoxidases. The major Phase II enzymes include hydroxylase,

methyltransferase, glutathione S-transferase, other sulfotransferase, N-acetyltransferase, amino acid N-acyl transferase and UDP- glucuronosyltransferase (Katzung et al. 2004). Xenobiotic enzymes, receptors, and transporters are unlike their classical forms; they may all exhibit substrate recognition and catalysis with broad substrate specificity, low turnover rates, atypical kinetics (substrate specific), multi-product formation, and other unusual properties (Ma and Lu. 2008).

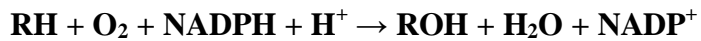
Hormonal changes caused by pregnancy, malnutrition, obesity, diabetes mellitus, systemic inflammation, and conditions of altered extracellular fluid volume or osmolality may also alter drug distribution (Sarlis et al. 2005) will affect the level of DMEs such as CYPs and transport protein such as P-glycoprotein (Pgp) through a nuclear receptor mediated mechanism (Foster 2009).

1.3.3 Cytochrome P450 system

Human CYP450 is the most important Phase I drug-metabolizing enzyme system, responsible for the metabolism of a variety of xenobiotics including therapeutic drugs and some important endogenous substances such as steroids, fatty acids, eicosanoids, vitamins, and environmental pollutants. CYP450 is a large and diverse super family of hemoproteins which have been identified from all domains of life, including mammals, birds, fish, insects, worms, sea squirts, sea urchins, plants, fungi, slime moulds, bacteria and Archaea. More than 7700 distinct CYP450 sequences have been identified (Sigel et al. 2007). Humans have 57 genes and 59 pseudo genes divided among 18 families of cytochrome P450 genes and 43 subfamilies (Al Omari et al. 2007). Human CYP450 are

primarily membrane-associated proteins, located either in the inner membrane of mitochondria or in the endoplasmic reticulum of cells (Pelkonen et al. 2008).

Oxidation is the most common reaction mediated by CYP450, this process generally requires energy and a free electron provided by reducing equivalents or nicotinamide adenine dinucleotide phosphate (NADPH). The oxidation happens in the heme domain of the CYP450 one of the oxygen atoms inserts into substrates (RH) and the other is reduced to water.



CYP450 catalyze N-, O-, S-dealkylation, aromatic and aliphatic hydroxylation, N-oxidation, nitro reduction, peroxidation, desaturation, and aldehyde and hydroxycarbonyl oxidation (Cederbaum 2006).

The CYP3A, CYP2D and CYP2C subfamilies have been reported to be responsible for the metabolism of 50% 25% and 20% of total therapeutic products, respectively (Bertz and Granneman 1997; Rendic and Di Carlo 1997). Fourteen individual CYP450 isozymes including 1A1, 1A2, 2A6, 2B6, 2C9, 2C8, 2C19, 2D6, 2E1, 2F1, 2J2, 3A4, 3A5 and 3A7 have been identified as major DMEs (Guengerich 2005). The most significant feature of these DME is that they are not substrate specific which means they can be involved in the metabolism of a large number of xenobiotics. For example, CYP3A4 contains multiple substrate binding domains (Schrag and Winekers 2001) and its substrates have been generally categorized into four distinct groups based on their patterns of activity variation by different CYP3A4 inhibitors (Kenworthy et al. 1999). CYP19 (aromatase – first stage

in steroid biosynthesis) and CYP2B13 are also considered important drug metabolism enzymes; however, both of them are regarded as substrate specific (Guengerich 2008). Most of the remaining human CYP enzymes are only involved in the metabolism of specific endogenous substrates. There is limited information on whether NHPs affect their activity.

Besides liver and intestine, CYP isoforms are also found in almost all other organs and tissues such as skin, placenta, lung, kidney, endothelium, brain, intestine, olfactory mucosa and heart and other cardiovascular tissue. They take part in the biotransformation of a large number of endogenous substrates which may involve the activation or deactivation of many important compounds such as arachidonic acid (CYP4A, CYP2C), vitamins D (CYP24A1), testosterone (CYP3A) and estrogen (CYP19)(Hsu et al. 2007; Okuda et al. 1995; Ghosh et al. 2009). They are believed to play important physiological roles in specific organs (Pelkonen et al. 2008).

Genetic polymorphisms of CYP450 are very common, particularly in the CYP2C and 2D subfamilies (Table 4). The most abundant CYP isozyme, CYP3A4, has also been found to have polymorphisms. At least 78 nucleotide sequence variations of CYP3A4 have been identified (Channa et al. 2004). These genetic polymorphisms may cause dosing problems of therapeutic products, especially in CYP1 and 2 families which involve approximately 40% of CYP450-mediated drug metabolism (Table 5).

Ten CYP450 enzymes from 5 CYP families and 7 sub families have been examined in

Table 4. Polymorphisms in CYP1 and 2 families (Phillips et al. 2001).

Enzymes	Variant alleles	Distribution of prevalence of variant alleles
CYP1A2	CYP1A2*1C	No data
CYP2C9	CYC2C9*2	8-20% Caucasian
	CYC2C9*3	6-9% Caucasian
CYP2C18	CYP2C18*3	27% Japanese
CYP2C19	CYP2C19*2A	13% Caucasian; 29% Chinese; 25% African American; 21% Korean; 14% Ethiopian
	CYP2C19*3A	0.3% of Caucasian; 12% Japanese and Korean
	CYP2C19*4	0.6% Caucasian
CYP2D6	CYP2D6*2A	28-30% Caucasian; 20% Chinese; 12% Japanese
	CYP2D6*3A	21% Caucasian
	CYP2D6*3B	2% Caucasian
	CYP2D6*4A, B	20-23% Caucasian; 7-9% African American% 9% African
	CYP2D6*5	2-5% Caucasian; 10-13% Japanese
	CYP2D6*6A	2% Caucasian
	CYP2D6*7	Less than 1-2% Caucasian
	CYP2D6*8	Less than 1% Caucasian
	CYP2D6*9	2% Caucasian
	CYP2D6*10A, B	2-5% Caucasian, 43-51% Chinese; 33-60% Japanese
	CYP2D6*11	Less than 1% Caucasian
	CYP2D6*12	Less than 1% Caucasian
	CYP2D6*17	26% African American; 9-34% African; 19% Korean
CYP2D6*36	9% Korean; 31% Chinese and Japanese	

Table 5. Adverse drug reactions caused by CYP2 family polymorphisms with reduced enzyme activity (Phillips et al. 2001).

Drug	Drug metabolism Enzymes
Carbamazepine	CYP1A2
Diltiazem	CYP1A2, CYP2D6.
Erythromycin	CYP1A2
Fluoxetine	CYP1A2, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1.
Ibuprofen sodium	CYP2C9
Imipramine	CYP1A2, CYP2C9, CYP2C18, CYP2C19, CYP2D6
Isoniazid	CYP1A2, CYP2C9, CYP2C19, CYP2E1.
Metoprolol	CYP2D6.
Naproxen	CYP1A2, CYP2C9
Nortriptyline	CYP2C19, CYP2D6
Phenytoin	CYP1A2, CYP2C9, CYP2C19
Piroxicam	CYP2C9, CYP2C18
Rifampin	CYP1A2, CYP2C9, CYP2C18, CYP2C19
Theophylline	CYP1A2, CYP2D6, CYP2E1.
Verapamil	CYP1A2, CYP2C9, CYP2E1.
Warfarin	CYP2C9, CYP2C19.

this study to determine the potential inhibitory effect of the selected NHPs. Some of these CYPs are major hepatic DMEs including CYP2C9/19, CYP2D6 (*1&*10), and CYP3A4/5/7. CYP19, CYP2J2/3 and CYP4A11 were also chosen because they also have an important role in the metabolism or biosyntheses of endogenous substrates such as estrogen (CYP19) and fatty acid (CYP2J2/3, CYP4A11).

1.3.4 The flavin-containing monooxygenase system

The flavin-containing monooxygenase (FMO) system is another important Phase I oxidase involved in drug metabolism. These reactions involve oxidation of heteroatoms, particularly nucleophilic atoms such as the nitrogen, phosphorus or sulfur. Unlike the CYP system, the FMO system cannot oxidize carbon atoms (Cashman 2008).

The FMO system is also distinct from the CYP system in that it is rarely-inducible and does not have a heme prosthetic group. However, both systems require NADPH and oxygen; multiple isozymes are known for both; and they are both localized in microsomes of the endoplasmic reticulum.

There are five families of FMO in mammals (FMO 1-5) and four of them exist in human (FMO 1-3 and 5). FMO1, absent in the adult liver, is primarily expressed in kidney. However it is also found in fetal liver. It is a highly selective in stereochemical S-oxidation. FMO2 is mostly expressed in human lung; its substrate specific is most similar to FMO1; however it cannot metabolize certain tertiary amines such as imipramine and chlorpromazine (Krueger et al. 2001). FMO3 is the most important member of the FMO

family as it is the most prominent FMO in adult human liver (Cashman 2000) and is involved in the mono-oxygenation of common drugs such as cimetidine, ketoconazole, ranitidine, clozapine and tamoxifen (Zhou and Shepard 2006; Cashman 2000).

1.3.5 Human carboxylesterase

Human carboxylesterase (CES) is a Phase I esterase. CES belong to the α/β -hydrolase family (Randy MW et al. 2007). Four main groups (CES1-4) have been found in mammals. CES1 and 2 are the major human CES groups. Although both CES1 and 2 hydrolyze ester containing compounds, their substrate specificity is distinctly different. CES1 mainly metabolize substrates in hepatic tissue that contain a small alcohol group and large acyl group. CES2 is mainly an intestinal enzyme and metabolizes substrates which have a large alcohol group and small acyl group (Imai 2006).

The CES1 gene is polymorphic with a non-conservative substitution at codon 143 (p.Gly143Glu) and a frameshift mutation at codon 260 (p.Asp260fs) (Zhu et al. 2008). Codon 143 frequency ranged between 3.7%, 4.3% 2.0% and 0% in the white, black, Hispanic, and Asian populations examined, respectively. *In vitro* testing suggests that the catalytic function of both polymorphisms is substantially impaired and substrate-dependant. Many therapeutic products such as aspirin, clopidogrel, deltamethrin, permethrin and oseltamivir are metabolized by this enzyme (Yang et al. 2009). The carboxylate metabolite is excreted into the urine by the organic anion transporter (He et al. 1999).

1.4 Metabolic Interactions

An interaction is a situation in which a substance affects either pharmacokinetic or pharmacodynamic activity of another substance. Interactions are not only found between drugs (drug-drug interaction), but may also exist between foods, NHPs, over-the-counter products (OTCs), and other xenobiotics. The impact of an interaction will depend on whether it is a result of an acute or chronic (repeated) administration of the products.

In the acute situation, a single or limited number of doses will be inhibitory. This will tend to result in increased plasma levels of the products. A decrease in the metabolic rate of a product may then lead to overdose. Aside from the immediate health concerns, such overdosing can result in reduced compliance with patients not taking their medications as scheduled.

The mechanism of inhibition can be divided in two categories: irreversible or reversible inhibition. The reversible inhibition can also be sub categorized as competitive, non-competitive (or uncompetitive), and mixed-type inhibition (Lin and Lu 1998; Hollenberg 2002; Madan et al. 2002). Competitive inhibition is when the inhibitor and the substrate for the enzyme share the same binding site, and there will be a competition of binding depending on their affinity. Non-competitive inhibition is when the inhibitor binds to another active site on the enzyme and prevents the substrate from binding to its own active site. Basically, neither competitive inhibition nor non-competitive inhibition requires biotransformation of the inhibitor, and the bonds to the enzyme are relatively weak which means it is easily formed and also easily broken down. Reversible inhibition

happens fast but only temporarily disables the enzyme (Lin and Lu 1998; Hollenberg 2002; Madan et al. 2002).

Irreversible inhibition, sometimes referred to as suicide-substrate inhibition or mechanism-based inactivation (MBI), is much more critical than the reversible inhibition. MBI occurs when the biotransformation product covalently bonds to the enzyme causing an irreversible structure change on the enzyme molecular. This permanently alters the shape and loss of activity of the enzyme. In some instances, the altered enzyme can trigger an immunological hypersensitivity response. It is estimated to take on average 8-12 hr for the human body to reproduce and replace the inactivated enzyme, and during this period the human body is vulnerable to all xenobiotics which are metabolized or eliminated through this pathway (Lilja et al. 2000; Takanga et al. 2000). Time, NADPH and concentration dependence are most important phenomenon of a MBI on CYP450, and are the most common characteristics to identify a MBI (Halpert 1995; Lin and Lu 1998). Several drugs have been identified to cause MBI on CYP450 such as CYP1A2 inhibitor furafylline (Sesardic et al. 1990; Kunze and Trager 1993) and the CYP3A4-inhibitor gestodene (Guengerich 1990; Back et al. 1991). Components from NHPs may trigger MBI as well. For example, the most documented CYP3A4 MBI inhibitors are bergamottin and dihydroxybergamottin found in grapefruit juice (Flanagan 2005, Mertens-Talcott et al. 2006). Other reported examples demonstrate that most of the CYPs involved in xenobiotic metabolism can be affected through MBI.

The inhibition of other DMEs such as FMO and CES will also cause metabolic drug

interactions. Inhibition and induction of FMOs have been rarely reported (Cashman 2000); however, there are some drugs that can affect FMOs activity. Anti-HIV agent tenofovir and tenofovir disoproxil fumarate were reported to have a strong inhibitory effect on FMO3 even at a very low level (below 100 μ M) (Matal et al. 2006).

Trifluoromethyl ketone (TFK)-containing compounds are the most potent inhibitors of human CES (Wheelock et al. 2001). The inhibition of human CES-1 may decrease the formation of active hydrolysates of many ester containing pro-drugs such as oseltamivir (Rui et al. 2010) which may lead to therapeutic failure.

Inhibition might be the most common reason for a harmful metabolic drug interaction.

Enzyme inhibition usually leads to an increase of drug's bioavailability by decreasing the first-pass effect or slowing the elimination of a drug which will enlarge the side effect as well. This could be serious for a drug which has a narrow therapeutic index; nevertheless the inhibition effect would be critical if a drug is only metabolized through a single pathway. Several drugs have been removed from the market due to adverse events associated with inhibition (Friedman et al. 1999; Lasser et al. 2002). NHPs also have the potential to cause the same serious interaction. As most NHPs contain a large number of components; the chance of causing an adverse effect is even greater than with single-active ingredient therapeutic products.

Chronic or repeated exposure generally results in the adaptive response through induction which may increase the rate of drug metabolism with a corresponding decrease of plasma drug concentration. This may cause reduced efficiency for most therapeutic products. The

inducer increases metabolism by modifying the expression of gene coding DMEs to produce more enzymes. The inducer is usually a ligand for a nuclear receptor regulating the expression of specific DMEs, and will stimulate the expression after binding.

Compared to inhibition, induction is considered as a slow regulatory process. As an example, the induction effect of rifampicin needs almost a week to reach its maximum effect, and may take more than two weeks to restore the normal enzyme level after discontinuing rifampicin (Fromm et al. 1996).

There are three major nuclear receptors involved in CYP-induction: aryl hydrocarbon receptor (AHR) for CYP1 family, and constitutive androstane receptor (CAR) and pregnane X receptor (PXR) for CYP2 and 3 families (Lin 2006). Many drugs such as ethosuximide, glutethimide, and rifampicin (Lin 2006) have been identified as CYP inducers. NHPs may be inducers as well. For example, hyperforin, an active compound found in St. John's wort, is a potent ligand for the PXR, an orphan nuclear receptor regulating expression of CYP3A4, 2B6 and Pgp. *In vitro* study demonstrated that the mRNA levels of CYP3A4, 2B6 and Pgp were significantly increased after the treatment with hyperforin (Mai et al. 2004). Clinical studies have also observed the increased metabolism of drugs metabolized by CYP3A4 and the reduced absorption of drug through Pgp efflux (Whitten et al. 2006).

Other DMEs such as FMO and CES may be also induced through nuclear receptor based mechanisms. The common environmental contaminant 2, 3, 7, 8-tetrachloro- dibenzo-*p*-dioxin (TCDD) was reported to induce FMO2/3 in mouse liver through an AHR-based

mechanism. Basal FMO3 mRNA was induced 6-fold at 4 h and 6000-fold at 24 h of TCDD treatment (Celius et al. 2008). *In vivo* studies suggest TCDD regulates CES expression. TCDD showed different effects on protein and mRNA levels for a CES. In the 3-mg/kg TCDD dosage group, the protein level of hydrolase B was increased by 30%; however the hydrolase B mRNA level was decreased by 45% in the same group. This suggests that TCDD may affect both translation and transcription.

Although the nuclear receptor-mediated induction is considered to be the major route of DEMs induction, there are other mechanisms of induction such as decreased enzyme protein degradation or increased mRNA stability (Watkins et al. 1986; Song et al. 1987).

Pro-drugs are becoming more clinically important for targeted therapy. They are pharmacologically inactive and need to be activated through metabolism, often by specific enzymes. Thus, the metabolic drug interaction may affect pro-drug safety and efficacy differently. An inhibitory effect may block the transformation from pro-drug to active metabolite and cause therapeutic failure. On the other hand, induction may increase the formation of active metabolite and this may lead to overdose or an adverse effect (Sorin et al. 2006).

The critical role of CYP450 played in the metabolism of most therapeutic drug makes them a major potential source of the metabolic drug interactions. The modification of enzyme activity may lead to harmful results such as therapeutic failure or adverse effects. “Grape fruit juice effect” is one of the most documented NHP-drug effects. It is a serious

NHP-drug interaction which was originally reported in 1989 and became well-publicized after being responsible for a number of deaths due to overdosing on medication (Bailey et al. 1991). Grapefruit juice contains flavonoids and furanocoumarins (Flanagan 2005); *in vitro* studies indicated that some of these furanocoumarin constituents such as bergamottin, dihydroxybergamottin and flavonoids such as naringin are potent inhibitors for CYP3A4 which will block the metabolism pathway of a large number of medicines including terfenadine, astemizole, felodipine and verapamil, this will increase the plasma drug concentration and leading to adverse effect (Flanagan 2005; Mertens-Talcott et al. 2006). Many studies have been done to investigate the potential risk of NHP-CYP interaction and have identified that many of NHPs can affect the metabolism and distribution of conventional medicine such as St. John's wort, ginkgo, Echinacea, kava, licorice root and garlic (Ernst 2002; Anke et al. 2004; Bocker et al. 1991; Foster et al. 2001). The metabolic drug interaction between FMO, CES and NHPs was rarely reported. However, the potential risks of a serious FMO-NHP or CES-NHP interaction still exist.

It is noteworthy that these interactions can be beneficial in some cases. The anticancer pro-drug, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin (CPT-11) is metabolized by CES to produce the active drug 7-ethyl-10-hydroxy-camptothecin. However the activation of CPT-11 in intestine by intestinal CES may cause delayed onset diarrhea, a dose-limiting side effect of this drug. A CES inhibitor can block the intestinal CES activity and decrease the risk of this side effect (Kyoung et al. 2006). Furthermore, there is anticipation that NHPs with a high inhibitory effect on the intestinal enzyme activity can be used as a dose adjusting agent; a pharmacoenhancer. These interactions

are expected to decrease the first pass effect and increase the absorption and concentration of oral formulations.

1.5 Type II Diabetes

Type II diabetes (T2D), previously called non-insulin-dependent diabetes mellitus (NIDDM), is a worldwide health problem. More than 150 million people were affected by this disease in 2000 (Zimmet et al. 2001) and this number rapidly increased to about 194 million in 2005 (International Diabetes Federation 2005). WHO is expecting a 39% increase of global rate of total diabetes from 2000 to 2030 (WHO data base 2006), and 90% of them would be T2D (Inzucchi et al. 2005). T2D prevalence rate is increasing at an even faster rate in Canada. According to a case study based on the population and validated diabetes database from the province of Ontario, the prevalence of T2D increased about 69% from 1995 to 2005 in the population over 20 years old (Lipscombe and Hux 2007).

T2D is a disorder caused by high blood glucose which is due to insulin resistance and relative insulin deficiency (Robbins and Cotran 2003). High blood glucose levels can lead to a condition called glucose toxicity. This leads to further damage in the pancreas which will decrease the insulin secretion, and the glucose levels may continue raise to levels that can cause damage to organs such as the eyes, nerves, and kidneys. The damage of high levels of blood glucose on small blood vessels and nerves may accelerate the development of atherosclerosis, which can result in a heart attack, stroke, or poor blood flow to the legs; the damage to small blood vessels can also affect eyes, kidneys, and it is

called diabetic retinopathy which causing blindness. Damage to the kidneys, called diabetic nephropathy, can lead to kidney failure and the need for dialysis. Damage to nerves can cause neuropathy. Some diabetics who develop peripheral neuropathy and have poor blood flow to the legs may eventually require amputation. If blood glucose levels become very high, especially when there are other stresses such as infection, patient may become confused, dizzy, and have seizures which may result non-ketonic hyperglycemia-hyperosmolar coma (Robbins and Cotran 2003).

Currently, there are 6 classic classes of pharmacologic agents to treated T2D and each of them usually regulates glucose through one or more major pathways. Insulin and insulin analogues are used on the correction of insulin deficiency; sulfonylureas and glinides can stimulate insulin secretion; biguanides mainly decrease the hepatic glucose production and it may also increase muscle glucose utilization, glitazones (thiazolidinediones) mainly increase muscle glucose utilization and also provide a weak effect on the decrease the hepatic glucose production. R-glucosidase inhibitors are used to retard carbohydrate absorption (Skyler 2004). Combinations of these products are also being used to enhance the therapeutic efficiency (Skyler 2004). Beside anti-diabetic agents, other medications such as cardiovascular drugs are also used to treated T2D symptoms. Recently, there has been increased interest in using of NHPs as anti-diabetic agents. Antioxidants such as vitamin C and E, essential fatty acids, l-carnitine, niacin, short-chain fructo-oligosaccharides and some herbal medicines such as Ginkgo and American ginseng have demonstrated some degree of anti-diabetic activity in clinic trials (Triggiani et al. 2006).

In Canada and The United States, T2D prevalence rates are much higher in the aboriginal population. The morbidity rate in Canadian First Nations is about twice higher than average in Canada (Harbilas et al. 2009). This may be due to rapid change in culture and life style such as lose of traditional diet and an increasingly sedentary lifestyle (Hegele et al. 2001). This impact of modern life style influences genetic factors which are adapted to fasting – high conversion dietary intake leading to obesity and associated health risks becoming more critical (Skyler 2004). The previous study is centered upon a request from the Cree Board of Health and Social services of James Bay to work with the Cree Nation of Eeyou Istchee (CEI), a group of First Nations located in northern Quebec where more than 16% of CEI's population suffer from T2D (Kuzmina and Dannenbaum 2004). This prevalence is approximately four times the provincial and national rates (Kuzmina and Dannenbaum 2004).

Due to the cultural differences, conventional drugs are not well accepted in CEI, and in many instances they prefer their traditional medicine to treated health problems, including the symptoms of T2D. With the purpose of providing a more culturally acceptable treatment for T2D in CEI, an evaluation of anti-diabetic activity of these traditional herbal medicines was undertaken by the Canadian Institutes of Health Research (CIHR) team in diabetic medicines. Based on ethnobotanical surveys of 34 healers and elders (Leduc et al. 2005) from Mistissini and 31 healers and elders in Whapmagoostui, identified 17 traditional plant species (Table 6) used to treated T2D symptoms. These botanicals were then pharmacologically examined for their potential anti-diabetic activity (Harbilas et al. 2009) The results showed that most exhibited anti-

diabetic activity including stimulating glucose uptake, accelerating adipogenesis, toxicity protection, glucose deprivation protection, exhibited high antioxidant activity, some even have multiple anti-diabetic activities (Spoor et al. 2006; Harbilas et al. 2009). They were also examined to determine if they could enhance insulin secretion, but none of them have been reported to have this ability (Spoor et al. 2006; Harbilas et al. 2009). The complex phytochemical compositions make these Cree anti-diabetic herbal medicine ideal complementary therapy for T2D. However, the healers were concerned that their patients may consume a number these products together with conventional drugs and increase the risk of NHP-drug interaction that could lead to a serious adverse effect. This concern is justified as there are reports suggesting that diabetic patients metabolize therapeutic products differently. As diabetes is in essence a hormonal disease, the abnormal hormone level would be expected to affect metabolism and possibly the nuclear modifiers which would also alter transport (Sarlis et al. 2005). This makes the potential interaction between Cree anti-diabetic herbal medicine and conventional drugs more complex and may increase the risk of adverse effects in diabetic patients.

In 2005, the CIHR Team in Aboriginal Anti-diabetic Medicines initialled this multipartite project investigated the anti-diabetic effects of plants used by aboriginals. Dr. Pierre S. Haddad from Fonds de la recherche en santé du Québec (FRSQ), is the leader of this CIHR team in Aboriginal Anti-diabetic Medicines. His laboratory in University of Montreal mainly focuses on the insulin-sensitizing action of plant extracts evaluated by bioassays of muscle, fat and liver tissues, in vivo animal studies are also carried out in his laboratory. Dr. John T. Arnason leading a team from University of Ottawa was in charge

of the preparation of all plant extracts; Dr. Arnason's team is also focus on the identification of useful marker substances and biologically active principles in order to standardize the extracts. Dr. Steffany Bennett heads the neural regeneration laboratory at the University of Ottawa. Her laboratory mainly focuses on the anti-apoptotic activity of plant extracts on retinal and nervous tissue. Dr. Timothy Johns' from University of McGill will be responsible for nutritional surveys with Cree community members to determine appropriate dosages and preparations of the standardized plants to be used in clinical studies. His team are also closely collaborated with Dr. Haddad evaluated anti-oxidant activity and the pharmacological activity on bioassays of vascular endothelium of the plant extracts. Dr Brian C Foster's team from University of Ottawa were in charge of the *in vitro* and *in vivo* safety of plant preparations and their potential interactions with therapeutic drugs. This multipartite project is closely collaborated with the Cree Nation of Eeyou Istchee in Northern Quebec (James Bay area).

