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The Effects of Cree Anti-Diabetic Natural Health Products on Drug Metabolism and Cardiomyocytes

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ABSTRACT

Seventeen Cree anti-diabetic medicinal plant were investigated for their capacity to cause adverse effects when used as alternative or complementary medicine. Two aspects of safety were studied using their extracts: the ability to affect the contraction rate of neonatal rat cardiomyocytes *in vitro* to determine if the plants can influence the human heart rate, and the ability to interfere with drug metabolism by inhibiting the cytochrome P450 enzymes *in vitro*. The results suggest that several Cree plants may cause a harmful effect through different mechanisms. The extracts of W2, W4, W5, and W9 did not affect the contraction rate of cardiomyocytes; however W9 extract was cardiotoxic at 10 μg/mL. Extracts of AD01, AD07, W2, and W4 had moderate or strong inhibitory potency towards the CYP isoforms involved in metabolizing common anti-diabetic drugs. Furthermore, AD02 extract was identified as a possible CYP3A4 mechanism-based inhibitor. Overall, the results suggest that several of the plant species can cause adverse events when used by diabetic patients.

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ABBREVIATIONS

AD	Anti-diabetic
ANOVA	Analysis of variance
AP	Action potential
BO	Bitter orange
Ca^{2+}	Calcium
CEC	3-cyano-7-ethoxycoumarin
CEI	Cree Nation of Eeyou Istchee
CYP	Cytochrome P450
DBF	Dibenzylfluorescein
DC	Diethyldithiocarbamate
DIV	Days *in vitro*
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulfoxide
EET	Epoxyeicosatrienoic acid
F12	Ham's F12 medium
FBS	Fetal bovine serum
FF	Furafylline
FMO	Flavin-containing monooxygenase
FP	Field potential
GS	Goldenseal
HPLC	High pressure liquid chromatography
IC_{50}	Median inhibitory concentration
ICH	International Conference on Harmonisation
ISI	Inter-spike interval
K^+	Potassium
KC	Ketoconazole
LDH	Lactate dehydronase
MBI	Mechanism-based inhibition
MEA	Microelectrode array
MeOH	Methanol
MFC	7-methoxy-4-(trifluoromethyl)-coumarin
Na^+	Sodium
NADPH	Nictotinamide adenine dinucleotide phosphate
NHP	Natural health product
PBS	Phosphate buffer solution
PBST	PBS with Tween 20
PI	Positive inhibitor
QD	Quinidine
ROS	Reactive oxygen species
SP	Sulphaphenazole
T2D	Type II diabetes mellitus
TC	Tranylcypromine
TCM	Traditional Chinese medicine
W	Whapmagoostui

1 INTRODUCTION

1.1 Introduction to Thesis

Natural health products (NHPs) are commonly used around the world as sources of medicine. They can be taken concomitantly with conventional pharmaceuticals as complementary medicine, or taken in replacement of conventional pharmaceuticals as alternative medicine. A group of First Nations in Quebec known as the Cree Nation of Eeyou Istchee (CEI) commonly use traditional NHPs to treat type II diabetes mellitus (T2D). Even though NHPs are commonly assumed to be safe to use because they are naturally rather than synthetically produced, NHPs can cause adverse effects in the human through various mechanisms such as directly affecting the contraction of cells that control the beating of the heart, or by interfering with the metabolism of conventional drugs. This thesis focuses on the Cree NHPs' ability to cause these adverse effects when used as complementary or alternative medicine.