This project was aimed to profile the inhibitory effect of Cree plant extracts on major DMEs. This study was conducted in parallel to studies undertaken by Ms. Teresa W. Tam to determine the inhibitory effect of the Cree anti-diabetic plants on 14 enzymes including 12 CYP enzymes (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, 4A11 and 19), FMO3 and CES1. Among them, enzymes CYP1A2, 2B6, 2C8, 2C9, 2C19 2D6, 2E1, 3A4, 3A5 and 3A7 were tested by Ms. Tam using a microtiter fluorometric plate assay. This thesis project was focused on the enzymes CYP3A4 (alternate-substrate study), CYP4A11, 19 and FMO3.

1.6 Traditional Chinese Medicine (TCM)

The Cree healers also informed us that TCMs are used in their community. TCM is one of the oldest and most widely used traditional medicines with an over 3000 year's history of practice. TCM has a unique system to diagnose and cure illness which is mainly based on the yin-yang and five elements theories. The understanding of the human body is based on the holistic understanding of the universe as described in Daoism, and the treatment of illness is based primarily on the diagnosis and differentiation of syndromes. Herbal medication is one of the most important parts of TCM which contains nearly 2000 therapeutic products including animal, microbial, mineral and plant material. Shen Nong Ben Cao Jing was the first book on medicinal plants in history, which was most likely written between about 300 B.C. and 200 A.D. This book recorded 365 medicinal materials including 252 plant materials, 67 animal materials and 46 minerals including many widely used herbal medicines such as Ginseng, Ginger, and Ma Huang (ephedra). It is considered as the foundation of TCM herbal medication. The Compendium of Materia Medica was written in 1590 by Li Shizhen, one of the greatest herbalists in TCM history, is the most important literature of TCM. 1892 herbal medicines and 11,096 prescriptions in TCM have been documented with detailed information such as identification, function, side effect and contraindication. It was translated into Latin and transmitted to Europe in late 17th century. Many TCMs have been studied under the western medicine system, and many compounds have been identified and demonstrated to have pharmacological activities, such as ginsenosides from ginseng (Li et al. 1996), ginkgolides and bilobalides from Ginkgo (Mahadevan et al. 2008), Ganoderan from Lingzhi mushroom

Table 6. List of the 17 Cree anti-diabetic plant species examined in this study.

Species	Common Name	Organ	Cree Name	Family	Code
<i>Abies balsamea</i> (L.) Mill.	Balsam fir	Bark	Innasht	Pinaceae	AD03
<i>Alnus incana</i> ssp. <i>Rugosa</i> (Du Roi) Clausen	Grey alder	Bark	Atushpi	Betulaceae	AD08
<i>Gaultheria hispidula</i> (L.) Muhl.	Snow berry	Fruit	Pieuminaan	Ericaceae	W8
<i>Juniperus communis</i> L.	Juniper	Fruit	Kahkachiminahtikw	Cupressaceae	W4
<i>Kalmia angustifolia</i> L.	Sheep-laurel	Leaves	Uishichipukw	Ericaceae	W2
<i>Larix laricina</i> K.Koch	Tamarack larch	Bark	Watnagan	Pinaceae	AD02
<i>Lycopodium clavatum</i> L.	Wolf's-foot clubmoss	Whole	Pashtanhoagin	Lycopodiaceae	W6
<i>Picea glauca</i> (Moench) Voss.	White spruce	Leaves	Minhikw	Pinaceae	W3
<i>Picea mariana</i> (Mill.) BSP.	Black spruce	Cones	Innahtikw	Pinaceae	AD06
<i>Pinus banksiana</i> Lamb.	Jack pine	Cones	Ushchishk	Pinaceae	AD11
<i>Populus balsamifera</i> L.	Balsam poplar	Bark	Mitus	Salicaceae	W7
<i>Rhododendron groenlandicum</i> (Oeder) Kron and Judd	Bog Labrador tea	Leaves	Kachichpukw	Ericaceae	AD01
<i>Rhododendron tomentosum</i> (Stokes) Harmaja ssp. <i>subarcticum</i> (Harmaja) G. Wallace	Marsh Labrador tea	Leaves	Wishichipikushh	Ericaceae	W1
<i>Salix planifolia</i> Pursh	Diamond leaf willow	Leaves	Pieuatikw	Salicaceae	W5
<i>Sarracenia purpurea</i> L.	Purple pitcher plant	Leaves	Ayigadash	Sarraceniaceae	AD09
<i>Sorbus decora</i> (Sarg.) Schneid.	Showy mountain-ash	Bark	Mushkuminanatikw	Rosaceae	AD07
<i>Vaccinium vitis-idaea</i> L.	Cowberry	Fruit	Wishichimna	Ericaceae	W9

(Boh et al. 2007). Some have been transferred into modern therapy as a single chemical entity. For example, artemisinin is an active compound found in Qin Hao (*Artemisia annua L.*) and now it is widely used as an anti-malaria drug. However, most of the TCM products are still only available in the form of multi-chemical entity which cannot be approved as a prescription medication in the western world.

Eight widely used TCM products have been chosen in this project (Table 7). Goji, the fruit of *Lycium barbarum L.* in the family Solanaceae, also known as Gou Qi Zi or wolfberry in the western world is a well-known traditional Chinese herbal medicine. TCM states that Goji can promote Yin energy, improve eyesight, protect the liver and it is an anti-aging agent. Modern medicine has promoted Goji for multiple beneficial effects, such as reducing blood glucose and serum lipids, immune-modulation, anti-cancer, anti-fatigue, and for male fertility-enhancement (Gao et al. 2000; Peng et al. 2001a, b; Wang et al. 2002a, b; Gan and Zhang 2003). Goji can be used to produce various types of health food products, including medicinal beverages, drinks, and healthy dietary soups (Li 2001).

Chrysanthemum flower (Ju Hua) is the flower of *Dendranthema morifolium (Ramat.) Tzvel.* In TCM theory, Chrysanthemum flower is believed to enhance the Yin energy, cooling, detoxification and improve eye sight. Modern medicine has determined that Chrysanthemum flower has beneficial effects including sedation, anti-fever, anti-bacterial, anti-viral activity, reduces blood pressure and coronary dilatation. It is traditionally used to prevent cold or treated fever, hypertension and coronary diseases (Huang 1999).

Functional tea and soup is the most common way to consume Chrysanthemum flower.

Du Huo is the root of *Angelica pubescens Maximowicz* in the Apiaceae family. It has sedative, hypnologic, pain release, and anti-inflammation activities and is usually used to treated rheumatism (Huang 1999).

Banlangen, the root of *Isatis tinctoria L.*, is a common TCM product used to prevent and treated influenza. TCM herbalists believe that Banlangen can clear away heat and toxic material and remove pathogenic heat from blood. Pharmacological study showed that Banlangen had a very strong anti-viral and anti-microbial agent, and can be used to treated disease such as influenza, encephalitis, pneumonia, hepatitis and erysipelas (Chen et al. 2006). Polysaccharides of Banlangen were reported have anti-endotoxin activities and can also enhance the immune system (Jing et al. 2007).

Ge gen is the root of *Pueraria lobata (Willd.) Ohwi*, which is also known as Pueraria Root or Kudzu root in North America and Europe. Ge gen extract can increase the blood flow in coronary and cerebral (Yue and Hu 1996) and have hypoglycemic, anti-fever and spasmolysis effects (Hao et al. 2006), it can also use as an anti-dipsotropic agents (Wing and Bert 1998).

Chai Hu, root of *Bupleurum Chinense DC* or *Bupleurum Scorzonerifolium Willd* is a TCM product with a usage of nearly 3000 years. Some other species such as the roots of *B. falcatum*, *B. bicaule* and *B. marginatum* var. have been also found in TCM practice and have similar pharmaceutical activity (Tian et al. 2009). Clinical study has shown that Chai hu can relieve fever, cure cough, enhance the immune system and also have anti-

viral, anti-bacterial, anti-inflammation and sedative activities (Zhengping 2007). Animal studies found that saikosapins and bupleurumol can decrease the blood fat, cholesterol, glycerin tripalmitate and phospholipid (Zhengping 2007). Chai hu products are usually used to treated common cold, cough, infectious hepatitis, liver cirrhosis, hyperlipidemia, erythema and globus hystericus (Huang 1999).

Fu Shen is the root of a Pinaceae family plant surrounded by *Poria cocos* (Schw.) Wolf (a fungus from the Polyporaceae family). Fu Shen has sedation and diuresis properties. The common use of Fu Shen is to treated insomnia (Huang 1999).

Dang shen is the root of *Codonopsis pilosula* (Franch.) Nannf, and belongs to the Campanulaceae family. It is mainly used as a nutritious medicine. Clinical and animal studies showed that Dang shen can suppress peristole and enterokinesia, and consequently heals stomach dysfunction and intestine peristalsis (Jiao 2005). Dang shen has multiple beneficial activities on the cardiovascular system including cardiogenic, anti-shock, two-way regulation of blood pressure and improving the coronary blood flow. Additionally, Dang shen can also enhance the immune system, improve hematopoiesis, improve the memory, relieve the symptoms of gastric ulcer and also have sedative effect (Jiao 2005; Huang 1999).

The modernization of TCMs started at the late 20th century in order to provide a simple and efficient way of TCM administration. Many TCM materials are now processed by modern pharmaceutical preparation technologies. These products are widely called “Near

Modern Chinese Herbal Drugs” and are usually available in capsules, infusions, tablets and oral liquid products (Chinese Pharmacopoeia 2010). Hence, many of the complex multi-component and rarely prepared TCM have become widely available. Most selected TCM products, except Banlangen (NRP267), have been reported to have nutritional or therapeutic effects for chronic disease, which suggest that long term treatment with these products would be common. Thus, they may have an increased risk of triggering NHP-drug interactions.

The TCM products have been categorized into two groups depend on their availability in Canada. Seven TCM infusions are rarely found in the market and are most likely to be used by the Asian population; they were considered lower risk because of lower uses and they were only tested with a small scale of major DMEs (CYP3A4 and CYP2D6). Goji products are widely available in the market with different form such as dried/fresh berry, juice or ingredient of other NHPs. They were considered more important as they had greater chance of being taken with drugs. They were tested with a large scale of major DMEs.

Table 7. List of Traditional Chinese Medicine products examined in this study.

Code	Spice Name	Common name	Chinese Name	Family	Organ	Products form
NRP265	<i>Dendranthema morifolium</i> (Ramat.) Tzvel	Chrysanthemum Flower	菊花	Asteraceae	Flower	Infusions
NRP266	<i>Pueraria lobata</i> (Willd.) Ohwi	Ge Gen	葛根	Fabaceae	Root	Infusions
NRP267	<i>Isatis tinctoria</i> L	Banlangen	板蓝根	Brassicaceae	Root	Infusions
NRP268	<i>Angelica pubescens Maximowicz</i>	Du Huo	独活	Apiaceae	Root	Infusions
NRP269	<i>Bupleurum Chinense</i> DC or <i>Bupleurum Scorzonerifolium</i> Willd	Chai Hu	柴胡	Apiaceae	Root	Infusions
NRP270	<i>Poria cocos</i> (Schw.) Wolf	Fu Shen	茯神	Polyporaceae	Root with fungi	Infusions
NRP271	<i>Codonopsis pilosula</i> (Franch.) Nannf	Dang Shen	党参	Campanulaceae	Root	Infusions
NRP272	<i>Lycium barbarum</i> .L	Goji	枸杞子	Solanaceae	Fruit	Fresh/dry berries

1.7 Rational, Hypotheses and Objective

1.7.1 Rational

NHPs contain a large number of different constituents that could be substrates, inhibitors, and/or inducers which modulate the activity of DMEs. The modulation of the DME system might significantly change the pharmacokinetic profile of many drugs. The increase or decrease of drug plasma concentration could lead to adverse reactions or therapeutic failure. Understanding the interaction between NHPs and DMEs may provide information for health care providers to identify potential NHP drug interactions which are a threat to human health. These studies may also reveal information about NHP metabolism which would be valuable for learning their mechanisms of action and how to enhance their effectiveness, as well as clarify any potential toxicity. Furthermore, NHP constituents may also interact with some endogenous chemicals since DMEs are involved in the metabolism and biosynthesis of a number of endogenous substrates. This may be helpful to understand some of the NHPs' beneficial effects.

1.7.2 Hypothesis

Cree traditional medicine and other traditional Chinese medicines may inhibit the activity of selected DMEs.

1.7.3 Objectives

In order to test the hypothesis, the objectives of this study were:

- 1). Examine if the selected natural health products (NHP) can affect the activity of major drug metabolism enzymes.
- 2). Examine if there is CYP3A4 mechanism-based inactivation (MBI).
- 3). Examine if selected NHPs can affect the bio-activation of oseltamivir (Tamiflu) in a human liver microsome system.

2 Materials and Methods

2.1 Chemicals and Reagents

HPLC grade methanol (MeOH), acetonitrile (ACN), ethyl acetate (EtOAc) and 95% ethanol (EtOH) were purchased from Fisher Scientific (Ottawa, ON). Testosterone and 6- β -hydroxytestosterone were purchased from Steraloids Inc. (Newport, RI, U.S.A.). 3-cyano-7-ethoxycoumarin (CEC), 7-methoxy-4-(trifluoromethyl)-coumarin (MFC), dibenzylfluorescein (DBF), 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin iodide (AMMC), and microsomes derived from Baculovirus infected insect cells expressing CYP450s, FMO3 with CYP-reductase and/or cytochrome b₅ and 50-pooled mixed gender human liver microsomes (HLM) were purchased from BD Biosciences (Mississauga, ON). Trifluoroacetic acid (TFA), methyl *p*-tolyl sulfide (MpTS), methyl *p*-tolyl sulfoxide (MpTSO), methyl *p*-tolyl sulfone (MpTSO₂) 1-pyrenyldiazomethane (PDAM), 98% lauric acid, terfenadine and β -nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) were purchased from Sigma-Aldrich (Oakville, On). Furaflavine, tranylcypropramine, sulphaphenazole, quinidine, diethyldithiocarbamate, bifonazole, and methimazole were purchased from Sigma-Aldrich (Oakville, ON). Ketoconazole was purchased from Calbiochem (Gibbstown, NJ, USA).

2.2 Sample Preparation

2.2.1 Cree plant material

The 17 anti-diabetic plants examined in this study were harvested with the help of elders and healers from the CEI in northern Quebec. All have been treated with respect throughout these studies. Plants were identified by Dr. A. Cuerrier and voucher specimens were deposited at the Marie-Victorin herbarium at the Montreal Botanic garden, Montreal, Quebec, Canada. Nine of the 17 plants were collected in Mistissini and given an accession code “AD” plus a number. AD represents “Anti Diabetic” (Spoor et al. 2006). The remaining 8 plants were collected in Whapmagoostui which were given an accession code “W” plus a number. W represents the source region “Whapmagoostui” (Despina et al. 2009). Air-dried samples were washed and separated by organ parts. The appropriate part was ground using a Wiley Mill with a 2 mm filter (A.H. Thomas Co., Swedesboro, N.J., USA) and extracted twice with 80% ethanol (10 mL/g) for 24 hr. The extracts were combined, filtered and reduced to dryness through rotary evaporation and then lyophilization (16). Dried ethanol extract was stored at -20°C. The plant extracts were solubilized in 95% ethanol or 100% MeOH for testing and used within 2 weeks.

2.2.2 Preparation AD01 and W1 tea sample for CYP3A4 inhibition study

AD01 and W1 Tea were prepared from dried plant leaves. One gram of dried material was added to 250 ml of boiling water. The water was maintained at a

boiling temperature for 5 or 30 min, the volume of tea was adjusted to 250 ml by adding water.

2.2.3 Preparation of AD01, W1 and W4 tea sample for CES1 inhibition study

Traditional Labrador tea (AD01) northern Labrador tea (W1) and Juniper tea (W4) was prepared by adding 1 gm of dried material to 250 mL of boiling water. The water was maintained at a boiling temperature and aliquots of the tea were collected after 20 or 60 min of boiling.

2.2.4 TCM infusions

Chrysanthemum Flower, Ge Gen, Banlangen, Fu Shen and Dang Shen were manufactured by Sanjiu Medical and Pharmaceutical Co (P. R. China), Du Huo and Chai Hu were manufactured by Guangdong Yifang Pharmaceutical Company (P. R. China) All were purchased in Ottawa and tested before their expiry date. TCM material came in sachets already in usable, ground form. Water/MeOH extracts were prepared by vigorously vortexing certain amount (5 mg, 20 mg, and 25 mg for water extraction and 100 mg for MeOH extraction) of ground material in 1 mL of the solvent for 1 min and then sonicated for 10 min. The extract was separated from the undissolved material by centrifugation for 15 min at 13.4*1000 g at room temperature. MeOH extract was 1:4 diluted by water before test. All solutions were freshly prepared before use.

2.2.5 Preparation of Goji extract for CYP inhibition study

Three Goji products were obtained from different sources: Dry Goji berry (DGB) was sun dried Goji cultivated in NingXia province, China, the major producing region of Goji was purchased commercially in Beijing, China; fresh Goji berries (FGB), a generous gift from Mrs. Rany Tang were privately cultivated in Ottawa, Canada; and 100% Himalaya Goji juice (HGJ, the source material was from NingXia province, China) was purchased from Nutrition house, Ottawa, Canada.

Samples were prepared by a simple extraction method based on the traditional or non-traditional Goji consumption patterns. FGB and DGB were crushed into powder after they were freeze dried.

Cold water extract: 50 mg powder sample was dissolved in 1 mL water then vortexed and extracted by shaking for 20 min then centrifuged for 10 min at 12000 rpm, supernatant fluid was collected for test.

Hot water extract: 50 mg powder sample was boiled in 1 mL water for 20 min and then centrifuged for 10 min at 12000 rpm, supernatant fluid was collected for test.

80% ethanol extract: 200 mg powder sample was dissolved in 1 mL 80% ethanol, then sonicated for 20 min and then centrifuged for 10 min at 12000 rpm, the supernatant was then 1:4 diluted by water before test.

Fresh Goji juice (FGJ) was also made from FGB by using a simple home use juice extractor.

2.2.6 Preparation of Goji extract for rat cardiac CYP2J2/3 study

The dried Goji was freeze dried and crushed into powder. Ground plant material was extracted twice in 5 mL 80% EtOH per gram dried material on a shaker at room temperature overnight. The supernatant was then filtered and then evaporated by using a rotor evaporator. Dried extract was collected and stored at 4°C until tested.

2.2.7 Preparation of Goji for HPLC fingerprint study

Freeze dried FGB and DGB powder (2.5 g for each) were macerated in 5 mL of water for 20 min and then transferred to a 50 mL tube. The volume was adjusted to 20 mL by adding water and then extracted for 10 min by sonication. The residue was collected and air-dried. The dried plant residue was pulverized and then 25 mL of ethyl acetate or 45 mL of MeOH was added and sonicated for 20 min, this process was repeated three times, the solution were combined and then filtered. The filtrate was dried under reduced pressure. The resulting residue was dissolved in 6 mL of methanol; this solution was then filtered through 0.2 µm filters for use in HPLC analysis.

2.2.8 Concentration selection for enzyme inhibition study

Since the enzyme inhibition study is a high throughput screen study, the concentration of extract need to fulfill the following term. Cree anti-diabetic plant

extract were standardized ethanolic extract, all extract will be made in the same stock concentration which the extract will be fully dissolved, and the percentage inhibition range need to be in the range from 0% to 95%. With these conditions, the inhibitory potential of different sample will be easy to compare. The concentration selection of TCM samples and several Cree plants which use in tea study are mainly base on the traditional usage.

2.3 Cytochrome P450 Inhibition Studies

2.3.1 Microtitre fluorometric assay

A microtitre fluorometric assay was used to assess the inhibitory capacity of plant extracts against 8 recombinant CYP isoforms: 2C9 2C19 2D6*1 2D6*10, 3A4, 3A5, 3A7, and 19. The procedure used was adapted and modified from Scott et al. (2006), and Ghosal et al. (2003). The assays were performed in 96-well plates with white walls and clear, flat bottoms under red-coloured light to minimize the exposure of fluorescent light to photosensitive material. The fluorescence was measured using a Cytofluor 4000 Fluorescence Measurement System (Applied Biosystems, Foster City, CA). The percent inhibition of each extract was calculated relative to the CYP activity with the MeOH control. The assayed samples were diluted ten-fold with water and the final in-well MeOH concentration was 0.5%. A final concentration of 10 µg/mL of extract was tested for all assays and tested in triplicate per assay. All diluted extracts in water were freshly made on experimental days and the remainder

discarded.

DBF was used as substrate for 3A4, 3A5, 3A7 and 19; CEC was used as substrate for 2C9; MFC was used as substrate 2C19; and AMMC was used as substrate for CYP2D6*1 and *10. All substrate were dissolved in ACN. The positive inhibitors, tranylcypramine (2C19), ketoconazole (3A4, 3A5, and 3A7), sulphaphenazole (2C9), quinidine (2D6), and bifenazole (19) were dissolved in MeOH.

Wells were designated as “control,” “control blank,” “sample,” or “sample blank.”

The control represented the MeOH vehicle control, whereas the sample represented the extract or positive control. Solution A contained 1.08 mM NADPH, and the substrate in 0.25 M potassium phosphate buffer solution (buffer, pH 7.4). Solution B contained the CYP in 0.13 M buffer solution. Solution C was identical to Solution B but instead contained heat denatured CYP rather than active enzyme (“blank”). A volume of 100 μ L of Solution A was added to each well followed by the addition of 10 μ L of the extract. Enzyme was thawed prior to its addition to Solution B or C which were then immediately aliquoted into the wells at a volume of 90 μ L. The total volume per well was 200 μ L. Then the plate was shaken for three seconds, and the initial fluorescence (T_{initial}) was measured. The plate was incubated at 37°C for 20 to 60 min and then final fluorescence (T_{final}) was measured. The following formula was used to determine percentage inhibition:

$$\left[1 - \frac{((Sample_{T_{final}} - Sample_{T_{initial}}) - (Sample\ Blank_{T_{final}} - Sample\ Blank_{T_{initial}}))}{((Control_{T_{final}} - Control_{T_{initial}}) - (Control\ Blank_{T_{final}} - Control\ Blank_{T_{initial}}))} \right] > 100\%$$

2.3.2 Testosterone hydroxylation assay

Microtitre fluorometric assay have many advantages such as fast and easy to operate.

However, this assay may be strongly interfered when tested extract have similar fluorescent absorption. In addition to the plate reader assay, a HPLC method with testosterone as an alternative substrate was used to further explore the potential effect of these products on the metabolic capability of recombinant P450 3A4/5 due to the importance of CYP3A subfamily. NHPs were incubated with 0.1 mM CYP3A4 (or CYP3A5), 35 μ M testosterone, 2.7 mM NADPH and 3.3 mM magnesium chloride ($MgCl_2$) in 0.125 M buffer (total volume of 200 μ L) for 30 min at 37°C. The reaction was stopped by adding 75 μ L of cold ACN. The mixture was centrifuged at 13.4*1000 g for 15 min and filtered with PTFE filters (0.45 μ m pore; Chromatographic Specialties Inc, Brockville, Canada). A volume of 20 μ L of the reaction mixture was injected into a Supelco LC-BD C18 column (5 μ m practical size 250 mm x 4.6 mm; Supelco, Ottawa, Canada) and analyzed using an Agilent 1100 Series HPLC system with a diode array detector (DAD). Testosterone and its metabolite, 6- β -hydroxy testosterone, were separated by a gradient eluted method with an initial 35:65 (v/v) ratio of ACN and water and a gradient change of 65:35, for 10 min at a flow rate of 1 mL/min, with a column temperature of 37°C and DAD set at 245 nm. Testosterone and its 6- β -hydroxyl metabolite eluted at 9.5 and 3.8 min respectively. Ketoconazole was used as a positive control.

Different groups has been set as “control”, “blank control”, “sample blank” and “sample”. Control represented the MeOH vehicle control and sample represented the extract or positive control. Denatured enzyme was used in blank control group instead of active enzyme to confirm there were no other constituent in the incubation matrix involved in the formation of the target metabolite. Sample blank group only contains extract (sample or positive control), buffer, NADPH and substrate (testosterone), this group was set to conform that extract cannot interact with substrate directly and no compounds form extract have a similar retention time as the target metabolite. Blank control and sample blank was only tested in the case to make sure the assay won't be interfered by anything from the incubation matrix and will not be used in the calculation of percentage inhibition. The following formula was used to determine % inhibition:

$$\{1 - [\text{Metabolite peak area}_{(\text{sample})} / \text{Metabolite peak area}_{(\text{control})}]\} * 100\%$$

This test and calculation method was used in all the enzyme inhibition study which requires HPLC-UV as detector.

2.3.3 Lauric acid ω -hydroxylation assay

The effects of NHPs on CYP4A11 catalyzed activity was identified by a lauric-acid ω -hydroxylation assay (Amet et al. 1996). Samples were incubated with 0.8 mM recombinant CYP4A11, 35 μ M lauric acid 2 mM NADPH and 3.3 mM magnesium chloride in 0.125 M Tris buffer at pH 7.5 with a total volume of 200 μ L. The

incubation was continued for 30 min at 37°C, and stop by adding 500 µL cold ethyl acetate. The mixture was extracted by 500 µL ethyl acetate twice and organic phases were combined and evaporated. The dried extract was resuspended by 100 µL fresh made 1mg/mL PDAM in ethyl acetate and derivatized at 25°C overnight. The mixture was centrifuged and filtered before HPLC injection. An Agilent 1100 Series HPLC-DAD was used to monitor the reaction. A 30 µL aliquots of reaction mixture was injected into a Supelco LC-BD C18 column (5 µm practical size 250 mM x4.6 mm; Supelco, Ottawa, ON, CA), lauric acid and its metabolite was separated by a gradient eluted method initial with a ratio of ACN-water at ACN-methanol-water at 40:60 (v/v) and gradient change to 100% ACN in 20 min with a flow rate at 1 mL/min, column temperature was set at 45°C and the DAD was set at 245 nm. Water was used as the volume control of water extract and juice sample and 20% ethanol was used as the volume control of ethanol extract. The following formula was used to determine % inhibition:

$$\{1 - [\text{Metabolite peak area}_{(\text{sample})} / \text{Metabolite peak area}_{(\text{control})}]\} * 100\%$$

2.3.4 Terfenadine hydroxylation assay

Terfenadine hydroxylation assay was performed in 100 mM potassium phosphate buffer (pH 7.4) containing 2.7 mM NADPH 3.3 mM MgCl₂, 0.1mM recombination human CYP2J2 and 0.1 mM terfenadine in a final volume of 0.2 mL. Incubations were carried out for 20 min at 37°C. Incubations were terminated with 50 µL of ACN. Incubations were centrifuged at 13.4*1000 g for three min and supernatants

were removed for HPLC analysis. The HPLC system was an Agilent 1100 with DAD detector. A Supelco C-18, (5 μ , 4.6 x 250 mm, and 5 μ m practical size) was used for the analysis; the column temperature was constant at 45°C. The alcohol metabolite of terfenadine was separated using HPLC mobile phases consisting of 0.1% TFA in H₂O (Solvent A), and 0.1% TFA in ACN (Solvent B). Initial HPLC solvent conditions were 90% A and 10% B, then linear increase to 100% B in 16 min; column was washed by 100% B for 5 min before returning to initial conditions. The flow rate was set at 1 mL/min. DAD was set at 212 nm. The retention time of hydroxy terfenadine was around 11.2 min and terfenadine was at 13.6 min. The following formula was used to determine % activity of CYP2J2:

$$[\text{Metabolite peak area}_{(\text{sample})}/\text{Metabolite peak area}_{(\text{control})}] * 100\%$$

2.3.5 Mechanism-based inactivation study

The mechanism-based inhibition was determined by time-dependent CYP3A4 inhibition assay and a NADPH dependent inhibition assay; both DBF and testosterone were used as substrate in these two assays. Briefly, the time-dependent inhibition CYP3A4 inhibition assay pre-incubated the sample with enzyme and NADPH in PBS buffer without substrate for different ranges of time and then the pre-incubation mixture was moved to the reaction mixture which contains substrate. The reaction was continued for a set period of time. For the fluorometric microtitre plate method (DBF as substrate): 10 μ L/mL Goji juice was pre-incubated with 1.08 mM NADPH, 0.25 μ M EDTA 100 pmol/ μ L CYP3A4 at 37°C in 140 mM PBS

buffer. After 1 min, 5 min and 10 min 20 μL pre-incubation mixture was added to a 180 μL reaction mixture containing 0.6 mM NADPH, 0.13 M PBS and 1 μM DBF, then incubated for another 20min. The reaction was real time monitored by a Cytofluor Series 4000 Multiwell Plate Reader with excitation/emission set at 460 nm/530 nm. For the Testosterone hydroxylation method: Sample (10 $\mu\text{L}/\text{mL}$ for Goji juice or 20 $\mu\text{g}/\text{ml}$ for Cree plant extracts) was pre-incubated with 0.8 mM NADPH and 50 nM CYP3A4 at 37°C in 0.16 mM PBS buffer. After 1 min, 5 min and 10 min, 40 μL pre-incubation mixture was added to 160 μL reaction mixture containing 4 mM NADPH, 0.15 M PBS, 3.3 mM MgCl_2 and 35 μM testosterone, then incubated for another 20 min, the reaction mixture was extracted twice with 500 μL cold ethyl acetate, the organic phases were combined and evaporated, dry extracts were resuspended by 120 μL 70% ACN and filtered before HPLC analysis.

In the NADPH dependent CYP3A4 inhibition assay, extracts were pre-incubated the with enzyme in PBS buffer for 10 min with or without the presence of NADPH, the assay condition and composition of pre-incubation and reaction mixture were the same as the time dependent CYP3A4 inhibition assay except the absence of NADPH in the comparison group. The reaction was monitored by Cytofluor plate reader or HPLC-DAD.

H_2O was used as volume control for Goji juice; ketoconazole, was used as negative control; goldenseal, a known CYP3A4 MBI inhibitor, was used as positive control

(Chatterjee and Franklin 2003). All samples (including positive and negative control) have been tested in triplicate and the test repeated at least once.

2.4 Flavin-containing Monooxygenase-3Inhibition Assay

The effect of Cree plant extracts on FMO3 catalyzing activity was characterized by using an S-oxygenation assay. Cree extracts (400 µg/mL) were incubated with 0.2 mg/mL FMO3, 3 mM MpTS, 1.44 mM NADPH and 3.3 mM MgCl₂ in 50 mM glycine buffer (pH 6.5) (total volume of 250 µL) for 40 min at 37°C. The reaction was stopped by adding 100 µL of cold ACN. The mixture was centrifuged at 13.4*1000 g and filtered with 0.45 µm PTFE filters. A volume of 20 µL of the reaction mixture was injected into an Agilent Zarbox C8 column (5 µm practical size 150 mm x 4.6 mm; Agilent, Ottawa, ON, CA), and analyzed using an Agilent 1100 Series HPLC system with a DAD. MpTS and its metabolite methyl *p*-tolyl sulfoxide (MpTSO) were separated by a gradient eluted method. From 0 to 2 min, the ratio of ACN–water was stay at 35:65 (v/v), and then changed to 65:35 (v/v) in 4 min. This ratio was maintained for 8 min then returned to the initial condition. System was recalibrated for 5 min before the next injection. The flow rate was of 1 mL/min with column heat to 45°C. The DAD was set at 237 nm. MpTS and MpTSO eluted at 9.3 and 2.9 min respectively. Methanol (10%) was used as the volume control of the extracts, and methimazole (3.5 µM) was used as a positive control. The following formula was used to determine % inhibition:

$$\{1 - [\text{Metabolite peak area}_{(\text{sample})} / \text{Metabolite peak area}_{(\text{control})}]\} * 100\%$$

2.5 Rat Cardiac CYP2J3 activity

2.5.1 Rat H9C2 cell line

H9C2 cell were obtained from Invitrogen (Ottawa, On.) and cultured in Dulbecco's modified Eagle's medium high glucose (Invitrogen, Ottawa, On) supplemented with 10% fetal bovine serum (Gibco, BRL) 2 mM glutamine (Invitrogen), and P.S (Invitrogen), and the incubator was set with 5% CO₂ in water-saturated atmosphere at 37°C. H9C2 cell were cultured in a 75 mL cells culture flask for at least one week under this condition, and then transferred to a 6 well plate with a concentration of 20,000 cells/mL; cells in plate were kept into culture up for 24 hr until confluent.

Goji extracts and terfenadine were dissolved in DMSO, and the final DMSO concentration was less than 0.3% in the medium. Pure DMSO was used as volume control in this study.

The assay was initiated when the cells in 6 well plate reached 100% confluency. The cells were divided into different groups including "blank control"; "control" and "test" which had been treated differently. The blank control only contained 25µM terfenadine, 0.2 mg/mL Goji extract, but no H9C2 cell in the medium. This group was to confirm that there was no chemical reaction with the medium, terfenadine and Goji.

Control group contained H9C2 cell, and 25 μ M terfenadine. Test group contain 25 μ M terfenadine, 0.2 mg/mL Goji extract and H9C2 cells.

Another assay was designed with a 72 hr pre-treatment with Goji extract. In this assay, cells were divided into 5 groups including “blank control”; “pre-incubation control”; “no-pre-incubation control”; “pre-incubation test” and “no-pre-incubation test”. Blank control was as same as mentioned previously. Pre-incubation control was cells pre-incubated with 0.2 mg/mL Goji extract and then incubated with 25 μ M terfenadine. No-pre-incubation control was cells that had been pre-incubated with DMSO and then incubated with 25 μ M terfenadine. Pre-incubation test cells were pre-incubated with 0.2 mg/mL Goji extract and then treated with 0.2 mg/mL Goji extract and 0.025 mM terfenadine together. No-pre-incubation test cells were treated with DMSO and then incubated with 0.2 mg/mL Goji extract and 25 μ M terfenadine together.

All groups were prepared in duplicate; one set was treated with lysis buffer (BD Gentest, Oakville, ON) (0.75 mL/well for 6 well plate and 0.3 mL/well for 24 well plate) at the end of incubation, equal volume of ice cold MeOH was added after most of the cells were lysed. The other set was not treated with lysis buffer, incubation was stopped by adding equal volume of ice cold MeOH.

2.5.2 Neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes (NRC) were kindly donated by Dr. Krantis, QBM

Science, University of Ottawa. NRC were cultured with 5% CO₂ in water-saturated atmosphere at 37°C in 24 well plates at a concentration of 50,000 cells/mL for at least 7 days before assay.

Goji extracts and terfenadine were dissolved in DMSO, and the final DMSO concentration was less than 0.3% in the medium. Pure DMSO was used as volume control in this study.

In the non pre-incubation assay, cells were divided into different groups including “blank control”; “control” and “test” which have been treated differently. The blank control only contained 12.5 µM terfenadine and 0.2 mg/mL Goji extract but no cell in the medium, this group was to confirm that there was no chemical reaction with the medium, terfenadine and Goji; control group contained cells and 12.5 µM terfenadine in medium; the test group contained 12.5 µM terfenadine, 0.2 mg/mL Goji extract and cells in the medium.

In the another assay with a 72 hr pre-treatment with Goji extract, cells were again divided in to 5 groups including “blank control”; “pre-incubation control”; “no-pre-incubation control” ; “pre-incubation test” and “no-pre-incubation test”. Blank control was as same as mentioned previously. In the pre-incubation control, cells were pre-incubated with 0.2 mg/mL Goji extract and then be incubated with 12.5 µM terfenadine. No-pre-incubation control is that cells were pre-incubated with DMSO

(vehicle control) and then incubated and 12.5 μ M terfenadine. In pre-incubation test, cells were pre-incubated with 0.2 mg/mL Goji extract and then incubated with a mixture of 0.2 mg/mL Goji extract and 12.5 μ M terfenadine. In the no-pre-incubation test, cells were pre-incubated with DMSO and then incubated with a mixture of 0.2 mg/mL Goji extract and 12.5 μ M terfenadine.

All groups were prepared in duplicate; one set was treated with lysis buffer (0.75 mL/well for 6 well plate and 0.3 mL/well for 24 well plate) at the end of incubation, equal volume of ice cold MeOH was added after most of the cells were lysed. The other set was not treated with lysis buffer, incubation was stopped by adding equal volume of ice cold MeOH.

2.5.3 Analysis of terfenadine and its metabolite

A Supelco Superclean C18 solid phase extraction tube was used for sample preparation. The tubes were pre-washed by 5 mL of 100% MeOH, 5 mL of H₂O and 5 mL of 0.2 M ammonium acetate respectively, and then 2.5 mL of medium sample was loaded on the tube, and then the tube was washed by 5 mL of H₂O and 2.5 mL of 50% MeOH, and terfenadine and its metabolite were eluted by 5 mL of 100% MeOH. The final 5 mL eluant was collected and dried. The residues were re-dissolving in 0.4 mL of MeOH and were filtered before HPLC injection. The HPLC method for analyze the medium sample was the same method which was mentioned in the CYP2J2 inhibitory assay (see 2.3.4).

2.6 Fingerprint Study of Goji

A volume of 5 μ L MeOH or EtOAc extract of DGB and FGB was injected onto a Waters C18 column (1.5 μ m practical size, 50 mM x2 mm; Waters, Ottawa, ON, CA) with an Agilent 1100 Series HPLC system and DAD. A gradient elution method was developed for the fingerprint study. From 0 to 5 min, the ratio of ACN–water was stay at 5:95 (v/v), and then changed to 95:5 (v/v) in 25 min. This ratio was maintained for 1 min then returned to the initial ratio in 5 min. The flow rate was of 1 mL/min with column heat to 40°C. The DAD was set at 210 nm.

2.7 HPLC-DAD analyze of AD01 and W1 tea samples

The tea sample was mixed with the same volume of 99% EtOH and kept in -20°C for 30 min, and then the mixture was centrifuged at 13.4*1000 g for 10 min. This process is in order to remove the polysaccharide from samples which may interfere with analysis.

The mixture was filtered before injected into an Agilent 1100 Series HPLC system with DAD. A volume of 50 μ L was injected into a Phenomenex Luna C18 column (3 μ m practical size 100 mM x 2 mm). A gradient method was used with a initial ratio of MeOH-0.1% TFA at 5:95 (v/v) and gradient change to MeOH-ACN-0.1% TFA at 30:30:40(v/v/v) in 25 min. The column then was washed by 100% ACN for 5min before the next injection. Flow rate was set at 0.3 mL/min and column temperature

was 50°C. Wave length 254nm, 280nm and 350nm have been monitored by DAD.

This HPLC method was developed in collaboration with Dr. Saleem. (Saleem et al. in press (a))

2.8 Human Carboxylesterase 1 Inhibition Study

2.8.1 CES-1 inhibition assay

HLM were thawed in 37°C water bath, and then placed on ice until required. A 5µl sample aliquot of the test extract was incubated in either 50 mM Tris (pH 7.4) with 2 mg/mL HLM and 1.6 mM oseltamivir for 30 min in a 37°C water bath with appropriate vehicle controls. The reaction was stop by 200 µL of cold acetonitrile, the mixture was then centrifuged at 13.4*1000 g for 5 min and the supernatant fluid was filtered through 0.2 micron PTFE disc filters (Chromatographic Specialties Inc, Brockville, ON, Canada) before analysis.

2.8.2 HPLC analysis

An Agilent 1100 Series HPLC-DAD was used to monitor the reaction. A 15 µL extract of reaction mixture was injected into a Supelco LC-BD C18 column (5 µM practical size, 250 mM x 4.6 mm; Supelco, Ottawa, ON, CA) and separated by a gradient eluted method initial with a ratio of acetonitrile-0.1% trifluoroacetic acid at 15:85 (v/v) for 2 min to 60:40 (v/v) at 7 min with a flow rate of 1.2 mL/min, column temperature was set at 35°C and the DAD was set at 230 nm.

2.8.3 HPLC-DAD-APCI-MSD analysis

Analysis was undertaken by injecting 20 μL via an autosampler into an Agilent 1100 series HPLC-DAD-ESI-MSD analytical system (Agilent Technologies, Santa Ana, CA, USA). The system consisted of an autosampler (with a 100 μL built in loop), a quaternary pump (maximum pressure limit of 400 bars), a column thermostat, a photodiode array detector, an ESI-MSD with a mass range of 100-1500 atomic mass unit (amu). Chemstation software (version B.03.01) was used for the acquisition and data analysis. For the analysis authentic oseltamivir and its metabolite were injected as a mixture into the analytical system to determine their separation and retention times followed by 15 μL injection of reaction mixture. The separations were achieved on a Supelco LC-18-BD, column (250 \times 4.6 mm I.D., 5 μ practical size, product number 58355C40, lot number 75251-06 (Supelco, distributed by Sigma-Aldrich, Brockville, ON, Canada) using a mobile phase comprising of A (water +0.1% trifluoroacetic acid, v/v) and B acetonitrile. The mobile phase composition, delivered at 1.2 ml/min, at a column thermostat temperature of 35 $^{\circ}\text{C}$, was 0-2 min 15% B isocratic, 2-7 min 15-40% B. The column was washed for 3 min with 100% B for 3 min and equilibrated at initial composition for 5 min before next injection. The signals were monitored at 230 nm (band width 4, reference off) with DAD with the range of 200-400 nm and peak width of 0.1 min. The mass spectrometric detection of target compounds was achieved with ESI operating in positive ionization mode within a scanning mass range of 100-700 amu. The optimized mass spectrometric conditions were: gas temp 350 $^{\circ}\text{C}$, drying gas (N_2) flow 10 L/min,

nebulizer pressure 50 bar, capillary voltage 3000 V.

3 Results

3.1 Enzyme Inhibitory Study of Cree Plant Extracts

3.1.1 Inhibition of CYP19, 3A4 and 4A11

The results indicate that most of the Cree plant extracts show no or low (lower than 30%) inhibition effect of CYP19 at tested concentration (50 µg/mL). AD07 (AD and W code for Cree plant extracts were listed in table 6) was the strongest inhibitor on CYP19; however it only had a moderate ($46.01 \pm 2.91\%$) inhibitory effect (Figure 1).

Most of the 17 extracts showed inhibitory effects on the CYP3A4-mediated testosterone metabolism ranging from low to high (Figure 2) at tested concentration (50 µg/mL), 7 of them including AD01, AD02, AD03, AD11, AD12, AD13 and W4 showed a strong inhibitory effect (more than 70%). W6, W7, W8 were identified as low inhibitors. W9 was the only Cree plant extract that showed no inhibitory effect on CYP3A4.

Most of Cree plant extracts had no or low inhibitory effect on CYP4A11 under these conditions (Figure 3). AD11 caused the highest inhibitory effect on CYP4A11 ($65.82 \pm 2.56\%$); next to AD11, AD06 ($42.21 \pm 1.33\%$), W5 ($38.77 \pm 2.93\%$) and W8 ($40.61 \pm 4.14\%$). These would be considered as the most inhibitory extracts, but this would only be rated as moderate inhibition effect on CYP4A11.

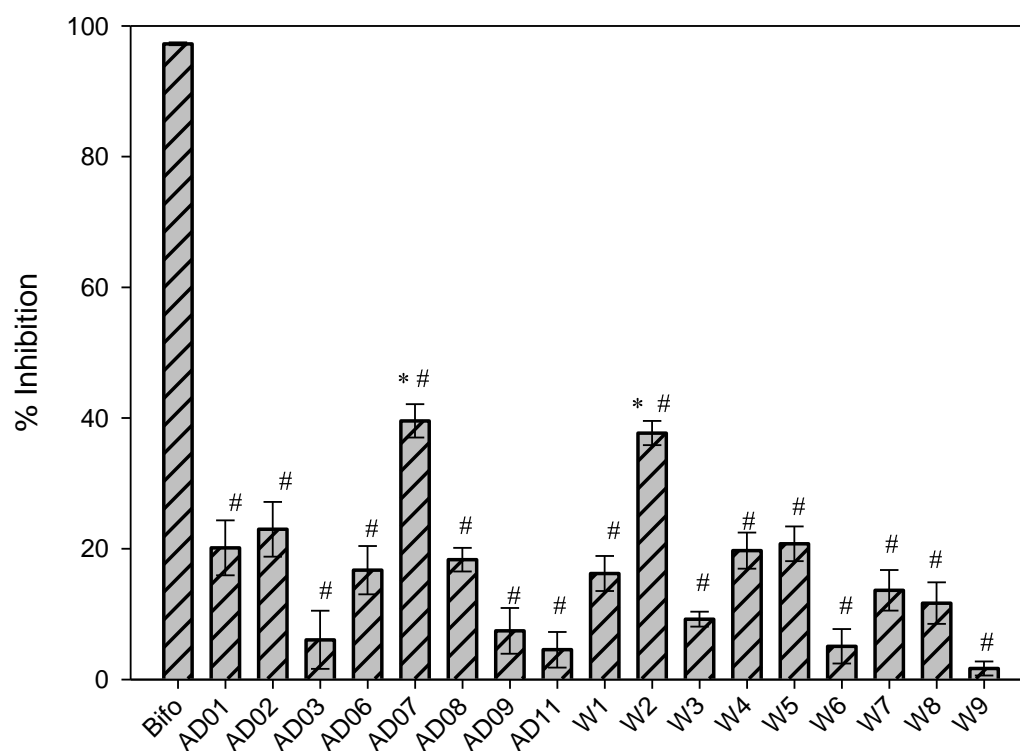


Figure 1. Inhibitory effect of Cree plant extracts on CYP19.

Cree plant extracts (50 µg/mL) were tested for their inhibitory potential on CYP19 by measuring the metabolism rate of substrate. DBF (1 µM) was used as substrate, 0.5 mM bifonazole (Bifo) was used as positive control; n=3, mean ± SEM. A Student *t*-test was used to determine the significance of percentage inhibition using SigmaPlot (Chicago, IL), * $p \leq 0.05$ with respect to vehicle control; # $p \leq 0.05$ with respect to positive control.

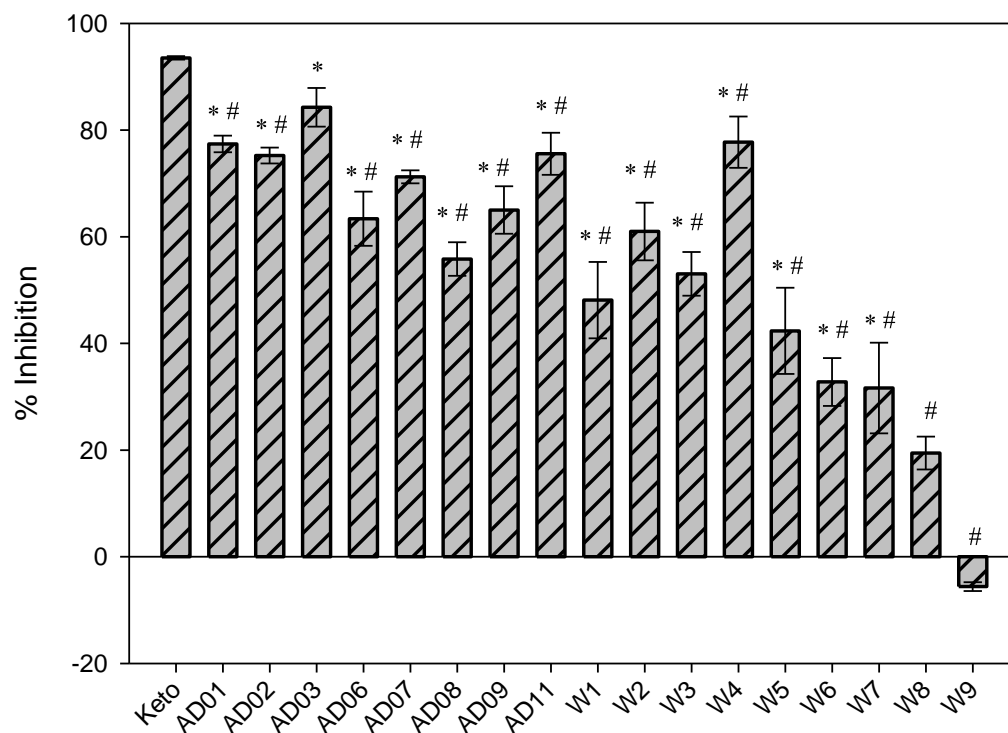


Figure 2. Inhibitory effect of Cree plant extracts on CYP3A4.

Cree plant extracts (50 µg/mL) were tested for their potency of inhibition on CYP3A4 by measuring the metabolism rate of substrate. Testosterone (35 µM) was used as substrate; ketoconazole (Keto, 1 mM) was used as positive control; n=3, mean ± SEM. The negative reading of W9 may due to the system error or fluorescent interference from the extract. A Student *t*-test was used to determine the significance of percentage inhibition using SigmaPlot (Chicago, IL), * $p \leq 0.05$ with respect to vehicle control; # $p \leq 0.05$ with respect to positive control.

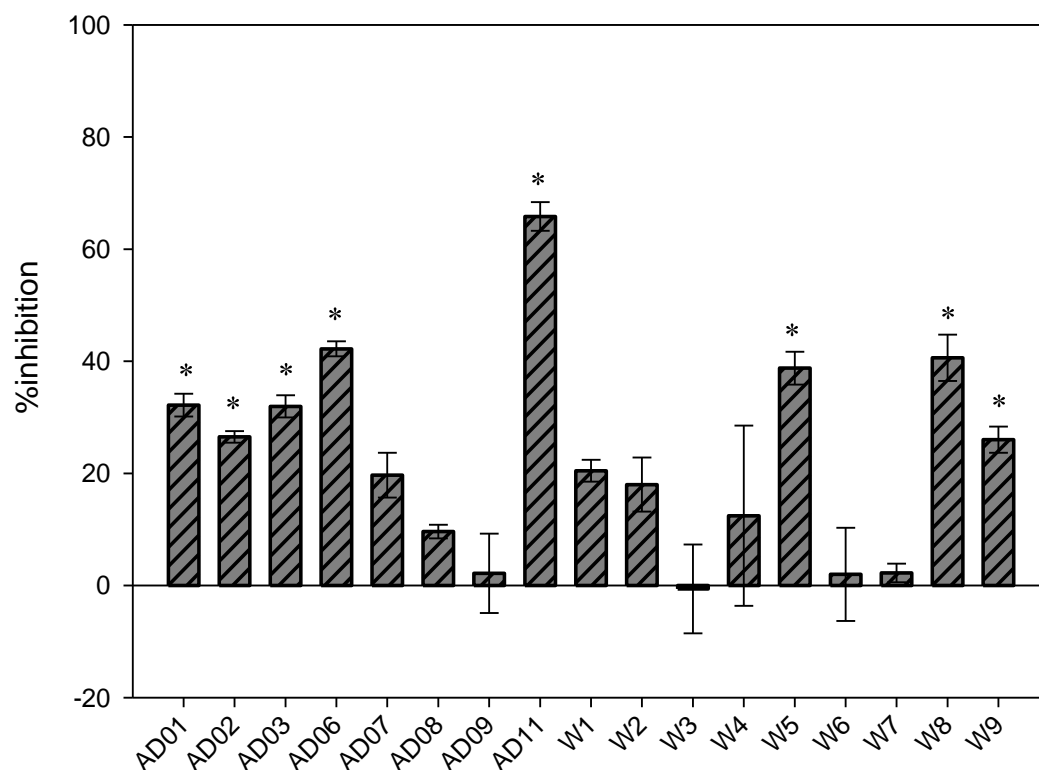


Figure 3. Inhibitory effect of Cree plant extracts on CYP4A11.