1.2 Diabetes in the Cree Nation of Eeyou Istchee

Diabetes mellitus is a metabolic disease in which the body does not adequately produce and/or respond to insulin, resulting in hyperglycemia. High glucose levels affect the health and function of various organs and systems in the body giving rise to microvascular (neuropathy, retinopathy, nephropathy, cardiomyopathy) and macrovascular (heart disease, stroke, peripheral vascular disease) dysfunction in diabetics. Macrovascular diseases are also commonly referred to as cardiovascular diseases (Laakso, 1999). A patient is diagnosed with diabetes when his or her fasting plasma glucose level is greater than or equal to 7 mM (World Health Organization, 2006). Diabetes has

reached an epidemic state, affecting more than 171 million adults worldwide and is estimated to increase to 366 million by 2030 (Wild *et al.*, 2004). T2D is the most common type of diabetes, comprising approximately 90% of all cases (Canadian Diabetes Association, 2008A) and is typically developed in adulthood when the pancreas does not adequately produce insulin, and/or the body does not respond to insulin. In Type I diabetes, the insulin producing cells in the pancreas (known as beta cells) are damaged through an autoimmune response, and can no longer produce insulin; however the body can still respond to insulin. Susceptibility to T2D is much greater in aboriginal populations compared to their local non-aboriginal populations in the majority of the regions of the world including Canada (Yu & Zinman, 2007). The recent increase of the incidence in aboriginal populations has been attributed to a combination of a genetic predisposition, the consumption of a non-traditional diet, and the adoption of a sedentary lifestyle (Young *et al.*, 2000).

The CEI inhabit the northern territory of Quebec with a population of 13,500 persons (Dannenbaum *et al.*, 2005). They are one of numerous Canadian First Nations affected by the increasing incidence of T2D in aboriginal populations. The prevalence of T2D in this population is approximately four times greater than the prevalence of T2D in Quebec and Canada (20.6% vs. 4.9% and 4.7% respectively) and is increasing by 0.5% per year (Kuzmina & Dannenbaum, 2005; Public Health Agency of Canada, 2008). A contributing factor to the increasing incidence of diabetes in the CEI is their low compliance to conventional pharmaceuticals, as this treatment mode is not cultural and traditionally practiced (Leduc *et al.*, 2006). The availability of alternative or complementary anti-diabetic treatments using traditional medicinal plants would be more

culturally acceptable and thus, would provide an accepted and effective strategy that can be integrated into Cree culture to treat T2D and its symptoms.

In 2003, a collaborative research group, CIHR Team in Aboriginal Antidiabetic Medicines, was created in hopes of identifying traditional Cree medicinal plants that can be used to treat T2D and its symptoms in the CEI. This group consists of scientists, CEI elders and healers, and members of the Public Health Board of the James Bay Cree Territory. An ethnobotanical survey involving interviews with CEI elders and healers identified 17 traditional plants used to treat T2D symptoms (Leduc *et al.*, 2006). The anti-diabetic properties of extracts of the plants have been studied *in vitro* through glucose uptake, insulin secretion, adipocyte differentiation, neuroprotection, and antioxidant assays, to pharmacologically identify effective anti-diabetic species that will be used for *in vivo* and clinical studies (Spoor *et al.*, 2006; Harbilas *et al.*, in press). The successful integration of traditionally used plants in the CEI to treat T2D will not only provide a culturally accepted method, it will also provide a novel approach that can be applicable to aboriginal groups worldwide to treat T2D or other diseases in a culturally, acceptable manner.

1.3 Natural Health Products

In a recent survey conducted in 2005 by Health Canada, it found that NHPs are regularly used by 71% of Canadians (Murty, 2007) as a way to prevent or treat illness. Examples of NHPs include vitamins, minerals, herbal products, traditional Chinese medicine (TCMs), probiotics, amino acids, and essential fatty acids (Health Canada, 2005). Although NHPs are widely available and frequently used, there is a lack of research on their safety and their potential to interact with conventional pharmaceuticals.

Even though NHPs consist of natural constituents (also referred to as phytochemicals) such as flavonoids, alkaloids, diterpines, quinones, carotenoids, and phenolics, they have the same potential to cause adverse effects as synthetic pharmaceuticals. In the same survey by Health Canada, it was reported that 12% of those who use NHPs had experienced an unwanted side effect or reaction upon their use (Health Canada, 2005). Pharmaceuticals and the constituents in NHPs are often referred to as xenobiotics. Xenobiotic is a term widely used to describe any compound that is foreign to the human body and also include chemicals, carcinogens, and pollutants.

It is common for herbalist practitioners such as aboriginal healers or Western herbalists, to combine various NHPs as a therapeutic treatment for a disease because it has been claimed that different constituents of the various NHPs work synergistically to improve the treatment efficacy (Moss *et al.*, 2007). In the CEI, multiple NHPs are used in combination to treat T2D; however, due to confidentially reasons the exact entities of these formulated products are unknown (The traditional knowledge about the plant identities and the symptoms they treat have been shared. Information on how the plants are prepared, and which plants are used together are not known).

There have been many documented cases of adverse effects associated with NHP use. For example, the TCM ephedra (also known as ma huang) was a common ingredient in natural weight-loss supplements before it was banned in 2004, because it contains alkaloids such as ephedrine, which have sympathomimetic activity that promote weight loss and increasing energy (Bent *et al.*, 2003). However, these alkaloids were associated with adverse effects such as hypertension, palpitations, tachycardia, and stroke, and resulted in at least 10 deaths from 1997 to 1999 (Haller & Benowitz, 2000). Other NHPs

that have been documented to cause adverse or harmful effects to the body are licorice root (induces hypokalemia) (Chung, 2004); kava (toxic to the liver) (Dasgupta, 2003); and gingko biloba (increases the risk in bleeding) (Sadler *et al.*, 2006).

A recent survey performed in 2004 showed that 46% of the participants were using NHPs concomitantly with conventional pharmaceuticals (Kuo *et al.*, 2004). NHPs can interact with pharmaceuticals by preventing their metabolism, and consequently raising their plasma concentrations to harmful levels. One of the most notorious NHPs that has been associated with causing adverse or fatal effects by increasing the plasma concentrations of concomitant pharmaceuticals such as terfenadine, astemizole, felodipine, nifedipine and verapamil, is grapefruit juice (Flanagan, 2005). Grapefruit juice contains flavanoids and furanocoumarins like bergamottin and 6',7'-dihydroxybergamottin that potently inhibit the enzymes involved in metabolizing more than half of the drugs on the market (Guo *et al.*, 2000; Naritomi *et al.*, 2004). There have been numerous studies that have investigated the potential for NHPs to inhibit drug-metabolizing enzymes. Many NHPs have proven to inhibit these enzymes, including cat's claw, garlic, St. John's wort, goldenseal, and licorice root (Williamson, 2006; Flanagan, 2005; Chatterjee *et al.*, 2003; Kent *et al.*, 2002; Budzinski *et al.*, 2000; Zou *et al.*, 2002A).

Manufactured NHPs are under regulatory control in Canada and require manufacturers to provide information about their products including their uses, recommended doses, ingredients and adverse effects (Health Canada, 2005). However, many NHPs are not manufactured, and thus are not subject to these controls. These include practitioner formulated products, raw plant material and traditional medicinal plants used by aboriginal communities (Moss *et al.*, 2007). These products still have the

risk to cause adverse effects and should be analyzed for their potential to cause these effects.

1.4 The Cardiac Action Potential

The heart is comprised of cardiac muscle (myocardium) that consists of millions of individual cells called cardiomyocytes which work together and allow the heart to function properly by controlling its contractile activity. Cardiomyocytes exhibit automaticity, in other words, the ability to contract spontaneously by the automatic depolarization of its cell membrane. The depolarization of the cell membrane results in an action potential (AP) and the contraction of the cell. The duration of an AP is the time required for depolarization and repolarization. Cardiomyocytes are coupled together electrically through gap junctions, and therefore depolarizing electrical current from one cardiomyocyte is transferred to an adjacent cardiomyocyte, to assist in the coordinated contraction throughout the heart.