Cree plant extracts (50 µg/mL) were tested for their inhibition of CYP4A11 by measuring the metabolism rate of substrate. Lauric acid (35 µM) was used as substrate; n=3, mean ± SEM. A Student *t*-test was used to determine the significance of percentage inhibition using SigmaPlot (Chicago, IL), **p*≤0.05 with respect to vehicle control.

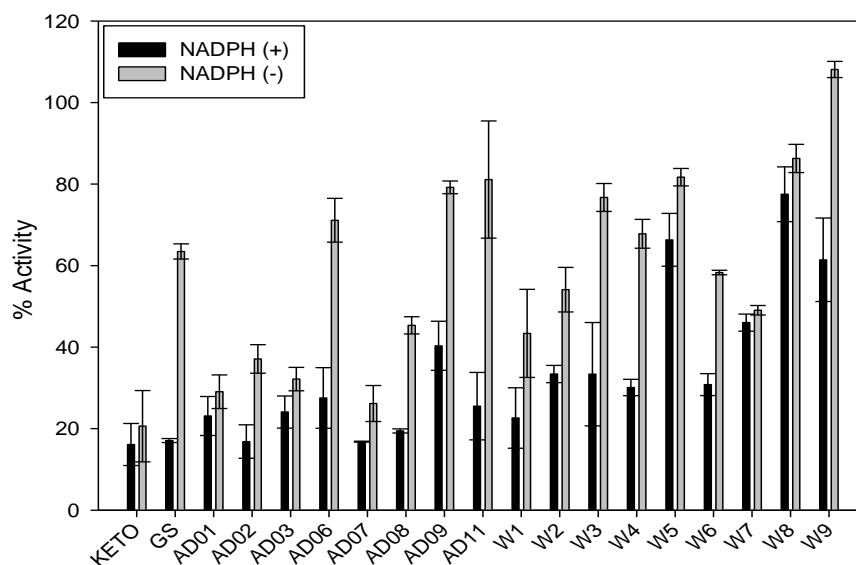
Overall, the inhibitory effects caused by Cree plant extracts are stronger toward CYP3A4 than the other two enzymes; this might be due to the large substrate range of CYP3A4.

3.1.2 CYP3A4 Mechanism-based inactivation

All 17 Cree plant extracts (Table 6) at a concentration of 20 µg/mL were studied for NADPH- and time-dependence, two requirements for MBI. In the NADPH-dependence assay, significant decreases ($p \leq 0.05$) in activity between samples pre-incubated with or without NADPH were observed with goldenseal extract (positive control, 11.5 µg/mL), AD06, AD08, AD09, W4, W6 and W9 (Figure 4A). AD03, AD11, W1, W2 and W3 caused more than a 20% difference of CYP3A4 inhibition between the two pre-incubation conditions, but it was not significant. Ketoconazole did not show NADPH-dependence of CYP3A4 inhibition as expected.

In the time-dependence assay, significant decreases ($p \leq 0.05$) in activity between the 1 and 5 min pre-incubation times were observed with goldenseal extract, AD08, AD09, AD11, W6, and W7 (Figure 4B). Between the 1 and 10 min pre-incubation times, significance was also observed for W2 and the aforementioned species except for AD09, W1 and W3 which caused more than a 25% difference of CYP3A4 inhibition between the 1 and 5 min pre-incubation times, but it was not significant. For the 1 and 10 min pre-incubation times, AD03, AD06, AD09, W1, W2 and W3 showed more than a 20% difference of CYP3A4 inhibition, but it was not significant.

(A) NADPH dependence



(B) Time dependence

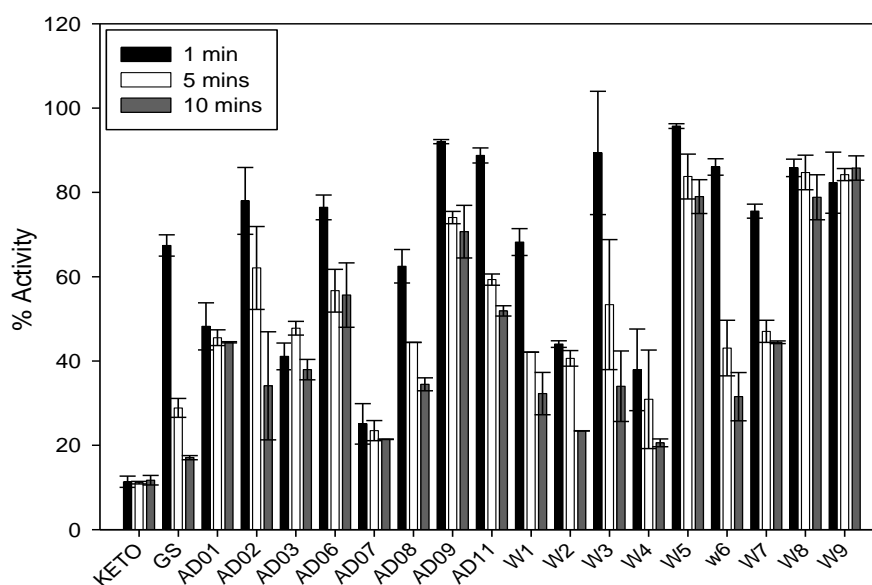


Figure 4. Identifying mechanism-based inactivation of CYP3A4 using testosterone hydroxylation assay.

The activity of CYP3A4 after a pre-incubation with each extract (20 $\mu\text{g/mL}$) (A) in the absence or presence of NADPH for 10 min, and (B) in the presence of NADPH for 1, 5, or 10 min. The results were expressed relative to a 10% MeOH vehicle control, as the mean CYP3A4 activity \pm SD ($n = 2$). Goldenseal (11.5 $\mu\text{g/mL}$) and ketoconazole (0.5 μM) were used as a positive and negative control respectively.

Ketoconazole did not show time-dependence of CYP3A4 inhibition as expected.

Overall, AD08, AD09, and W6 showed significant NADPH- and time-dependence of CYP3A4 inhibition. AD03, AD06, AD11, W1, W2, and W3 showed indications of NADPH- and time-dependence of CYP3A4 inhibition were using testosterone as substrate.

3.1.3 Inhibition of FMO3

Most of the 17 Cree plants extract showed inhibitory effects on the FMO3-mediated metabolism. Four (AD06, W5, AD03, and AD11) showed a strong inhibitory effect, even a very stronger than the positive control (>68.1% inhibition). W3, W7 and W8 showed a very low inhibitory effect, while AD09 and W8 showed no inhibition at all (Figure 5).

3.1.4 CYP3A4 Inhibition study and HPLC-DAD analysis of AD01 and W1 tea samples

Ethanol extracts from ground leaves of AD01&W1 had been examined previously for their ability to inhibit CYP3A4-mediated metabolism (Tam et al. 2009). As boiling is a common preparation method for these medicinal plants, whole leaves were boiled for up to 30 min and aliquots were collected and examined if teas can affect the CYP3A4 activity by using microtitre fluorometric assay. The result indicated a progressive increasing inhibition towards CYP3A4 activity with longer

boiling time (Figure 6). W1 tea was more inhibitory than the AD01; after 5 to 10 min of boiling, it had twice the inhibition than AD01 and inhibited at least $75.3\% \pm 5.5\%$ of CYP3A4 activity. The progressive increase in inhibition was higher with CEI-2 but started to slow after 20 min of boiling; whereas for the other collections the progressive increase was steady and linear. After 30 min of boiling, both tea samples exhibited very strong inhibition (higher than 90% relative to the water vehicle control).

Five marker compounds in the plants (cetachin, epi-catechin, chologenic acid, rutin, quercetin-3O-galactoside) have been identified in both AD01 and W1 in the previous study in Dr. Arnason's lab by using HPLC-DAD-ACPI-MS (Saleem et al. in press (a)). Identification of marker compounds in the tea was obtained by comparison of retention time of known markers in a standard mix and verified by its spectral absorbance. All five marker compounds have been found in both AD01 (Figure 7) and W1 tea (Figure 8) boiled for 30 min. For the tea sample boiled for 5 min, the concentration was much lower and only part of markers compounds were found (Figures 7 and 8).

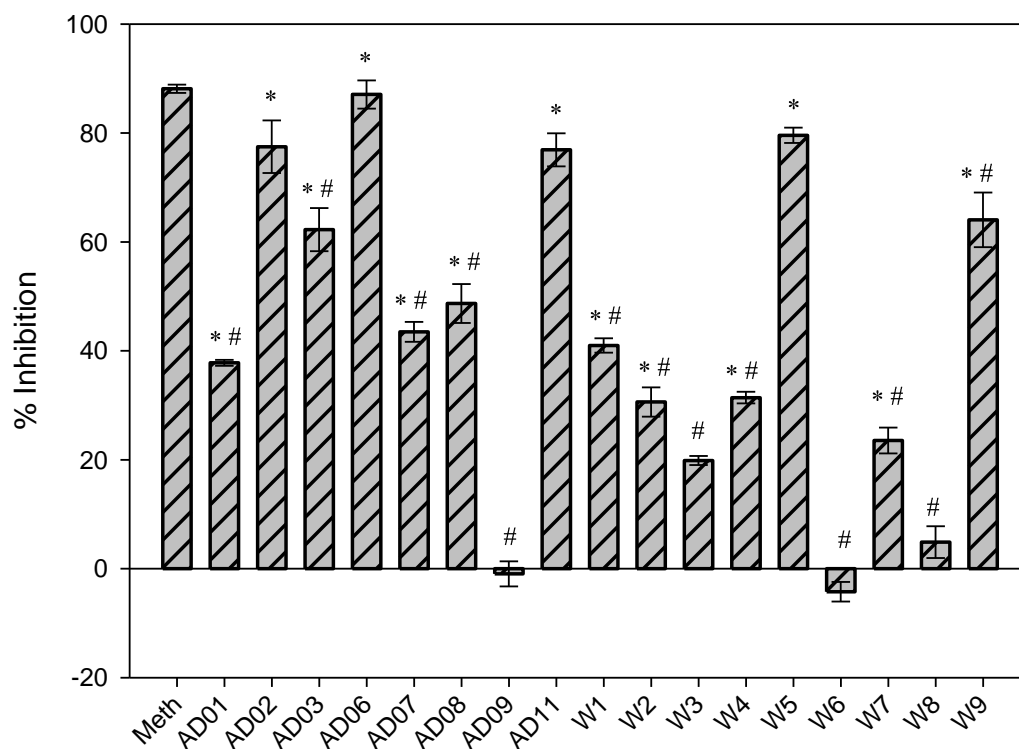


Figure 5. Inhibitory effect of Cree plant extracts on FMO3.

Cree plant extracts (50 $\mu\text{g}/\text{mL}$) were tested for their inhibition on FMO3 by measuring the metabolism rate of substrate. Methyl *p*-tolyl sulfide (3 mM) was used as substrate, 0.5 mM methimazole (Meth) was used as positive control; $n=3$, mean \pm SEM. The negative reading of AD09 and W6 may due to the system error or fluorescent interference from the extract. A Student *t*-test was used to determine the significance of percentage inhibition using SigmaPlot (Chicago, IL), * $p \leq 0.05$ with respect to vehicle control; # $p \leq 0.05$ with respect to positive control.

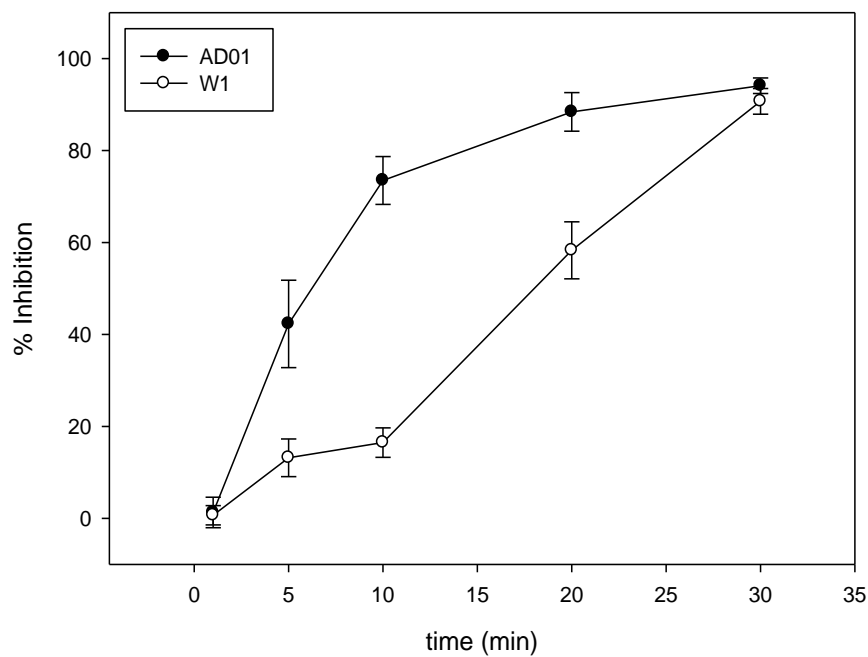


Figure 6. CYP3A4 inhibition of AD01&W1 tea sample boiling for different time
The progressive inhibition on human cytochrome P450 3A4-mediated metabolism by 10 μ L aliquots of a decoction prepared by the extended boiling of whole AD01 and W1 leaves (n = 3; mean \pm SEM).

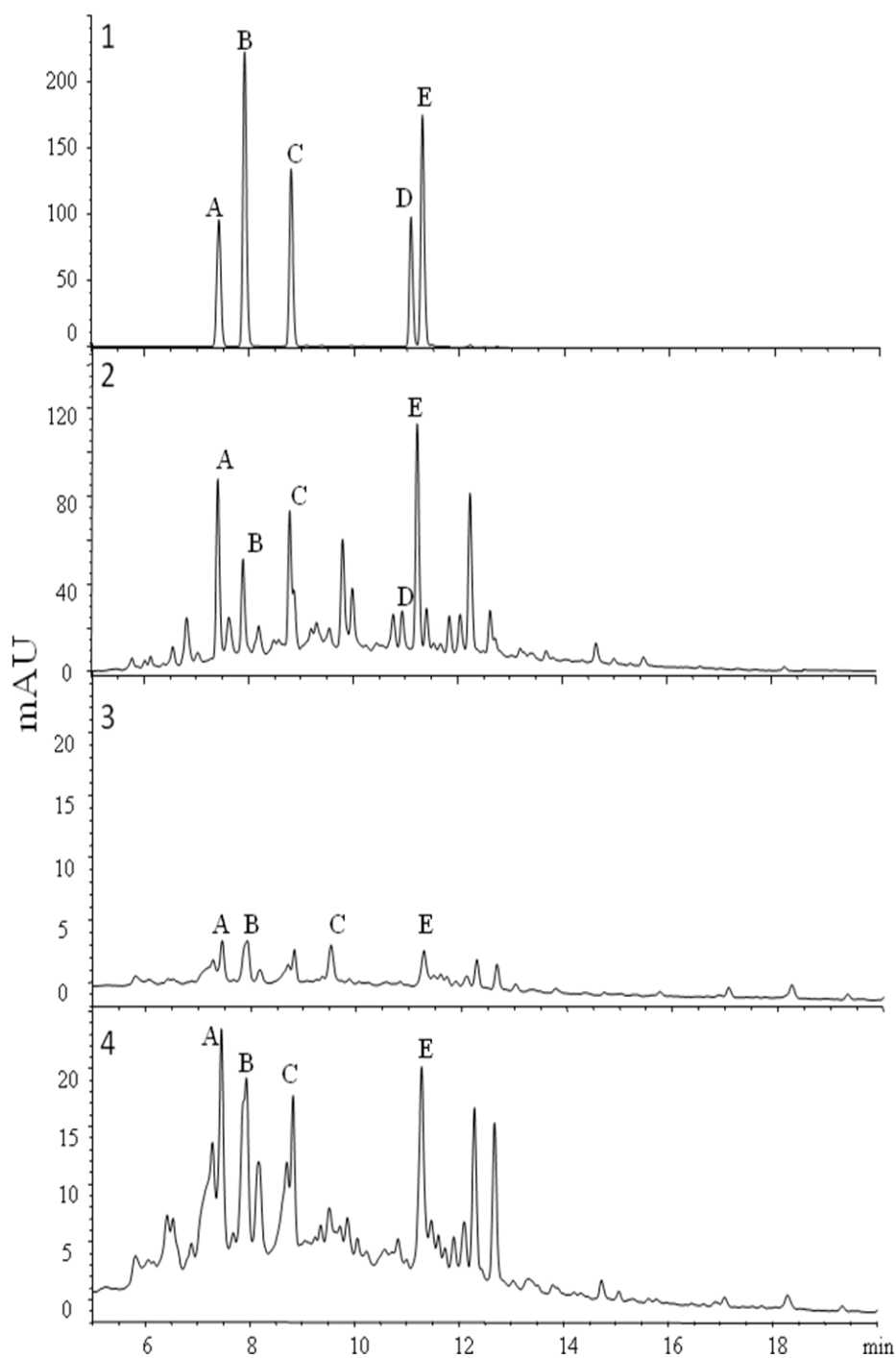


Figure 7. HPLC-DAD analysis of AD01 tea sample

Tea samples were analyzed by Agilent 1100 Series HPLC system with DAD. 1: standard compound mix, A: Catechin; B: Cholorgenic acid; C: Epi-catechin; D: Rutin; E: Qucertin-3O-galactoside. 2: 20 mg/mL AD01 80% EtOH extract. 3: AD01 tea boiling for 5 min. 4: AD01 tea boiling for 30 min. DAD was set at 280 nm.

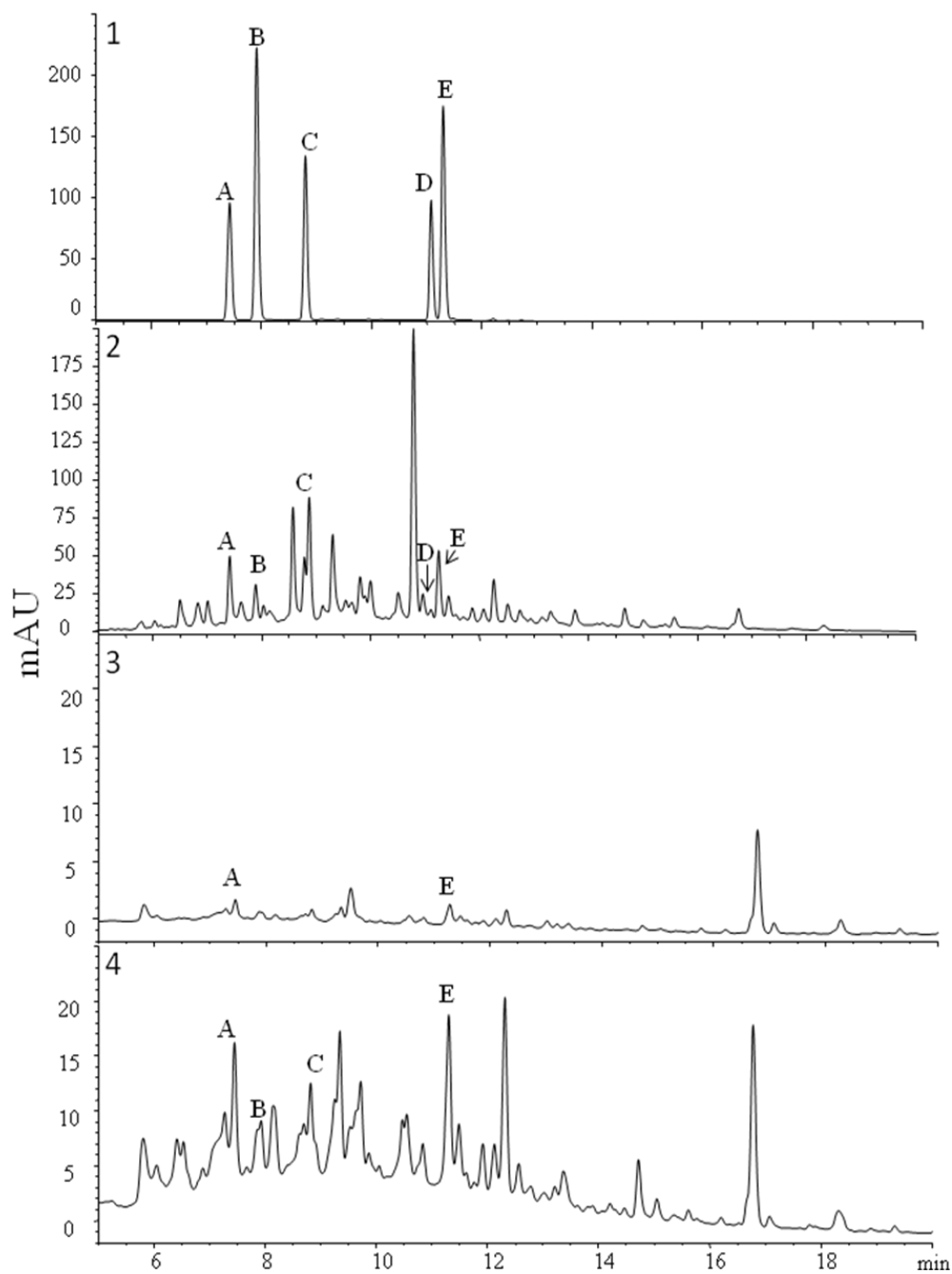


Figure 8. HPLC-DAD analysis of W1 tea sample

Tea samples were analyzed by Agilent 1100 Series HPLC system with DAD. 1: standard compound mix, A: Catechin; B: Chlorogenic acid; C: Epi-catechin; D: Rutin; E: Quercetin-3O-galactoside. 2: 20 mg/mL AD01 80% EtOH extract. 3: W1 tea boiling for 5 min. 4: W1 tea boiling for 30 min. DAD was set at 280 nm.

3.2 Enzyme Inhibitory Study of TCM Infusions

3.2.1 Inhibition of CYP3A4

All TCM infusions (Table 7) aqueous showed inhibitory effect on CYP3A4-mediated metabolism ranged from moderate to strong (Figure 9A) and the inhibition effects were enhanced along with increase of TCM infusion concentration. Aqueous extracts of NRP265, 266, 268, and 269 (NRP code for TCM were listed in Table 7) showed a strong (>70%) inhibitory effect at high concentration (1.25 mg/mL) while they had moderate (31-69%) inhibitory effect in low concentrations (0.25 mg/mL). NRP 267, 270, and 271 showed moderate (31-74%) inhibitory effects in high concentration (1.25 mg/mL) while they had weak or no (0-30%) inhibitory effects in low concentrations (0.25 mg/mL) (Figure 9A). Aqueous extracts of NRP265-269 showed stronger inhibition of CYP3A4 than those methanolic extracts at the same concentrations; however, the methanolic extracts of NRP270 and 271 showed stronger inhibition than their corresponding aqueous extracts (Figure 9B).

3.2.2 CYP2D6 polymorphism study

Both aqueous and methanolic extracts (5 mg/mL) of NRP265 exhibited strong inhibition on both 2D6 *1 and 2D6*10 (Figure 10A and B); while both aqueous and methanolic extracts (5mg/mL) of the other TCM infusions exhibited low or moderate inhibition (>40%) (Figure 10A and B).

A comparison study has been done to determine if the TCM infusions can affect the

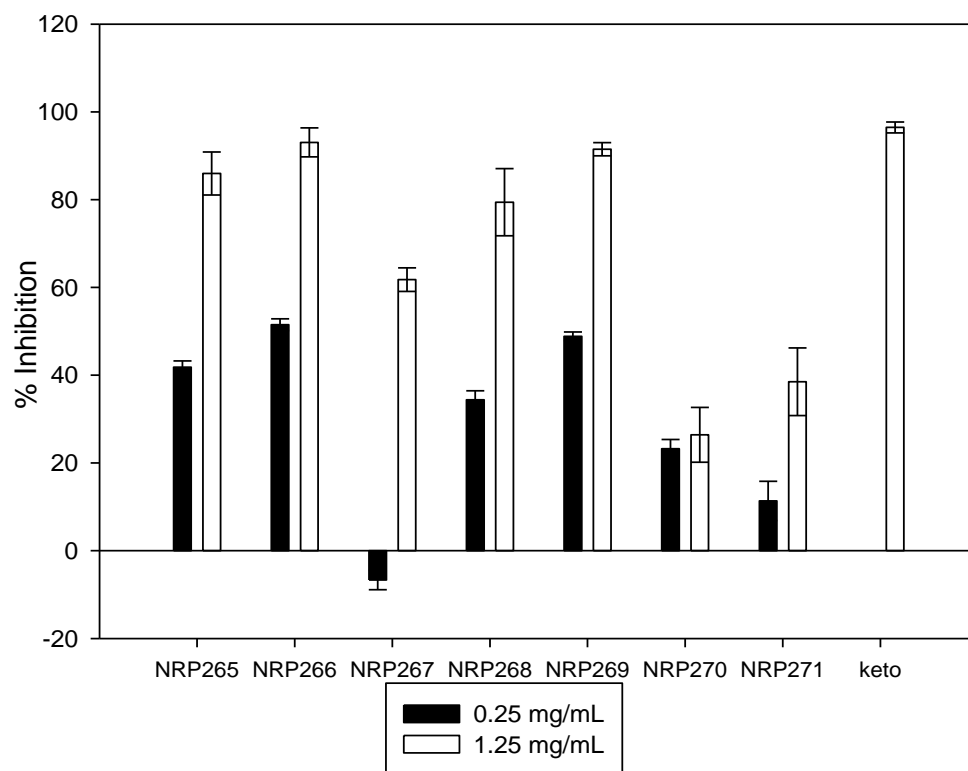


Figure 9A. Inhibitory effect of traditional Chinese medicine infusions aqueous extracts on CYP3A4 with different concentration.

TCM infusions aqueous extracts (0.5 mg/mL or 1.25 mg/mL) were tested for their potency of inhibition on CYP3A4 by measure the metabolism rate of substrate. DBF (1 μ M) was used as substrate, 1.9 μ M ketoconazole (keto) was used as positive control; n=3, mean \pm SEM.