Ion currents form the basis of the depolarizing and repolarization phases of an AP and are controlled by various ion channels. In brief, a typical cardiomyocyte AP begins with the spontaneous depolarization of the cell membrane which triggers the opening of voltage-gated sodium (Na^+) and calcium (Ca^{2+}) channels to allow Na^+ and Ca^{2+} ions to enter the cell resulting in further depolarization. The Na^+ channels quickly close; however the Ca^{2+} channels are slower to close, and allow the cardiomyocyte to sustain a long, but decreasing depolarized state. The Ca^{2+} ions activate the contractile apparatus within the cell. After full depolarization, potassium (K^+) channels open and allow K^+ ions to leave the cell causing repolarization and restoration of the resting membrane potential to -85 mV. A simple illustration of an AP is depicted in **Figure 1**. The resting membrane

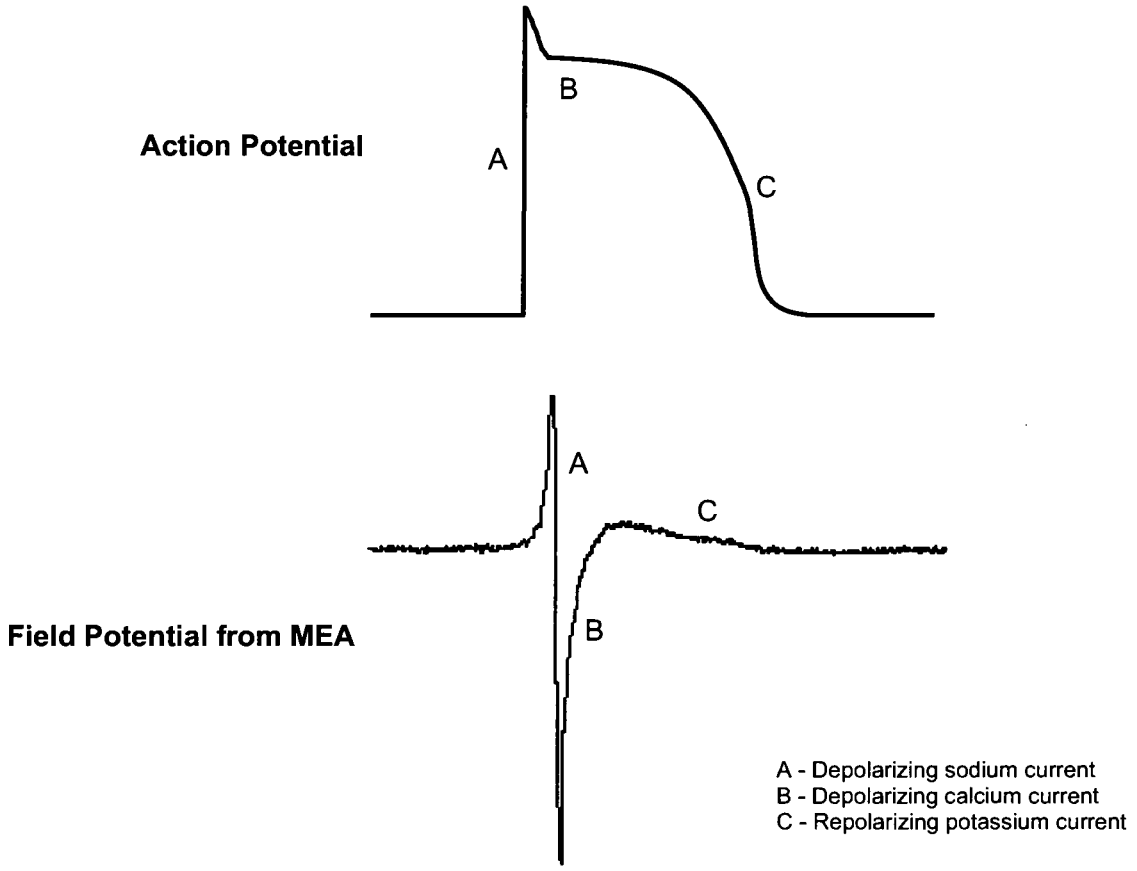


Figure 1: The relationship between an action potential and a field potential measured from a MEA. The changes in the waveforms for both the action and field potential reflect the activity of the different ion currents (A – sodium; B – calcium; C – potassium). The FP duration is correlated to the AP duration. The AP waveform is an illustration (Adapted from Meyer *et al*, 2004B).