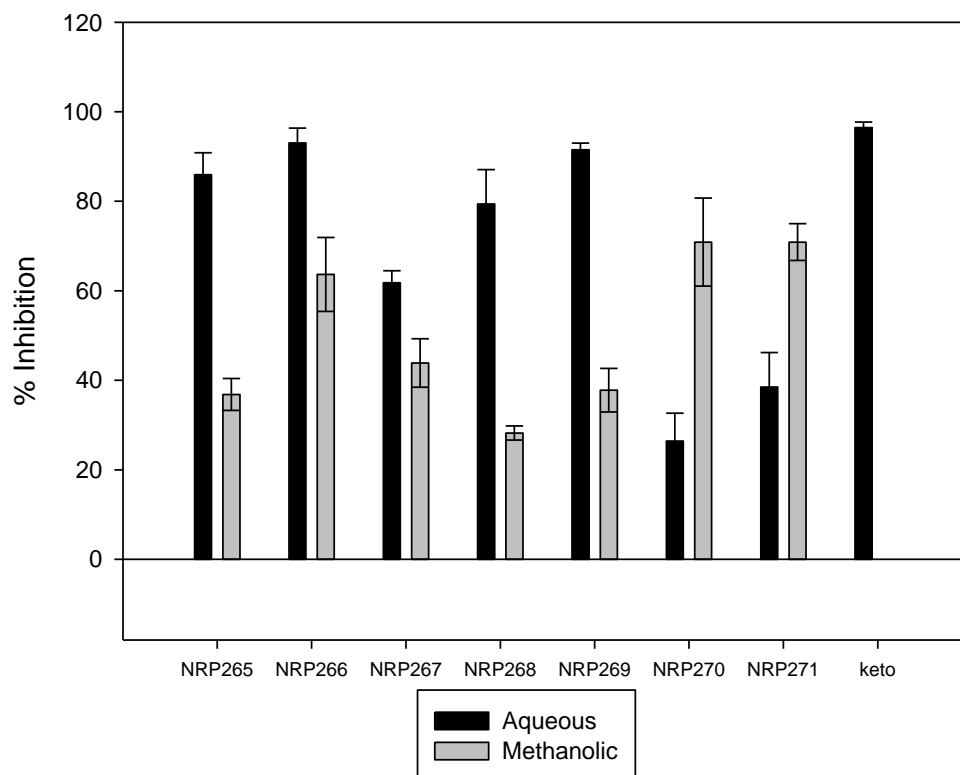


Figure 9B. Inhibitory effect of traditional Chinese medicine infusions aqueous and methanolic extracts on CYP3A4.

TCM infusions aqueous and methanolic extracts (1.25 mg/mL) were tested for their potency of inhibition of CYP3A4 by measuring the metabolism rate of substrate. DBF (1 μ M) was used as substrate, 1.9 μ M ketoconazole (keto) was used as positive control; n=3, mean \pm SEM.

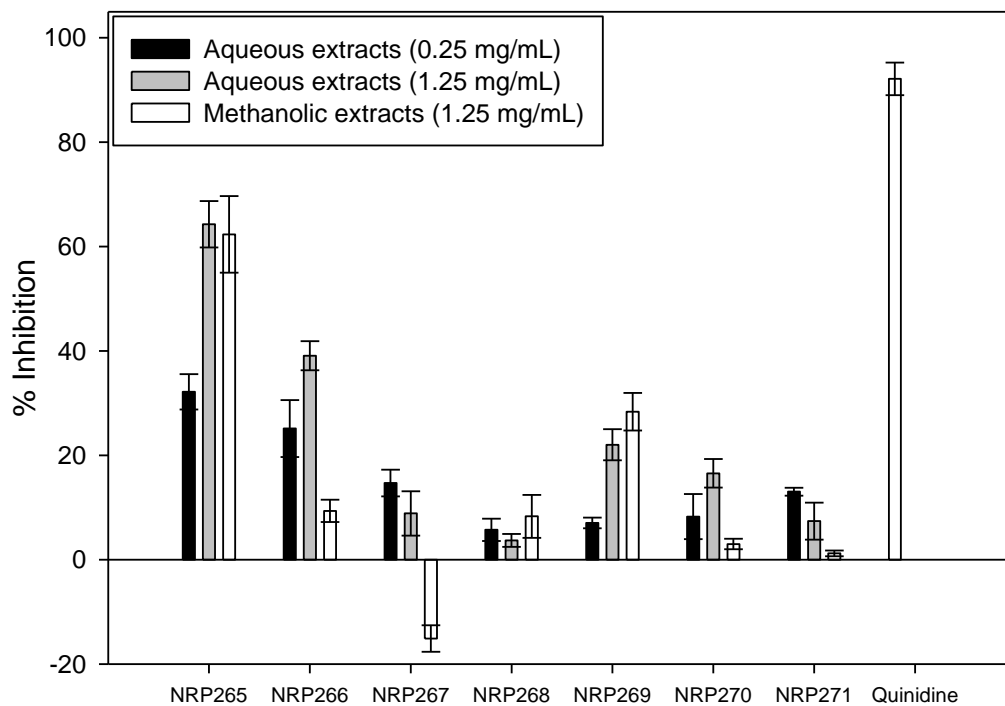


Figure 10A. Inhibitory effect of traditional Chinese medicine infusions aqueous extracts on CYP2D6*1 with different concentration.

TCM infusions aqueous and methanolic extracts (0.25 or 1.25 mg/mL) were tested for their potency of inhibition on CYP2D6*1 by measuring the metabolism rate of substrate. AMMC (0.12 μ M) was used as substrate, 2 μ M quinidine was used as positive control; n=3, mean \pm SEM. The negative reading of NRP267 methanolic extract may due to the system error or fluorescent interference from the extract.

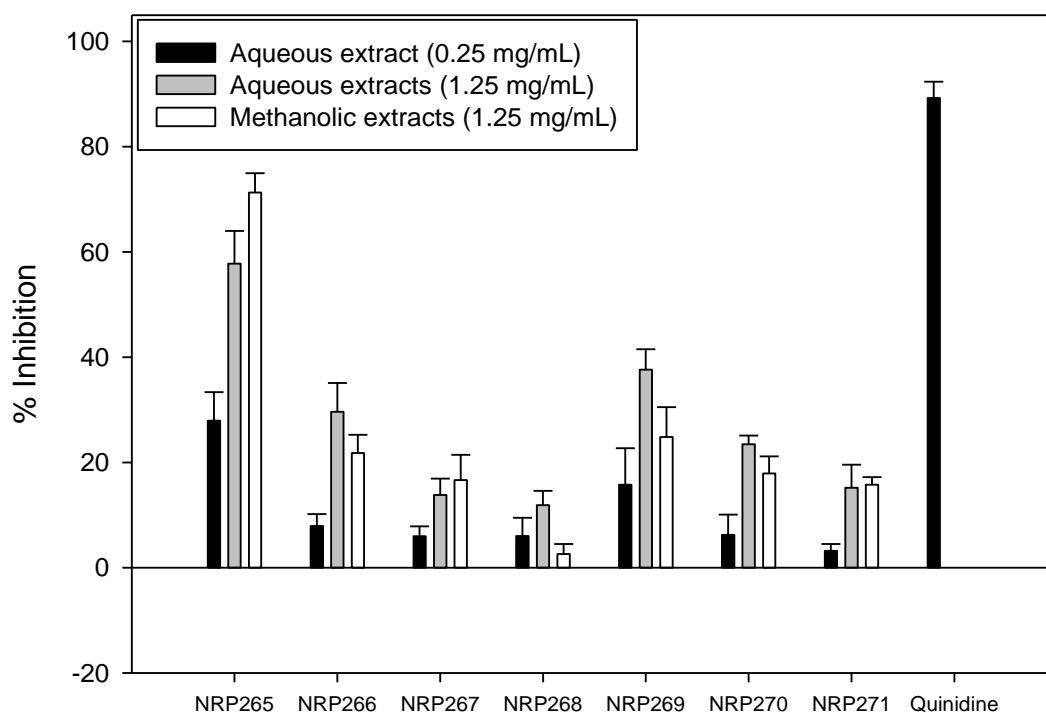


Figure 10B. Inhibitory effect of traditional Chinese medicine infusions aqueous extracts on CYP2D6*10 with different concentration.

TCM infusions aqueous and methanolic extracts (1.25 mg/mL) were tested for their potency of inhibition on CYP2D6*10 by measuring the metabolism rate of substrate. AMMC (0.12 μ M) was used as substrate, 2 μ M quinidine was used as positive control; n=3, mean \pm SEM.

Code	Statistically significant between CYP2D6*1 and *10 inhibitory potency (<i>p</i> -value)		
	Aqueous extracts (0.25 mg/mL)	Aqueous extracts (1.25 mg/mL)	Methanolic extract (1.25 mg/mL)
NRP265	0.24	0.11	0.07
NRP266	0.03	0.09	0.03
NRP267	0.47	0.73	0.23
NRP268	0.59	0.11	0.21
NRP269	0.85	0.06	0.44
NRP270	0.51	0.29	0.06
NRP271	0.07	0.18	0.11

Table 8. Statistical analysis of variation between CYP2D6*1 and CYP2D6*10 enzyme activity with the presence of TCM extracts.

A Student *t*-test was used to determine the significance between the two groups for each extract using SigmaPlot (Chicago, IL). A *p*-value ≤ 0.05 would indicate significance.

activity of 2D6*1 and 2D6*10 differently. Most percentage inhibition value of enzyme activity between 2D6 *1 and *10 are not significantly different except the 0.25 mg/ml aqueous extract of NRP266 (p value ≤ 0.05). This suggests that the TCM infusions may affect 2D6*1 and *10 differently, but most of these differences are not significant.

3.3 Enzyme Inhibitory Study of Goji

3.3.1 Inhibition of CYP450

The cold water extracts of fresh Goji berry (FGB) did not show a strong inhibitory effect on any of the enzymes which were studied (Figure 11A). CYP2C19 (44.2 ± 2.65), CYP2D6 (39.6 ± 3.86) and CYP3A5 (31.0 ± 0.34 , testosterone as substrate) being the top 3 inhibited enzymes (Figure 7A). The cold extract of dried Goji berry (DGB) showed a similar inhibitory effect as FGB, the top 3 inhibited enzymes were CYP2C19 (54.9 ± 3.59), CYP3A7 (35.5 ± 1.55) and CYP2D6 (24.3 ± 5.56).

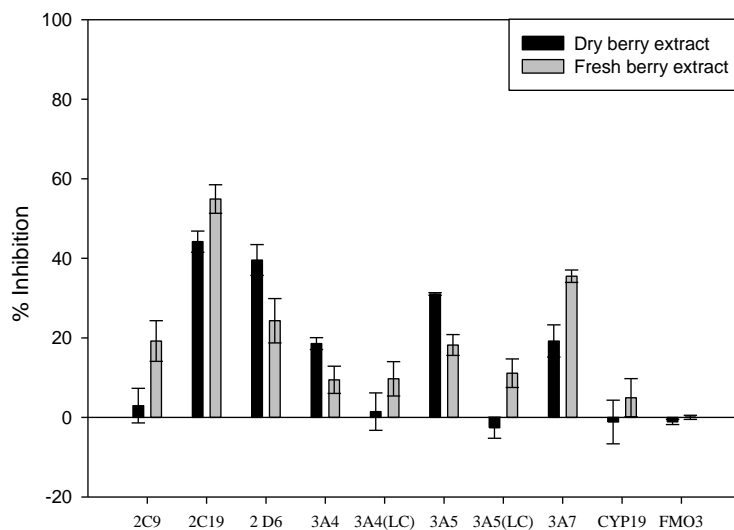
The hot water extract of FGB showed moderate (30%-70%) inhibitory effect on the CYP2C19 (65.3 ± 6.32) and CYP2D6 (41.4 ± 6.61), and weak inhibitory ($\leq 30\%$) effect of the rest of enzymes studied (Figure 11B). The hot water extract of DGB showed moderate inhibitory effect on CYP2C19 (64.3 ± 4.49), CYP2D6 (32.3 ± 6.93) and CYP3A5 (32.3 ± 6.93 , DBF used as substrate), and weak inhibitory effect of the rest enzyme studied. Compared to the inhibitory result of DGB and FGB hot water

extract, they showed a similar pattern of inhibitory effect on these enzymes, and the DGB caused higher inhibitory effects than FGB on most of the studied enzymes except CYP2C19 and CYP2D6.

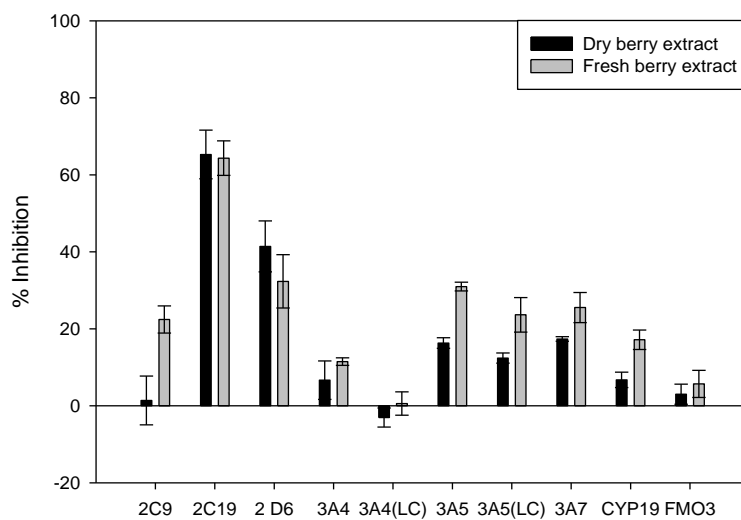
The ethanol extract of FGB exhibited a strong (>70%) inhibitory effect on CYP2C9 (87.9 ± 2.15) and CYP2C19, (90.3 ± 5.22), and caused weak inhibitory effect on the rest of enzymes tested (Figure 11C). The inhibitory effect of DGB ethanol extract showed similar results. Strong inhibition were found with CYP2C9 (102.4 ± 3.53) and CYP2C19 (98.8 ± 3.06), moderate inhibition were found with CYP3A5 (32.5 ± 3.06 , testosterone as substrate) and CYP3A7 (45.9 ± 3.49). Based on the comparison of DGB and FGB results, they had a similar enzyme inhibitory profile, but DGB exhibited stronger inhibitory effects.

Both Himalaya Goji juice (HGJ) and fresh made Goji juice (FGJ) had the strongest inhibitory effects among all Goji samples (Figure 10D). HGJ exhibited strong inhibitory effect on CYP2C9 (90.2 ± 0.3), CYP2C19 (89.3 ± 0.08), CYP3A4 (80.5 ± 1.81 , DBF as substrate), CYP3A5 (78.9 ± 0.65 DBF as substrate), CYP3A7 (89.1 ± 1.61) and CYP19 (76.6 ± 2.13), moderate inhibitory effect on CYP2D6 (50.3 ± 5.29), CYP3A4 (32.5 ± 3.21 , testosterone as substrate), CYP3A5 (44.0 ± 1.84 , testosterone as substrate) and CYP4A11 (34.5 ± 4.92). Compared to HGJ, FGJ showed relatively weaker inhibitory effect, it exhibited strong inhibition on CYP2C19 (70.7 ± 1.29), CYP3A7 (84.7 ± 1.01), CYP19 (75.2 ± 0.71) and CYP4A11 (70.6 ± 2.43), moderate

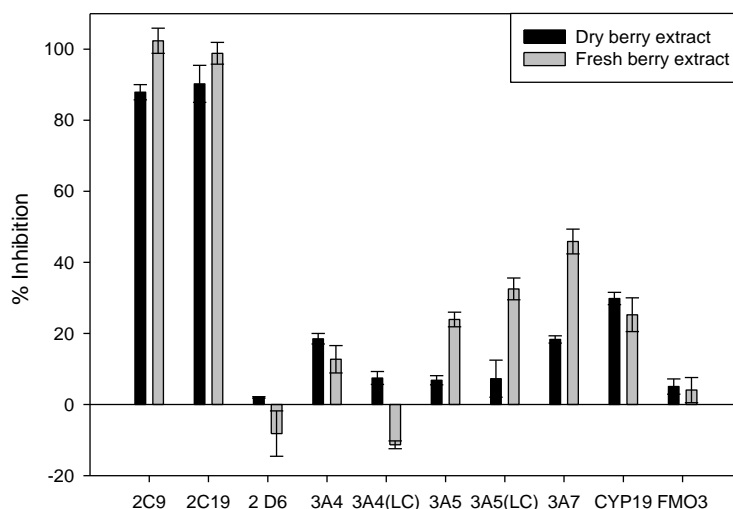
(A) Inhibitory effect of cold water extract on CYP450



(B) Inhibitory effect of hot water extract on CYP450



(C) Inhibitory effect of 80% ethanol extract on CYP450



(D) Inhibitory effect of juice sample on CYP450

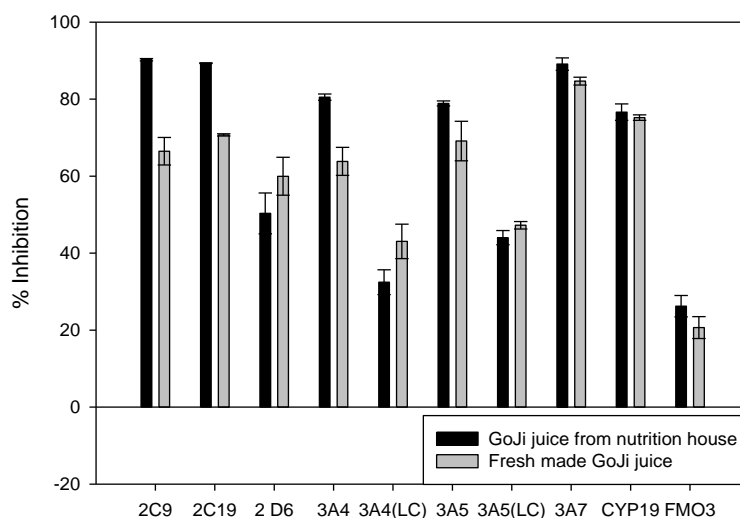


Figure 11. Inhibitory effect of Goji berry on CYP450.

Extracts and juices (the final concentration for all powdered sample extracts were 2.5 mg/mL and 50 uL/mL for juice sample) were tested for their potency of inhibition of CYP19, CYP2C9/19, CYP2D6, CYP3A4/5/7 and FMO3 by measuring the metabolism rate of fluorometric substrate (1 μ M DBF for CYP3A4/5/7 and CYP19; 0.116 μ M AMMC for CYP2D6; 0.1 μ M 7-MFC for CYP2C19, 25 μ M CEC for CYP2C9 and 3 mM methyl p-tolyl sulfide for FMO3, 35 μ M testosterone were used as an additional substrate than DBF for CYP3A4/5); n=3, mean \pm SEM.

inhibition on CYP2C9 (66.5 ± 3.57), CYP2D6 (60.0 ± 4.93), CYP3A4 (63.8 ± 3.64 , DBF; 43.1 ± 4.47 testosterone) and CYP3A5 (69.1 ± 5.14 , DBF; 47.2 ± 1.96 testosterone).

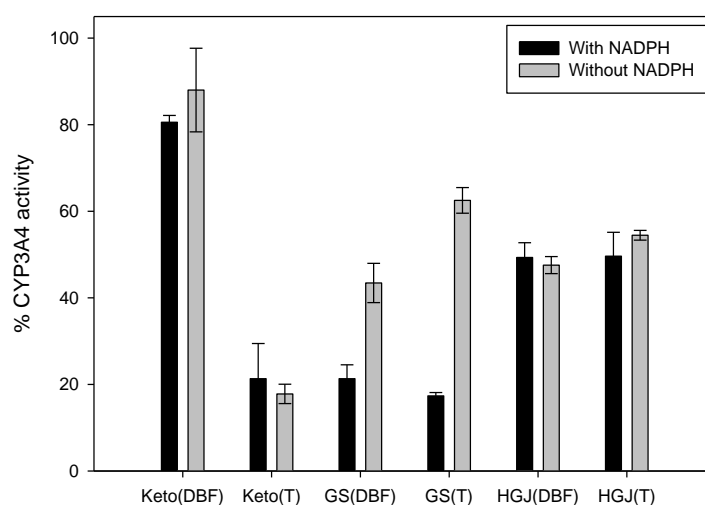
3.3.2 Inhibition of FMO3

All Goji samples tested showed weak or no inhibitory effect on FMO3 activity. The top 2 inhibitors are HGJ and FGJ which only exhibited weak inhibitory effects (26.2 ± 2.77 and 20.7 ± 2.84 respectively) (Figure 11).

3.3.3 CYP3A4 Mechanism-based inactivation

HGJ was the most inhibitory Goji product against CYP3A4; it was further studied for its inhibitory mechanism on CYP3A4 in the order to identify if it was a MBI inhibitor. In the NADPH dependence test, the CYP3A4 enzyme activity did not show a significant difference between the group with or without NADPH during the 10 min pre-incubation by using 2 different substrates (DBF and testosterone) (Figure 11a). In the time-dependence test, the inhibitory effect caused by HGJ on CYP3A4 activity of DBF metabolism was not altered with extension of pre-incubation time and NADPH was present in the reaction mixture, in another assay, the testosterone hydroxylation activity of CYP3A4 was not altered as well (Figure 11b). Overall, the inhibitory effect caused by HGJ exhibited no significant NADPH or time dependence, and would not be considered as a MBI.

(A) NADPH-dependence test



(B) Time-dependence test

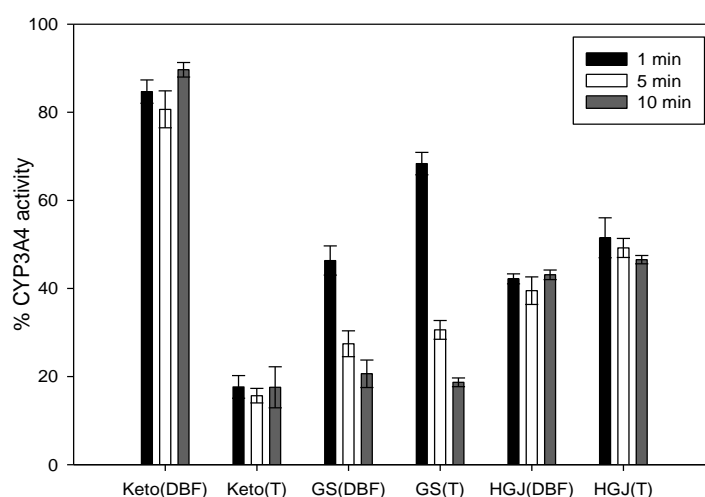


Figure 12. Identifying mechanism-based inactivation of CYP3A4 by Goji juice.

The activity of CYP3A4 after a pre-incubation with each extract (A) in the absence or presence of NADPH for ten mins, and (B) in the presence of NADPH for one, five or ten min(s). The results were expressed relative to vehicle control. No significant differences in activity ($p \leq 0.05$) observed between the two conditions tested (without NADPH vs. with NADPH, 1 min vs. 5 or 10 mins) for the same sample using Student's t-test. Mean \pm SEM ($n = 3$). DBF: Microtitre fluorometric assay use DBF as substrate; T: testosterone hydroxylation assay use testosterone as substrate. GS: goldenseal (11.5 $\mu\text{g}/\text{mL}$) was used as a positive control. Keto: ketoconazole (0.2 μM for MF assay and 0.5 μM for testosterone assay) was used as negative control. HGJ: Himalaya Goji juice.

3.4 Effect of Dried Goji Berry 80% Ethanol Extract on Cardiac CYP2J Activity

3.4.1 Inhibition of CYP2J2

The *in vitro* terfenadine hydroxylation assay indicated that 80% ethanol extracts of dried Goji berry (DGB) showed no inhibitory effect (-7.96 ± 3.94) on CYP2J2 activity (Figure 13).

3.4.2 Effect on CYP2J3 activity in rat H9C2 cell line

Hydroxyl terfenadine and carboxyl terfenadine are the two major metabolites of the probe drug terfenadine through CYP2J2/2J3 hydroxylation and Hydroxyl terfenadine is the predominantly one. Hydroxyl terfenadine has been detected in medium sample by HPLC-DAD after incubation with H9C2 cell line for at least 24 hr. However, carboxyl terfenadine was absent (Figure 14). Identification of metabolite were obtained by comparison of retention time of markers in a standard mix and verified by its spectral absorbance. The result also indicated that CYP2J3 activity in H9C2 cell was low, only 2-3% of the total terfenadine was metabolized during a 24 hr incubation and 5-7% during 72 hr incubation.

The result of a 24 hr incubation of DGB ethanol extract and terfenadine together with H9C2 cell line show that DGB extract did not alter CYP2J3 activity and another assay with 72 hr incubation exhibited a similar result (Figure 15A).

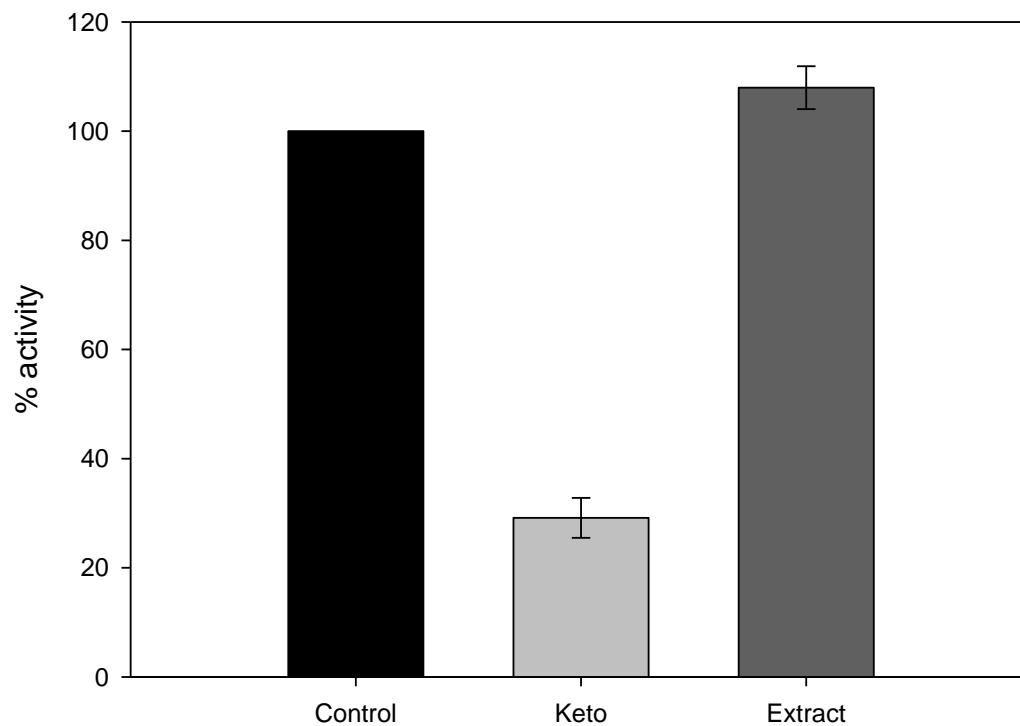


Figure 13. Inhibitory effect of Goji berry extract on CYP2J2. Goji berry extract (50 $\mu\text{L}/\text{ml}$) were tested for their potency of inhibition by measuring the metabolism rate of terfenadine (0.1 mM). Ketoconazole (1.9 μM) was used as positive control; $n=3$, mean \pm SEM.

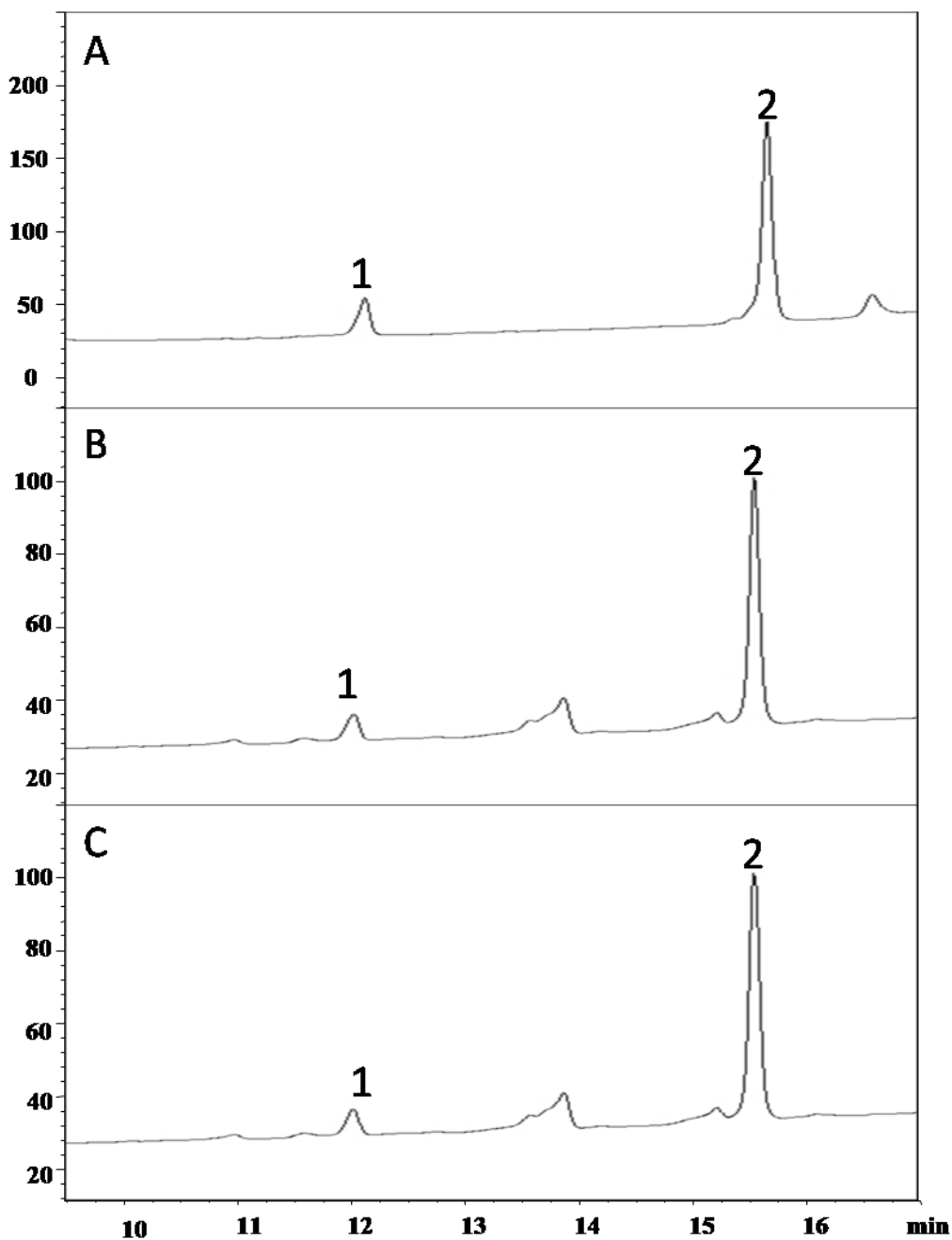


Figure 14. HPLC-UV result of terfenadine hydroxylation assay

Terfenadine was incubated with CYP2J2 enzyme (A), H9C2 cell line (B) and neonatal rat cardiomyocytes (C) in buffer system or medium for a range of time. Compound 1 is metabolite: hydroxyl terfenadine and compound 2 is terfenadine. Identification of metabolite were obtained by comparison of retention time of markers in a standard mix and verified by its spectral absorbance.

Another assay had been carried out with a 72 hr pre-incubation with DGB ethanol extract and no significant change of CYP2J3 activity had been found. The group which was pre-incubated with DMSO (volume control) and then incubated with extract and terfenadine is the only group showed a weak inhibitory effect on CYP2J3 activity (19.91 ± 12.57) (Figure 15B).

3.4.3 Effect on CYP2J3 activity in neonatal rat cardiomyocytes

Similar to the H9C2 cell line result, the assay using neonatal rat cardiomyocytes only one metabolite, the hydroxyl terfenadine, was detected in the medium sample by using HPLC-DAD, and carboxyl terfenadine was absent as well (Figure 14). The CYP2J3 activity in neonatal rat cardiomyocytes was low; only 1-2% of total terfenadine had been metabolized for 24 hr incubation and less than 4% for 72 hr incubation.

The assay with 24 hr incubation (no pre-incubation) showed that DGB extract slightly decrease the CYP2J3 activity (19.28 ± 7.44), however another assay with 72 hr incubation exhibited a slight induction (15.86 ± 10.02) (Figure 16A).

Another assay carried out with a 72 hr pre-incubation with DGB ethanol extract showed that the extract may slightly alter the CYP2J3 activity. The cells of which was pre-incubated with DMSO and then incubated with extract and terfenadine showed a weak inhibitory effect on CYP2J3 activity (19.91 ± 12.57) while the cells which was

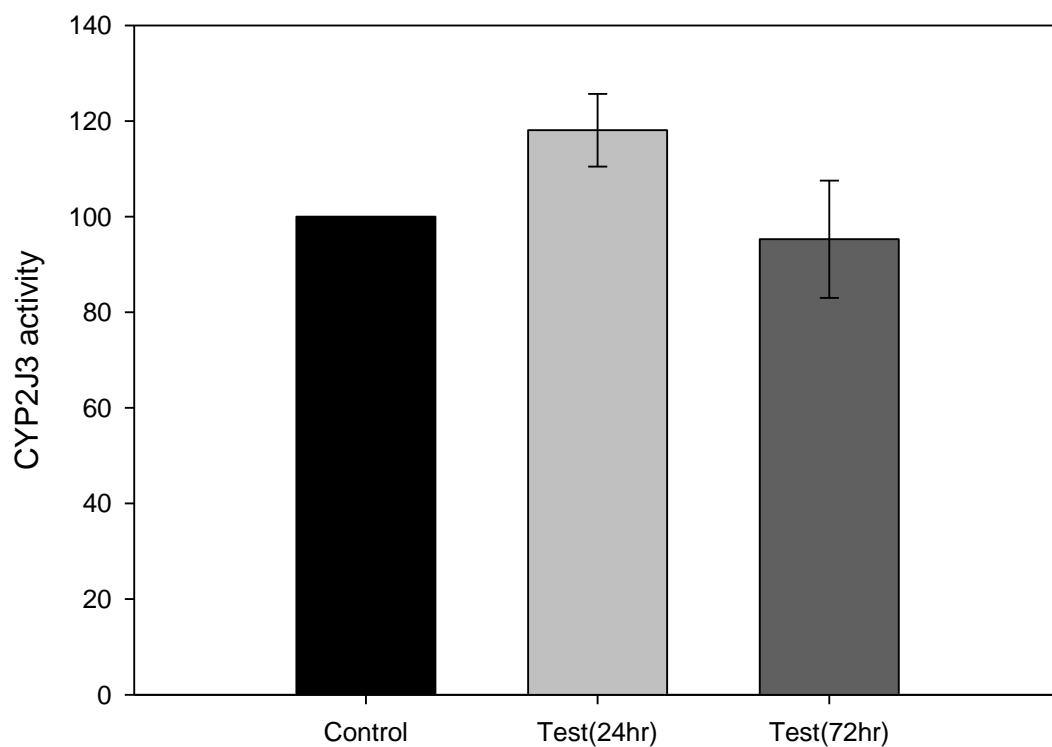


Figure 15A. Terfenadine hydroxylation assay using H9C2 cell line.

Two groups of cells were tested, the control group was only treated with terfenadine with the test group was treated with DGB 80% ethanol extract and terfenadine at the same time. Both group had been incubated for 24 or 72 hours; n=3, mean ± SEM.

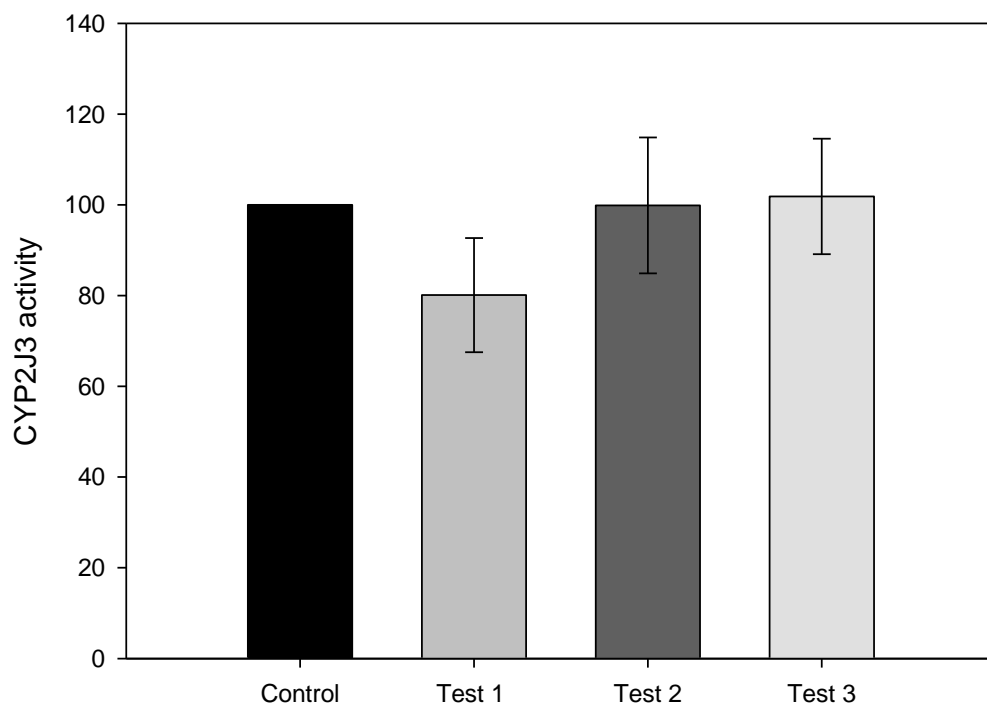


Figure 15B. Terfenadine hydroxylation assay using H9C2 cell line.

Four groups of cells were tested; the control group and test 1 group were pre-incubated with DMSO for 72 hours before medium change, the control group was then treated with terfenadine only while test 1 group was treated with terfenadine and DGB 80% ethanol extract together for 24 hours. Test 2 and 3 groups were pre-incubated with extract for 72 hours before medium change, then test 2 group was treated them with terfenadine while the test 3 group was treated with extracts and terfenadine together; n=3, mean \pm SEM.

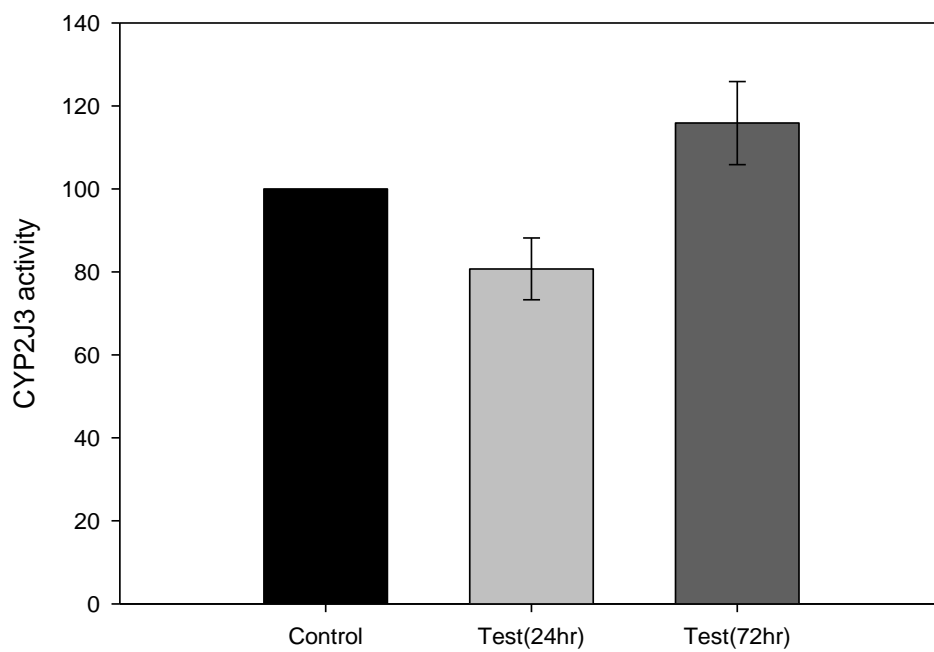


Figure 16A. Terfenadine hydroxylation assay using neonatal rat cardiomyocytes. Two groups of cells were tested, the control group was only treated with terfenadine with the test group was treated with DGB 80% ethanol extract and terfenadine at the same time. Both group had been incubated for 24 or 72 hours; n=3, mean ± SEM.

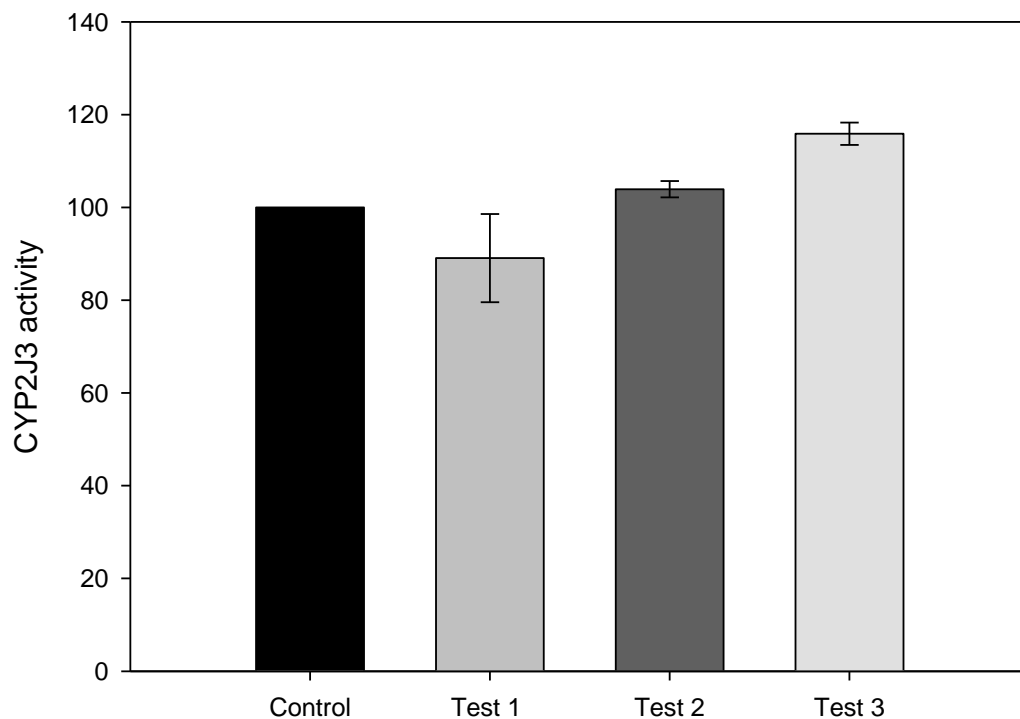


Figure 16B. Terfenadine hydroxylation assay using neonatal rat cardiomyocytes.

Four groups of cells were tested; the control group and test 1 group were pre-incubated with DMSO for 72 hours before medium change, the control group was then treated with terfenadine only while test 1 group was treated with terfenadine and DGB 80% ethanol extract together for 24 hours. Test 2 and 3 groups were pre-incubated with extract for 72 hours before medium change, then test 2 group was treated them with terfenadine while the test 3 group was treated with extracts and terfenadine together; n=3, mean \pm SEM.

pre-incubated with DGB ethanol extract and then treated with extract and terfenadine. Showed a weak induction effect on CYP2J3 activity (15.87 ± 2.4) (Figure 16B).

3.5 Goji HPLC-UV Fingerprint Study

The yield rate of fresh Goji berry (FGB) and dried Goji berry (DGB) with ethyl acetate extraction was 1.4% and 3.5%, respectively. The HPLC-UV fingerprint profile showed that FGB extract contained 3 major compounds which were also found in the DGB extract. The DGB extract contained more phytochemical compounds; however, most of the compounds were also found in FGB extract, the difference between FGB and DGB is the relative amount of those compounds (Figure 17).

The yield rate of FGB and DGB by methanol extraction was 5.68% and 12.32%, respectively. The HPLC-UV fingerprint profile showed that DGB extract contained more phytochemical compounds than FGB. Most of compounds can be found in the FGB extract but in lower concentration, however some of them were absent in the FGB extract. Compound C and D were found in DGB extract, however, none of them were found in FGB extract.

Over all, phytochemical differences were found between FGB and DBG. The concentration of compound A and B are much higher in FGB; however, DGB contained more compounds than FGB. Compound A, B were found in all extracts, and would be considered as maker compounds for Goji.

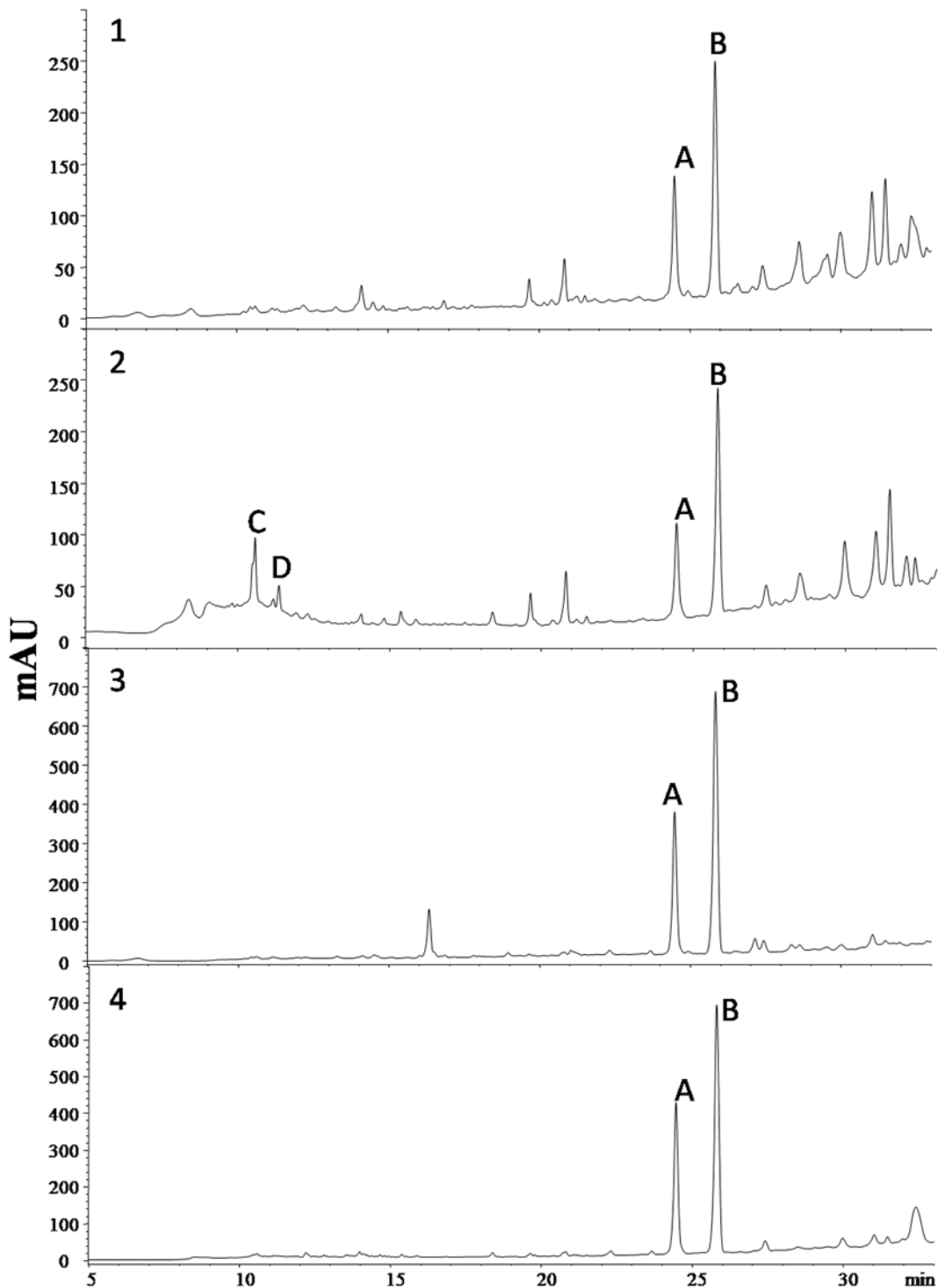


Figure 17 HPLC-DAD fingerprint study of Goji berry of methanol and ethyl acetate extracts.

An Agilent 1100 Series HPLC system and a diode array detector (DAD) was used to fingerprint Goji extracts: 1. Ethyl acetate extract of DGB; 2. Methanol extract of DGB; 3. Ethyl acetate extract of FGB; 4. Methanol extract of DGB. Compounds were given codes as A, B, C and D.

3.6 Effect of Selected Natural Health Products on Human Carboxyesterase I Activity

3.6.1 Identification of major metabolites of oseltamivir

Initial studies used methanolic stock solutions of oseltamivir phosphate and oseltamivir carboxylate. Figure 18 shows the HPLC-DAD profile of a mixture of authentic oseltamivir carboxylate (#2) and its parent compound oseltamivir (#4) eluting at respective retention times 6.8 min and 8.9 min. The mass spectrometric fragmentation pattern of both compounds followed similar pattern in the positive electrospray ionization. In the positive ionization mode both showed the loss of m/z 88 (pentan-3-ol moiety) followed by the loss of m/z 59 (acetamide moiety) (Figures 19 - #2 and 2 - #4). However $[M+Na]^+$ with an m/z of 313 was also observed in case of 4.

HPLC analysis of a HLM incubation mixture with Tris buffer pH 7.4 found the parent compound and a single large peak with a retention time between that of the carboxylate metabolite and parent compound (Figure 18C – #3a). Mass spectral analysis of the major unknown peak (compound #3a) found an $M+1$ of 299 (Figure 19 - #3a). This mass was m/z -14 lower than the parent compound and higher by the same amount than the carboxylate. The mass spectral fragmentation profile was similar to that of the parent compound with the exception that all major ions had an m/z -14 mass suggesting formation of the carboxylate metabolite with subsequent methylation of the decarboxylated side chain. Incubation of an aliquot of the methanolic stock solutions of oseltamivir or the carboxylate in denatured HLM did not result in the formation of this peak. The peak was also absent after overnight incubation of oseltamivir or carboxylate with methanol in reaction buffer at 37°C. The initial LC-MS sample at room temperature

in a light protected injection vial was re-run after a 3 week period and only a single peak was detected with a mass and retention time identical to that of the methyl derivative.

When deuterated methanol was used in the HLM incubation, an M+1 of 302 (Figure 18D - #3b) was detected. The three extra mass was from the deuterium methyl group which confirmed that the product (#3b) was formed by methylation of the decarboxylated side chain.

In order to check the effects of different solvents the incubation was then repeated with acetonitrile, ethyl acetate, 95% ethanol, and methanol compared to an aqueous control sample (Figure 20). EtOH (0.25%) had the least inhibitory effect.

Oseltamivir was then prepared as an aqueous stock solution. In the HLM incubation matrix (Tris, pH 7.5) a new peak with a retention time of 6.5 min that eluted prior to authentic carboxylate was detected (Figure 18B) with the corresponding decrease in the intensity of 4 which indicate the conversion of 4 into 1 (non-quantitative observation). Its mass spectral profile was similar to 2 but with the absence of an amino-benzoic acid moiety with mass fragment of m/z 138 (Figure 19-1).

3.6.2 Inhibitory effect of selected NHPs on CES1

Oseltamivir is an anti-viral prodrug that requires metabolic activation through carboxylesterase 1-mediated biotransformation to the active oseltamivir carboxylate metabolite for the prevention and treatment of uncomplicated acute illness due to influenza infection. The aim of this study was to determine if natural health products can

prevent the biotransformation of oseltamivir by the inhibiting carboxylesterase 1. Natural products were prepared at 20 mg/mL in 95% EtOH or water. The ethanolic extracts were diluted to 1 mg/mL with water to reduce the solvent effect. The aqueous extracts were examined directly. Cree plant extracts were examined as above for the formation of the carboxylate (Figure 21). Of the 17 samples examined, 6 (AD01, 02, 07, 09; W1, 2) reduced formation of the carboxylate by greater than 20%. Three of these extracts (AD01, 02 and W1) had an inhibitory effect of about 40 to 55%. Another 6 products (AD03, 11; W4, 6, 8, 9) reduced formation by about 10 to 20%. A similar pattern was observed when the effect on formation of the carboxylate and metabolite #3 was examined. Labrador tea (AD01 and W1) and juniper tea (W4) were then prepared in the traditional manner and aliquots of these preparations taken at 2 time points were not inhibitory (Figure 22).

Three traditional Chinese medicines, Chrysanthemum Flower (NRP 265), Du Huo (NRP 267) and Chai Hu (NRP 269) were examined because these TCMs are commonly used to treat flu and influenza infection. Of these 3 products, NRP 265 and 267 were not inhibitory and the third, NRP 269 had minimal inhibitory activity under these test conditions (Figure 22). Goldenseal had strong inhibitory activity and reduced carboxylate formation by about 75% (Figure 23).

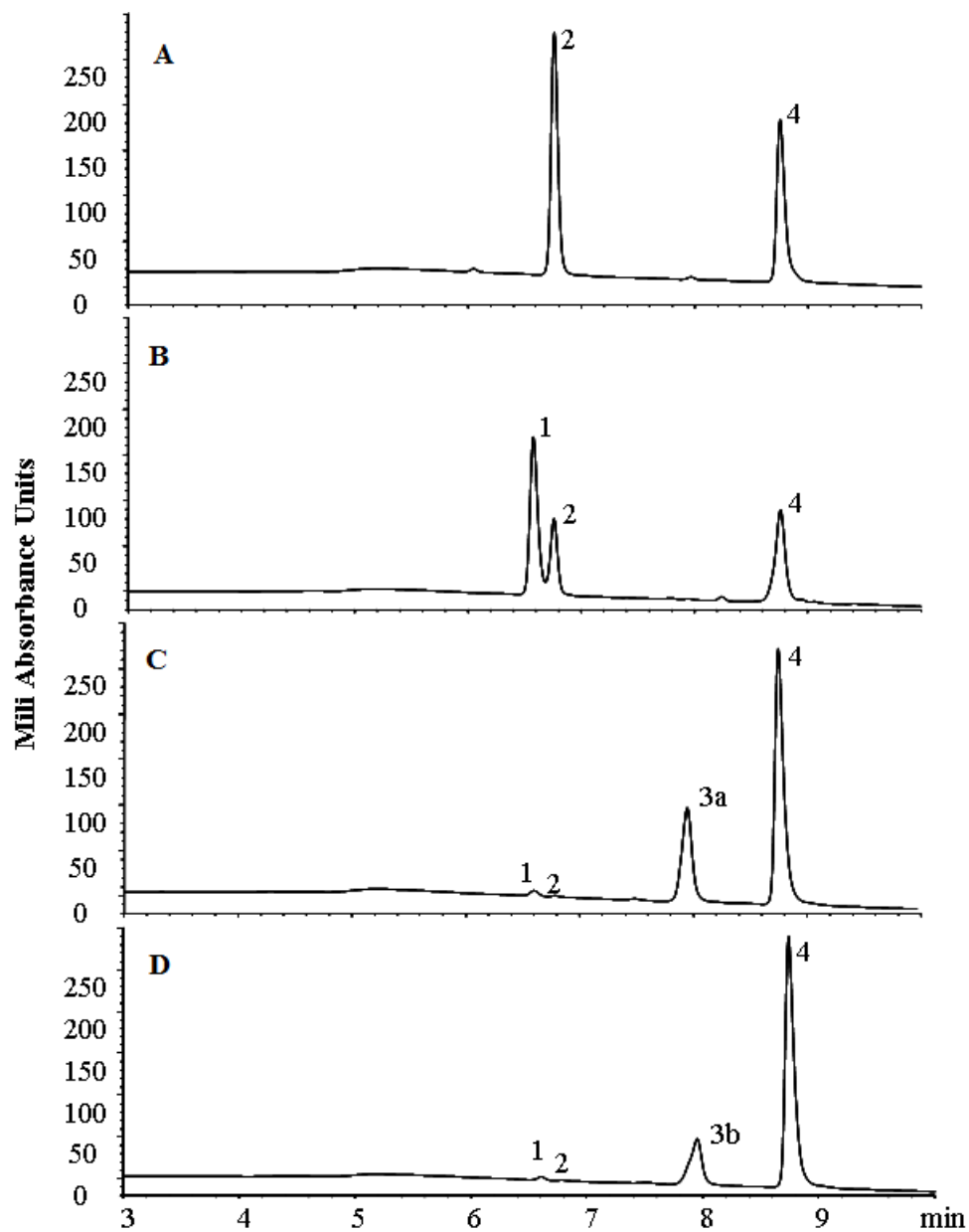


Figure 18. HPLC-UV examination of oseltamivir phosphate incubated under different conditions.

HPLC-DAD profiles of authentic standards and oseltamivir phosphate incubated under different incubation conditions: (A) authentic oseltamivir carboxylate (2) and oseltamivir phosphate (4); human liver microsome incubation in Tris buffer (pH 7.4) with oseltamivir phosphate (4) prepared in H₂O (B), in methanol (C) and CD₃OD (D).

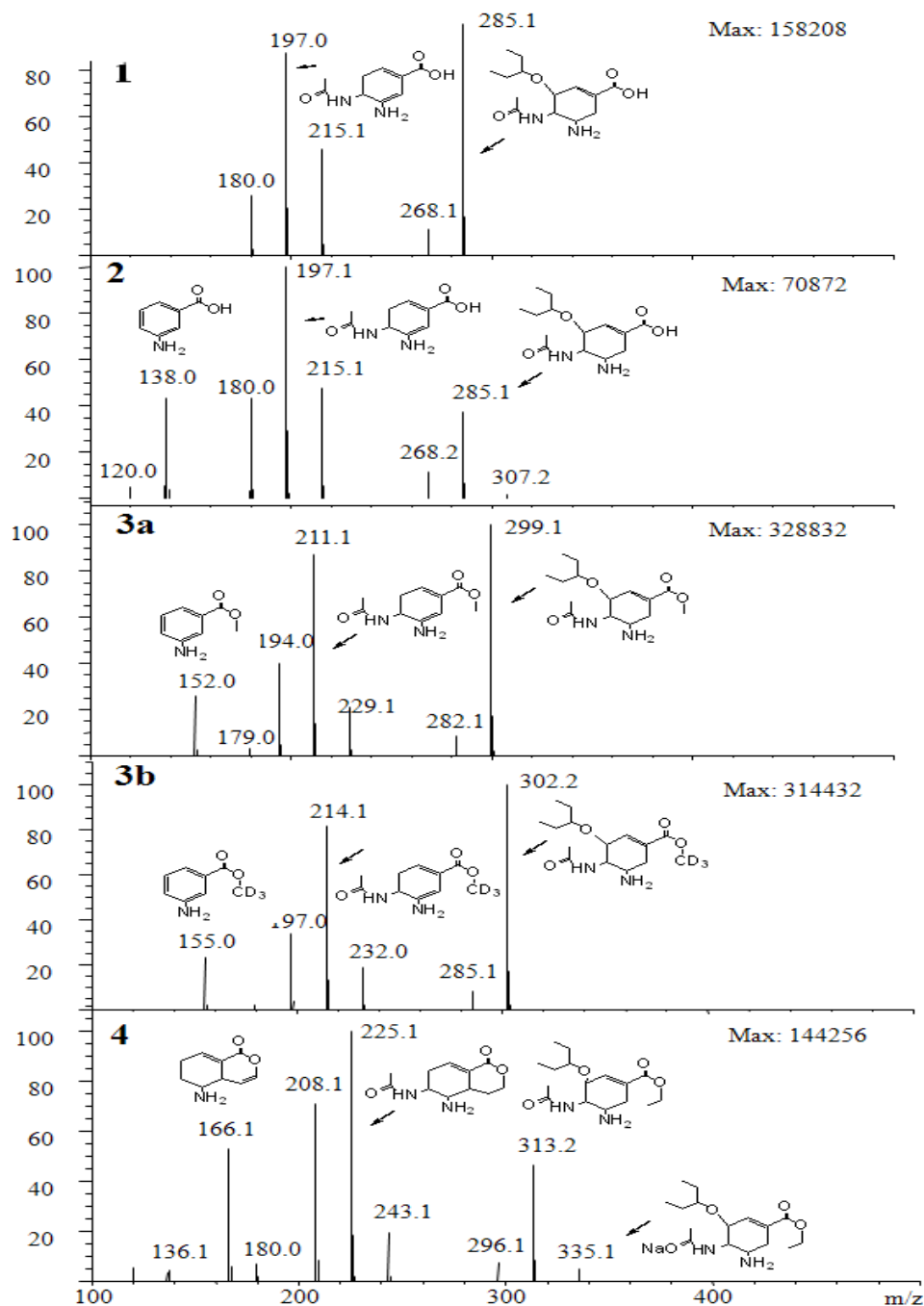


Figure 19. The Mass spectral analyze of Oseltamivir phosphate, oseltamivir carboxylate and other metabolites.

Mass spectra and structures of major fragments of oseltamivir phosphate (4), oseltamivir carboxylate (2), and the methanolic metabolites 3a and 3b (deuterated form of 3a) and an unknown isomer (1). The spectra are presented with mass range of 100-500 m/z (x-axis) and relative abundance of fragments (y-axis).

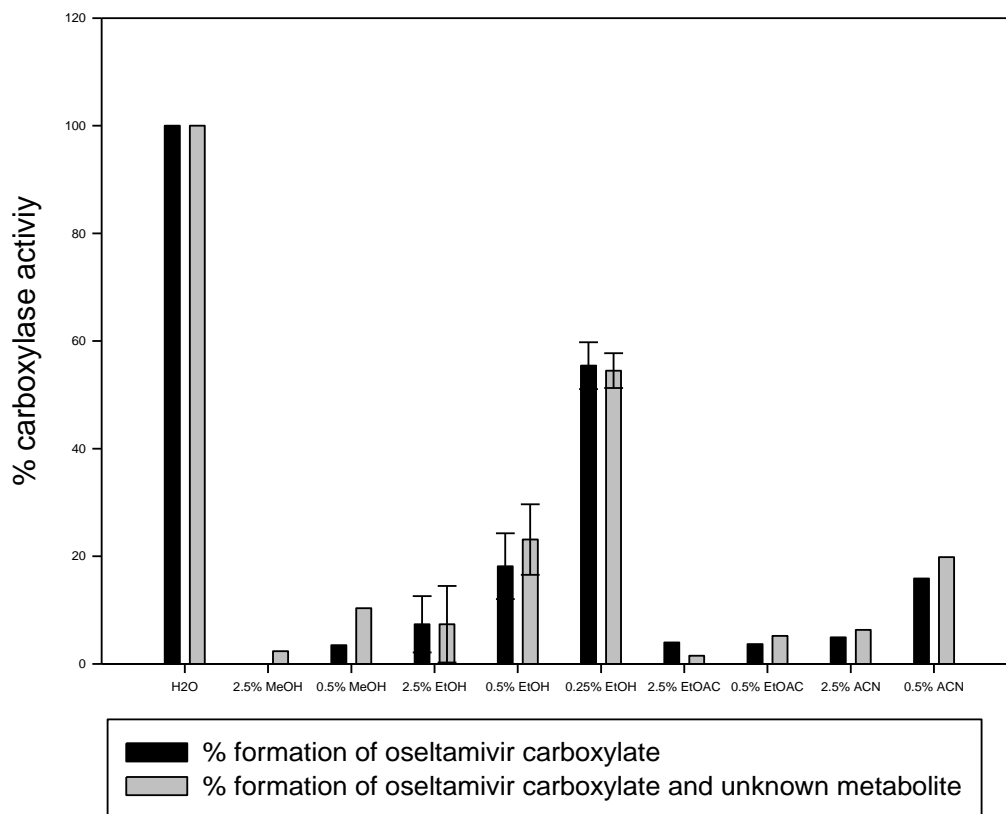


Figure 20. Effect of different solvent on the formation of oseltamivir metabolites.

A comparative study of the effect of methanol (MeOH), ethanol (EtOH), ethyl acetate (EtOAC) and acetonitrile (ACN) on the formation of the active oseltamivir carboxylate alone or together with an unknown metabolite relative to the aqueous control by the human liver microsome incubation mixture in Tris buffer pH 7.4; n=1-3, mean \pm SEM.

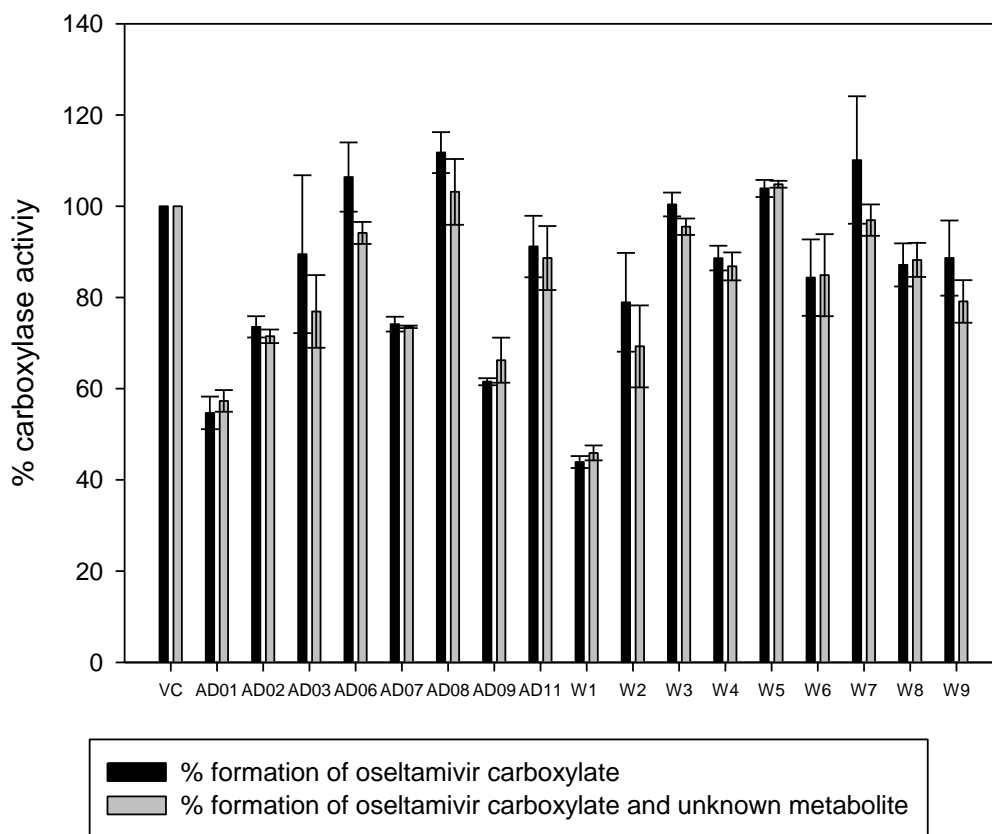


Figure 21. Effect of Cree anti-diabetic herbal medicine on the formation of oseltamivir metabolites.

A comparative study of the effect of several ethanolic extracts of ground plant material on the formation of the active carboxylate alone or together with an unknown metabolite relative to the ethanolic vehicle (V) control by the human liver microsomes incubation mixture in Tris buffer pH 7.4. Ethanolic extracts were made at 20 mg/mL in 95% EtOH, and then diluted with water to the final concentration in the incubation matrix of 2.5 µg/mL. n=3, mean ± SEM.

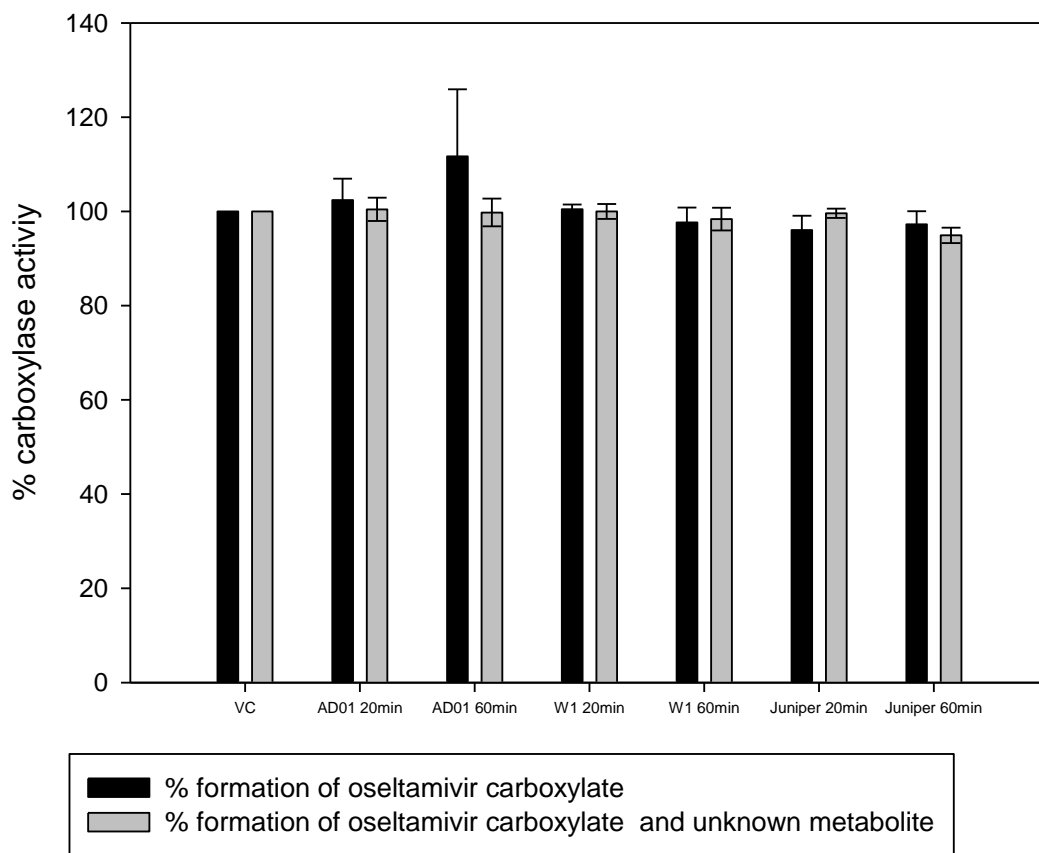


Figure 22. Effect of Labrador tea (AD01&W1) and juniper (W4) on the formation of oseltamivir metabolites.

A comparative evaluation of the effect of aliquots from traditionally prepared Labrador tea (AD01 and W1) and juniper (W4) on the formation of the active carboxylate alone or together with an unknown metabolite relative to the aqueous vehicle control (VC) where 1 gm of dried material was boiled in 250 mL water for 20 min or 60 min. Aliquots were taken and then analyzed by HPLC. n=3 mean \pm SEM.

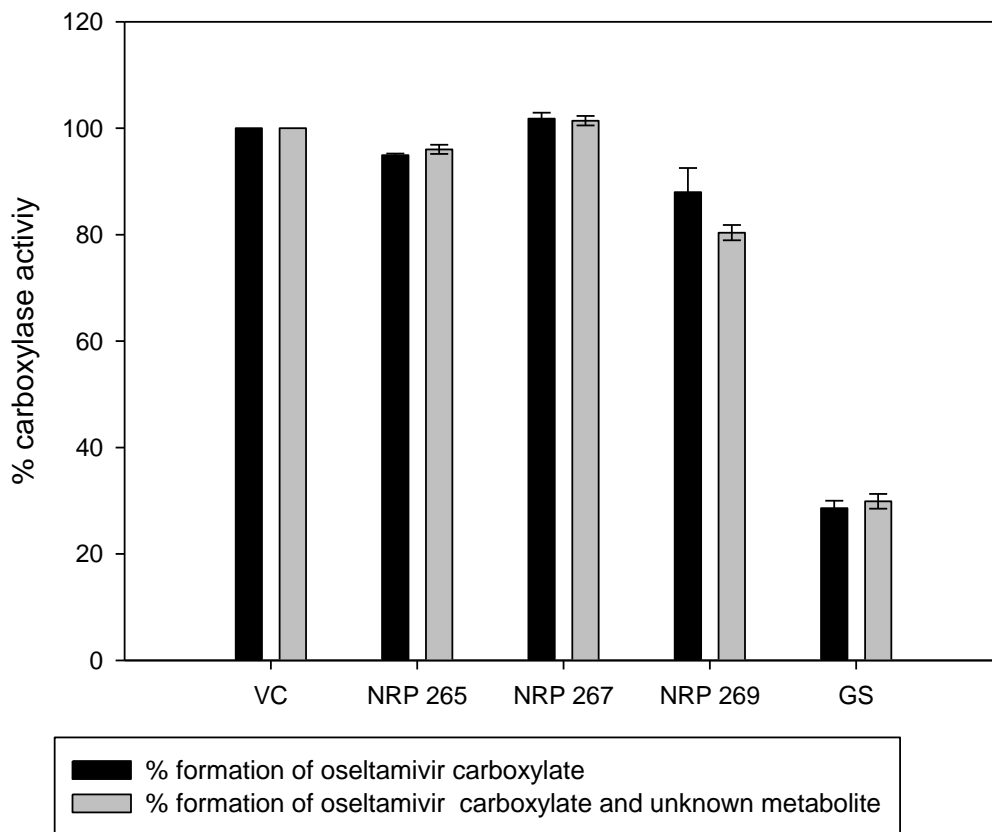


Figure 23. Effect of goldenseal and selected TCMs on the formation of oseltamivir metabolites.

A comparative study of the effect of extracts from Goldenseal (GS); and Chrysanthemum Flower (NRP 265), Du Huo (NRP 267) and Chai Hu (NRP 269) medicinal granules on the formation of the active oseltamivir carboxylate alone or together with an unknown metabolite relative to the vehicle control (VC) by the human liver microsome incubation mixture in Tris buffer pH 7.4, n=3, mean \pm SEM.

4 Discussion

Cree medicines are being used as both alternative and complementary medicines. However the potential for inhibitory actions on major DMEs had not previously been determined. This study examined the inhibition of DME's by Cree medicines and hence the potential for drug interactions to affect the safety and efficacy of the traditional Cree medicines. All 17 traditional plant species studied have been widely used in Cree nation of Eeyou Istchee (CEI) and highly valued for their anti-diabetic activities selected based on ethnobotanical surveys of healers and elders in Mistissini and Whapmagoostui. Anti-diabetic activities of these traditional plants have been supported by *in vitro* study (Harbilas et al. 2009). For this thesis project, 13 enzymes including 12 CYP enzymes (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, 4A11 and 19) and FMO3 were tested. This study was conducted in parallel to studies undertaken by Ms. Teresa W. Tam to determine the inhibitory effect of the Cree anti-diabetic plants. Among them, enzymes CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5 and 3A7 were tested by Ms. Tam using microtiter fluorometric plate assay. The study reported here focused on the enzymes CYP4A11, 19 and FMO3. CYP3A4 was also tested using another substrate to identify if the inhibitory effects were substrate specific. Results from both studies were combined and published in 2009 (Tam et al. 2009). Furthermore, Tam et al. (2009) examined the potential of these products to cause CYP3A4 MBI. Therefore, studies were undertaken to study 2 different substrates to determine the MBI on CYP3A4, and further examined if the MBI is substrate specific (Tam et al. 2011).

Other popular NHPs on the market, such as TCMs, may also be used by the Cree community. Therefore 8 other products were also tested for their potential to affect drug metabolism. Product selection was based upon reports that these products have nutritional or therapeutic effects for simple conditions such as flu or more chronic conditions including diabetes and its complications. Among them, there are case reports suggested that Goji products can cause metabolic interactions. Leung et al. (2008) documented a case of an 80 year old Chinese woman who consumed several cups of herbal tea containing Goji daily while being treated with warfarin. This interaction leads to a significant increase of the serum concentration of warfarin.

4.1 Potential for Metabolic Interaction

Type 2 diabetes (T2D) itself is not a fatal disease; however the patient suffers serious illness from the complications of T2D including renal disease, angio-cardiopathy, cerebrovascular disease, hypertension, neuropathy, blindness, or thanatosis on limbs (Skyler 2004; WHO 2006). Depending on the T2D complications, patients are usually treated with a number of prescription therapeutic products including antihyperglycemic, hypertension or nephropathic, lipid-lowering, cardiovascular and neuropathic drugs (Triplitt 2006; Siest et al. 2007; Castberg et al. 2005). An additional threat to diabetes patients is that T2D alters drug disposition which then increases the risk for developing serious adverse events when different health products are taken together.

CYP3A subfamily is the most important subfamily for drug metabolism. CYP3A4 is the dominant hepatic isozyme and it is also considered as the most important DME. It is

reported to be responsible for the metabolism of more than 50% of total therapeutic products (Zhou et al. 2005). Many drugs taken by diabetics such as angiotensin-converting enzyme inhibitors, calcium channel blockers, and some statins and anti-hyperglycemic agents are metabolized by CYP3A4 (Siest et al. 2007; Triplitt 2006) which emphasizes the importance of determining whether there is any inhibition by traditional medicines of this isozyme.

CYP3A4 is reported to contain multiple substrate binding domains (Schrag and Winekers 2001). On his basis, the extracts from the plant medicines may have different inhibition profiles depending on the health product combinations or substrates tested. Two different substrates were examined in the CYP3A4 inhibition studies to unequivocally examine the potential for interaction. The substrates, DBF and testosterone, were selected from two of the four different CYP3A4 substrate categories identified by Kenworthy et al. (1999). The use of two substrates should provide information on the inhibitory potential of these test products as well as providing information about binding domains.

Most of the Cree plant extracts tested showed an inhibitory effect on CYP3A4-mediated metabolic activity. AD01, AD02, AD03, AD11, and W4 were identified as strong inhibitors and as such, are expected to interact with drugs metabolized through CYP3A4 pathway. AD06, AD07, AD08, AD09, W1, W2, W3 and W5 had a moderate inhibitory effect and therefore may cause an interaction, but with lower potential than the strong inhibitors. W6, W7, W8 and W9 showed low or no inhibitory effect and were considered to be the safest of the agents tested as alternative and complimentary medicines.

The fact that the inhibition effect on CYP3A4 of TCM aqueous extract was concentration dependent, suggests that strong inhibitors (NRP265, 266, 268 and 269) may also initiate drug interaction in relative low concentration, and moderate inhibitors (NRP267, 270 and 271) may also have high potency to interact with drug when giving at high dose. The comparison between aqueous and methanolic prepared extracts indicates that the inhibitory effect of NRP270 and 271 methanolic extracts were higher than the aqueous extract. Since the recommended consumption method is a hot water solution, NRP270 and 271 infusions would be considered to have low potency to interact with drugs. However, the tincture products of NRP270 and 271 are expected to have high drug interactions potency.

Most of the Goji products such as Tea and tincture products would be considered safe to take with drugs metabolized by CYP3A4; they are not expected to cause metabolic drug interactions. However, the juice products should not be taken with drug treatment.

The potential risk of drug interaction from CYP3A4 inhibition can be greatly increased with MBI compared to other forms of inhibition. At least 8 hr is required to restore basal CYP activity from MBI. However, some clinical studies suggest that it may take up to 3 days for a substantial recovery if there is no additional exposure to the inhibitory product (Lilja et al. 2000; Rogers et al. 1999 Greenblatt et al. 2003). A longer recovery period may be required if there is repeated exposure to the product. All 17 Cree plant and 100% Himalaya Goji juice were examined to identify if their inhibitory effect is MBI. Among

them, AD08, AD09, and W6 were identified as potent CYP3A4 MBI inhibitors; AD02, AD06, AD11, W1 and W2 were categorized as less potent CYP3A4 MBIs. However, some of these MBIs did not exhibit strong inhibitory potency in the general CYP3A4 inhibition test. For example, AD06, AD08, AD09 have been only identified as moderate inhibitor and the potent MBI inhibitor W6 only showed low inhibitory potency. Hence, AD06, AD08, AD09, W1, W2 and W6 will be categorized in the group which have high potency to interact with drugs and should be treated carefully.

The patterns of CYP 3A4 inhibitory potential by these NHP products were different when different substrates were used. This is consistent with the observation by Kenworthy et al. (1999). The differences in inhibition by the extracts may be due to the affinity differences between substrates, or the multiple substrate binding domains of CYP3A4 (Schrag and Winekers, 2001), or multiple substrates binding within single active site (Kenworthy et al. 2001). The inhibitory potency for individual extract is altered when using different substrates. However, it is interesting that similar inhibition patterns were observed for the majority of the extracts with different substrates.

CYP3A4 MBI may also show substrate selectivity. For example, *trans*-resveratrol, a natural compound commonly found in red wine is a MBI of CYP3A4 when using testosterone as substrate (Chan and Delucchi 2000; Piver et al. 2001). However, in the study using DBF as substrate, it was not identified as a CYP3A4 MBI (Chang and Yeung 2001). Similar results were observed with Cree plant extracts. Ms Tam's identified no MBI inhibition when DBF was used as substrate (Tam et al. 2011); however, a total of 11

Cree plant extracts showed MBI potency when testosterone was used as substrate. The notion is that the structure change of enzyme caused by MBI may only disable the metabolism activity to certain groups of substrate, and the enzyme may still be capable of metabolizing other substrates.

CYP3A5 metabolizes the same range of substrates as CYP3A4 due to similar substrate specificity (Andrew Williams et al. 2002) and contributes less to drug metabolism in most individuals due to lower hepatic and intestinal concentration, and enzyme activity (Andrew Williams et al. 2002; Lin and Lu 1998). Hence, the potential risk of metabolic drug interactions caused by CYP3A5 inhibition may not be as clinically important as CYP3A4. However, CYP3A5 selectivity is different and may result in the formation of new or altered metabolic profile. This can mean differences in the safety of products that are metabolized by this isozyme. For example, if CYP3A4 were inhibited, some of CYP3A4 substrate may be forced to be metabolized by CYP3A5, thus there may be new metabolites formed which may cause adverse effect if the new metabolites are toxic. The inhibitory pattern of CYP3A5 by Goji products was quite similar to CYP3A4. Only Goji juice products are expected to interact with drug metabolized by CYP3A5.

CYP3A7 is mainly expressed in fetal liver, and in about half of the adult population (Lin and Lu 1998; Tateishi et al. 1999). CYP3A7 has a similar substrate specificity as CYP3A4, but the liver concentration and enzyme activity are much lower than CYP3A4 (Andrew et al. 2002, Tateishi et al. 1999). As a result, inhibition on CYP3A7 may not be expected to have a clinically significant effect. However, since it is a major fetal CYP

enzyme, the inhibitory potency may be critical for pregnant women. As the results of this study show that Goji juice may affect the fetal drug metabolism system, this product should be used carefully by pregnant women taking any drug metabolized by CYP3A subfamily.

The inhibitory pattern toward CYP3A4/5/7 is quite similar under the *in vitro* test conditions used herein which suggest a single inhibitor may deactivate the whole CYP3A subfamily; however, due to the different distributions of these enzymes, the result from *in vivo* or clinical study are expected to be more complex. CYP3A7 is mainly a fetal isozyme and the inhibition is not expected to be clinically significant except in pregnant women. CYP3A4/5 are the major functional adult enzymes in CYP3A subfamily, CYP3A4 are mainly expressed in intestine and liver; CYP3A5 is also expressed in human kidneys (Haehner et al. 1996). Thus, the potential to trigger a metabolic drug interaction through the inhibition of CYP 3A subfamily may be altered by the disposition of drugs and inhibitors.

The CYP2D subfamily is of second most important DME family. CYP2D6 is the dominant enzyme in CYP2D subfamily and is involved in the metabolism of approximately 25% of the currently available conventional drugs (Ingleman-Sundberg 2005). CYP2D6 is important in the metabolism of central nervous system drug such as adrenergic β -blockers, anti-arrhythmic drugs, and anti-coagulant drugs (Castberg et al. 2005; Chico et al. 2009). For T2D patients, CYP2D6 metabolizes many drugs such as adrenergic β -blockers used to treat cardiac complications (Triplitt, 2006). Six of seven

TCM infusions, Goji tea and tincture are not expected to interact with drugs metabolized by CYP2D6 due to low inhibitory potency. However, NRP265 and Goji juice showed moderate inhibitory effect; they should be carefully used with drugs metabolized by 2D6 especially at high dose. Most Cree plant extracts are identified as weak or non-inhibitors of CYP2D6 except AD01 (Tam et al. 2009); this suggests that the combination use of these TCM products and most Cree anti-diabetic medicine are less likely to interact. However, the combination use of Goji juice, NRP265 and AD01 should be avoided since there is an increased risk of interaction with some central nervous system drugs.

Polymorphisms are quite common in CYP2D6 (Phillips et al. 2001). More than 44 alleles identified (Jian et al. 2004). 2D6 polymorphism was also found in the Cree population. Hegele et al. (2000) reported a genotype of CYP2D6 G1934A polymorphisms in Oji-Cree population at Lake Winnipeg; metabolism capabilities of this genotype have not been studied and currently, enzyme samples of this genotype are not available for *in vitro* study. CYP2D6*10, a very common Asian genotype was selected in this study instead of G1934A because there are evidences suggest that ancestors of Cree nation may come from Asia. We hypothesize that the G1934A or other native genotypes maybe similar to CYP2D6*10.

CYP2D6*10 is a varied genotype of CYP2D6 highly expressed in East Asian area. It was found in 43-51% of Chinese and 33-60% of Japanese (Phillips et al. 2001). In general, this genotype was categorized as an unstable enzyme with reduced activity (Jian et al. 2004). However, Ramamoorthy et al. (2001) report that the catalytic activity of

CYP2D6*10 related to the 2D6*1(wild type) may be varied due to different substrates. Thus, differences on enzyme inhibitory potency between polymorphisms are expected. However, the result of this study indicated that the pattern of inhibitory potency related to the vehicle control are quite similar, no significant different ($p \leq 0.5$) were observed. However, since CYP2D6*10 was a poor metabolizer, the enzyme activity would be even lower than the normal CYP2D6*1 when inhibited by NHPs. Overall, strong or moderate inhibitor such as AD01, Goji juice and NRP265 should be more carefully mixed with drugs metabolized by 2D6. The populations which carry CYP2D6*10 or other CYP2D6 polymorphisms may have reduced enzyme activity.

The CYP2C subfamily is also a very critical in drug metabolism. CYP2C9 is the most important isozyme in CYP2C subfamily which accounts for approximately 60% of the hepatic CYP2C (Furge and Guengerich 2006). It mediates the metabolism anti-hyperglycemic agents such as sulfonylureas, nateglinide, and rosiglitazone; the angiotension-II type 1 receptor blockers; the diuretic torsemide; the statin fluvastatin; the anti-coagulant warfarin; and several drugs used to treated neuropathies (Triplitt 2006). CYP2C9 activity was highly inhibited by the ethanol extract of both fresh and dry berry, and Himalaya Goji juice. This finding may suggest that Goji products may be expected to interact strongly with drugs metabolized by CYP2C9. This result may provide a mechanistic explanation of the case report of the interaction between Goji tea and warfarin (Leung et al. 2008). For T2D patients, anti-hyperglycemic agents are the most important drugs metabolized by CYP2C9, the inhibitory on CYP2C9 activity may result in hypoglycaemia.

Most of the Cree plant extracts, except W1, W5 and W9, showed at least a moderate inhibitory effect on CYP2C9 (Tam et al. 2009). This suggested that Goji products especially juice and tincture should not be used with most of Cree plants since their combined use may increase the risk of metabolic drug interaction, especially for drugs with a narrow therapeutic index such as warfarin, where the adverse effect could be fatal (Zhou et al. 2003).

Although less important than CYP2C9, CYP2C19 is a polymorphic isozyme with known null alleles that is involved in the metabolism of approximately 8% of drugs on the market including: proton pump inhibitors, anti-epileptics, tricyclic antidepressants, and some cardiac medications (Lewis 2003; Klotz et al. 2004; Goldstein 2001). CYP2C19 was the most inhibited enzyme by Goji. The tincture and juice products of Goji should be used in caution with other therapeutic products metabolized by CYP2C19. Tea made from both fresh and dried berry would be expected to cause interactions but with a relatively lower potency compared to tincture and juice products.

CYP2C19 is also the most inhibited enzyme by the Cree plants extracts (Tam et al. 2009), which suggested that combination uses of Cree plants and Goji product should be avoided since risk of metabolic drug interaction may be increasing. However, most of the anti-diabetic drugs are not expected to be affected by them.

FMO family metabolizes a number of nitrogen-, sulphur-, and phosphorus- containing

drug such as cimetidine, (S)-nicotine, tamoxifen and olanzapine (Dolphin et al. 1992, Woolf 1999). AD03, AD06, AD11 and W5 extracts exhibited a strong inhibitory effect on FMO3 activity. The pharmacological effect in diabetic patients of this interaction is more difficult to determine as FMO3 does not metabolize the commonly used anti-diabetic drugs. However, if this enzyme is inhibited, it may affect how the body metabolizes other drugs and plant medicines that may be used by this patient population. For the Goji products FMO3 is not or only weakly inhibited, they are not expected to interact with drugs metabolized by FMO3. However, a risk may develop with some drugs that are also metabolized by the CYP pathway. The combination use of Goji products with Cree plants are not expected to increase the potency of triggering a metabolic drug interaction by these potent inhibitors.

The final enzyme examined was the human carboxylesterase 1. CES1 plays an important role in both drug metabolism and pro-drug activation. Several drug including heroin, cocaine, meperidine and lidocaine were metabolize by CES1 (Redinbo et al. 2003); many pro-drug such as CPT11 and oseltamivir (Tamiflu) require CES1catalyzed hydrolysis to be activated. This study was focus on if NHPs can change the metabolism profile of oseltamivir by altering the activity of CES1. The extracts from the Cree botanicals were prepared under identical conditions allowing a comparative evaluation of their potential to affect this enzyme. The findings in this study can be grouped into poor, fair and moderate to strong potential for interacting with this enzyme. It is noteworthy however than when 3 of these traditional medicines were made as infusions (teas) they did not affect the formation of the active carboxylate product. As this initial screen looked at low

concentrations of only 3 traditional Chinese medicines, the findings can only suggest that there may be a potential for Echinacea and Chai Hu (NRP 269) to interact. Interestingly, Goldenseal had a very strong inhibitory effect. These findings would then suggest that under some conditions, several of these medical botanicals could cause an adverse interaction but additional studies are required to extend and clarify the significance of these findings. As with many medications, the actual amount and the rate that the medicine is consumed will affect the response.

In addition, this study observed that minor differences in incubation conditions can markedly affect the metabolic profile. The findings have been confounded however by the detection of three previously unreported metabolites. Although compounds 1 and 3a have similar chromatographic properties to the rat metabolites reported by Sweeny et al. (2000), their mass spectral properties are markedly different.

The major peak obtained when oseltamivir was incubated with active but not denatured HLM in the presence of MeOH may be a metabonate resulting from condensation or trans-esterification of the carboxylate with methanol to give the corresponding methyl-ester. This was confirmed by the CD₃OD study suggesting that in addition to a slow chemical conversion, trans-esterification is also be mediated in HLM by an enzyme such as O-methyltransferase. Further studies are required to determine if the carboxylate can be trans-esterified and if these mechanisms could proceed with other small chain alcohols as the donor.

Some of these Cree plant extracts have been analyzed for their phytochemical composition. Results showed that flavonoids, triterpenoids, polyphenols, and alkaloids are quite common among those plant extracts (Saleem et al. in press (a) and (b)). Many of these compounds have been reported to affect the DME especially CYP activities. Based on the phytochemical study of AD01 and W1, catechin, chlorogenic acid, quercetin, etc (Saleem et al. in press (a) and (b)) have been identified in AD01 and W1 extract and they have been proved to affect the activity of major DMEs especially CYPs. Catechin is reported to be an inducer for CYP1A1, 1A2 and 2B (Zhou et al. 2003); chlorogenic acid can inhibit CYP1A2, 2C9/19, 2D6 and 3A4 at a relative high concentration ($IC_{50} > 100$ mM); quercetin can also inhibit CYP1A2, 2C9/19, 2D6 and 3A4 (Zhou et al. 2003), but it also an inducer for CYP1A2 (Canivenc-Lavier et al. 1996). In the combination study by Ms Tam, AD01 extract have been identified as strong inhibitor to CYP3A4, 2C19 and moderate inhibitor for CYP1A2, 2C9 and 2D6 which is identical to the signal compound inhibition profile (Tam et al. 2009a)..

Tea samples of Cree plants have also been examined for their phytochemical compositions. Similar to ethanol extract, most of the marker compounds can be found in a tea sample but with a lower concentration. The concentration of these compounds was significantly increased with cooking time, as well as the inhibitory effect on CYP3A4 (Tam et al. in press). The inhibitory effect was increased from less than 15% of 5 min boiling to over 90% of 30 min boiling. Associated with current results, chlorogenic acid is expected to be the major inhibitor for CYP3A4 in AD01&W1 tea sample.

Many drugs such as fluoxetine, imipramine, diltiazem and terfenadine (Phillips et al. 2001) are not only metabolized by a single DME, they have multiple metabolism pathways. This suggests that the inhibition on a single DME by NHPs may not cause significant metabolic drug interaction; however, since different pathway may form different metabolites, the safety profiles of drugs are still expected to be modified by the NHPs. This could be dangerous if the unusual metabolites are toxic.

On the other hand, many NHPs have high potential to interact with multiple DMEs due to their complicated phytochemical composition. As has been showed in this *in vitro* study, most of the Cree plant extracts and Goji juice can inhibit at least 2 DMEs from different sub families (Tam et al. 2009). Patients who take multiple medications (including diabetes) are expected to have adverse drug effect, because there is risk that one NHP may trigger multiple metabolic drug interactions.

Although the combination use of multiple NHPs with drugs may provide a boost in the therapeutic effects, this may also increase the risk of causing metabolic drug interaction since it may enhance the inhibition effect on single DME and it may also enlarge the range of DME inhibition.

Many NHPs were shown to inhibit DMEs under the test conditions. However, clinical events of NHP-drug interaction are not only related to the inhibitory potency of NHP. NHP may also contain inducers of DMEs, and they are also expected to affect the function of transporters. The polymorphism of DMEs or transporters is another common

factor which may alter the safety profile of drugs. Nevertheless, the adverse effect may also be altered by the toxicity profile or therapeutic index of conventional drug. For a drug with a narrow therapeutic index a weak inhibitor may cause a serious adverse effect. However, if a drug has almost no side effects or a wide therapeutic index, they will be less risky taken with strong inhibitors.

In summary, the findings of this project provide a high throughput survey study focussed on the inhibitory potency of selected NHPs on major DMEs. It provides useful information and guidance for further *in vivo* and clinical study; however, different results of metabolic drug interaction are expected in the *in vivo* and clinical study.

4.2 Endogenous Substrate-NHP Interaction Based Toxicity or Beneficial Effects

Of the 57 CYPs present in human, only a handful are considered to be major DMEs, partly because there is little information on the role or effect of compounds on the remaining isozymes. The constituent's from NHPs may also be inhibitors or inducers of these CYP enzymes, and the effect may be both beneficial and harmful.

CYP19, also called aromatase, is only known to metabolize a few conventional drugs, and none of the anti-diabetic drugs. However it is extremely important in the biosynthesis of estrogen. The modification of CYP19 enzyme activity may have a significant effect of the estrogen level in human body. As a result, inhibition may cause estrogen level dependent side effects (Simpson et al. 1994). This has been observed in the continuous consumption of some antiepileptic drugs that have side effects such as hyperandrogenism,

menstrual disorders and the formation of ovarian cysts (Jacobsen et al. 2008). The inhibition of CYP19 activity may result in estrogen level dependent disease. However, it may also provide beneficial effect. CYP19 was found to be highly expressed in the breast cancer cells and surrounding tissue cells. The increased CYP19 enzyme activity can produce more estrogen which can promote growth of hormone-dependent breast tumours (Miller et al. 1997; Dowsett et al. 1993). CYP19 inhibitors may counter this action and slow down the tumour development. According to our results, both Goji juices were identified as strong CYP19 inhibitor and they would be expected to show an anti-breast cancer activity. Accordingly, long term consumption of strong inhibitors, including AD07 and Goji juice, may be expected to cause estrogen level dependent side effects, but they also has the potential to be used as anti-breast cancer agents.

CYP4A11 is not associated with the metabolism of anti-diabetic drugs; however its role in the fatty acid metabolism may have physiological significance in T2D patients. In liver, fatty acid oxidation (combustion) occurs in 3 sub-cellular organelles; with β -oxidation confined to mitochondria and peroxisomes, and CYP4A catalyzed ω -oxidation occurring in the endoplasmic reticulum (Songtao et al. 2003). The expression of CYP4A family is increased in obesity and diabetes in an attempt to restore the normal blood fat level (Enriquez et al. 1999). The inhibition of hepatic CYP4A11 may reduce capacity of fatty acid metabolism in liver, and this will lead to an increase of lipid storage in liver. Excess accumulation of lipid in liver may lead to the development of non-alcoholic steatohepatitis, which is often the early clinical manifestation of insulin resistance, with its complications of high blood pressure, coronary heart disease and T2D (Songtao et al.

2003). However, CYP4A11 was also expressed extrahepatically, and this expression may have a beneficial effect since the substrates or metabolites of CYP4A11 are usually lipid messengers which can alter the function of specific organ or tissue. For example, 19 and 20-HETE is the major metabolite of arachidonic acid through the CYP4A family pathway and 20-HETE has a role in the regulation of vascular tone in many tissues (Gabor 2000). It could be possible that the anti-hypertension activity is related to the modulation of 20-HETE metabolism.

AD11 extract and Himalaya Goji juice showed a strong inhibitory effect on CYP4A11. AD06, W5 and W8 had moderate inhibition. Although this may be expected to provide beneficial effects on T2D and T2D complications, these products should be used carefully in obese and diabetic individuals since long term treatment with these products may lead to the development of insulin resistance.

Beside drug metabolism, CYP2 family isozymes are also involved in the activation or deactivation of many important compounds such arachidonic acid (AA) by different CYP isoforms. CYP2J2 is highly expressed in heart, and believed to play a key role of the AA metabolism in the human cardiovascular system. CYP2J3 is expressed in rat liver and heart, based on the deduced amino acid sequence, CYP2J3 is approximately 70% homologous to human CYP2J2. H9c2 cell line is a commercially available myogenic cell line derived from embryonic rat heart ventricle. Beshay et al. (2007) studied the CYP gene expression in the H9C2 cell line and reported that the expression of multiple CYP genes in H9c2 cells were at comparable levels to those expressed in the rat heart.

Neonatal rat cardiomyocytes were isolated from neonatal rat heart. These two systems offered unique *in vitro* models to study the metabolic activity of the heart.

Terfenadine is the recommended *in vitro* substrate of CYP2J2 (BDGentest), although it can be metabolized by several CYP enzymes (CYP3A4, CYP2J2, CYP4F12) and forms 3 metabolites: Hydroxyterfenadine, carboxyl terfenadine and azacyclonol, respectively.

Hydroxyterfenadine was identified as the dominate metabolite of these enzymes (Jurimari-Romet et al. 1998). However, in both H9c2 cell line and neonatal rat cardiomyocytes, CYP3A and 4F were not expressed which suggest that CYP2J3 would be the only enzyme involved in terfenadine metabolism (Beshay et al. 2007). The results found in this study showed that both H9c2 cell line and neonatal rat cardiomyocytes can metabolize terfenadine under these test conditions. Although the metabolic capability was poor under these test conditions with less than 10% of the terfenadine metabolized, the findings demonstrate that this can be a useful model to further examine these interactions. According to this study, Goji 80% ethanol extract showed no inhibition or induction effect on cardiac CYP2J3 activity *in vitro*. This result suggested that Goji extract under these conditions may not modify AA metabolism in cardiac tissue or it may modify AA metabolism through another pathway.

In summary, CYPs are found in extrahepatic tissues such as lung, kidney, gastrointestinal tract, endothelium, and heart. They play a very critical role of the oxidation of endogenous substrates which may involve the activation or deactivation of many important compounds. Inhibition will alter the metabolism or biosynthesis of many

important endogenous substances including fatty acid and estrogen, which may be involved in the development of insulin resistance and estrogen level dependent side effects respectively or contribute to some beneficial effect such as anti-hypertension and anti-breast cancer. As these CYPs are expressed in many organ and tissues, effects caused by modification on enzyme activity may be altered by the distribution of inhibitors and inducers.

4.3 Summary

In order to standardize these studies with other efforts being undertaken elsewhere in the research group, the Cree-anti diabetic herbal medicine samples were not prepared by the traditional method but as ethanolic extracts that were highly concentrated inactive secondary metabolites and the composition may differ from the traditional medicines.

The findings reported here clearly demonstrate that constituents in these herbal medicines have sufficient pharmacological activity to modify drug metabolism. Although the overall effect on drug disposition was not studied, these finding suggest a potential for interaction but are limited by the *in vitro* nature of the test systems used.

The findings of this study with extracts show that most of the traditional Cree and other NHPs have an inhibitory effect on one or more of the DMEs. Eleven Cree herbal medicines have been shown to be a MBI of CYP3A4. Combined, the results suggest that these products have the potential to affect the safety and efficacy of other Cree traditional medicines and most other conventional therapeutic products.

The TCMs and the Cree traditional medicines are prepared and used in a traditional context under the direction of a trained healer. The dose and composition of the product may be dependent upon the patient which would limit the risk of an interaction. However, if the patient self administers other health products or uses these products complementary to conventional therapeutic products, the risk of an interaction will increase.

In conclusion, most of the NHPs examined have the potential to cause drug interactions and they should be used carefully when taken together with conventional drugs. The absence of an interaction, or a low potential for an interaction in the test systems used do not assure their safety as there are numerous factors that could affect their safety and efficacy profile when consumed in the traditional form by the patient. The inhibition profiles of these traditional medicines provide important insights and warrant clinical studies to determine the clinical significance of these findings.

4.4 Future Directions

As many drugs have multiple metabolism pathways, an enzyme pool system such as the human liver microsome pool should be used in further inhibition test instead of single enzyme. Clinical drugs should also be used in the inhibition studies instead of using only pharmacologically inactive substrate.

There are still no firm conclusion regarding substrate dependent-CYP3A4 mechanism based inactivation, and only few substrates have been tested. The CYP3A4 MBI assay should be repeated with different probe substrates. Other major DMEs such as CYP2C9,

CYP2C8 and CYP2D6 need to be examined to determine if they can be deactivated by any NHPs via MBI.

Drug metabolism enzyme induction studies need to be undertaken as well, many compounds can act as both inhibitor and inducer such hyperforin from St John's wort. For these compounds they may exhibit an inhibition based NHP-drug interaction for short term expose, but they may cause an induction based NHP-drug interaction via PXR regulated gene expression. Examination of the interaction between NHPs and transporter proteins are also important. Overall, the NHPs have the potential to affect the whole drug distribution system, so a systemic study of inhibition and induction effects of both enzymes and transporters will be a great help to understand the complex natural of drug-NHP interaction.

In addition, further examination is particularly required on 'traditionally' prepared samples which are considered to have greater clinical relevance.

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6. Appendix

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