potential is maintained through various ionic pumps and exchangers such as the Na^+-K^+ pump (Opie, 2004). The electrical currents of a cardiomyocyte can be measured using various electrophysiological methods such as patch-clamp, which measures the intracellular changes in electrical activity, and tissue culture plate microelectrode arrays, which measures the extracellular field potentials (FPs) produced by electrical activity.

1.5 Cardiac Chronotropic Effects of Xenobiotics

A xenobiotic which has an affect on the heart rate is described as having a cardiac chronotropic effect and is also referred to as a chronotrope. Many pharmaceuticals with chronotropic activity are used specifically to regulate the heart rate. The ß-antagonists such as atenolol and propranolol are used to decrease a rapid heart rate (also medically referred to as tachycardia), by inhibiting the sympathetic adrenergic-ß receptors in the heart or blood vessels. Pharmaceuticals that activate the parasympathetic muscarinic acetylcholine receptors such as carbachol, can also have a negative chronotropic effect. Xenobiotics which are able to activate these receptors are considered to have parasympathomimetic activity. Conversely, there are pharmaceuticals which can increase a slow heart rate (bradycardia) because they have sympathomimetic activity, such as the ß-agonists (isoproterenol and dobutamine). Several NHPs have been documented to have chronotropic effects. For example, ephedra (mentioned earlier) contains ephedrine and related alkaloids which possess sympathomimetic activity and has a positive chronotropic effect (Haller & Benowitz, 2000). Bitter orange (BO) contains a similar sympathomimetic alkaloid, synephrine, which has been associated with positive chronotropic properties (Bui *et al.*, 2006; Firenzuoli *et al.*, 2005; Health Products and Food Branch, 2004). Atropine, an alkaloid isolated from the plant *Atropa belladonna*, is

an antagonist of muscarinic acetylcholine receptors, and has been used medically for bradycardia among other health problems (Williams *et al.*, 1990).

There are many documented cases where a pharmaceutical is used to treat a non-cardiac health problem such as allergies or gastrointestinal disorders, but as a side effect have an influence on the activity of the heart (Vandenberg *et al.*, 2001; Malik & Camm, 2001). The prolongation of the QT interval on an electrocardiogram is one of the most common side effects. It has been estimated that approximately 3% of the drugs on the market can unintentionally cause a prolongation of the QT interval (Viskin *et al.*, 2003). In addition, there are several pharmaceuticals which can be considered toxic to the heart such as the anti-cancer drugs Gleevec and doxorubicin, and the anesthetic bupivacaine (Kerkela *et al.*, 2006; Olson & Mushlin, 1990; Mather & Chang; 2001). Recently in 2007, it was shown that the anti-hyperglycemic drug rosiglitazone, (Avandia®) a drug used to treat T2D, was found to be associated with a significant increase in the risk of myocardial infarction and death from cardiovascular disease (Nissen & Wolski, 2007).

Testing for cardiac chronotropic effects is routine during drug development because one of the three core studies in safety pharmacology is with the cardiovascular system (ICH, 2001). NHPs have the same potential as drugs to cause adverse chronotropic effects, and should undergo similar studies.

1.6 Microelectrode Arrays

Microelectrode arrays (MEAs) are used to detect the extracellular electrical activity of electrogenic cells such as cardiomyocytes. Isolated cells or sections of tissue are plated directed on the microelectrodes which measure the collective extracellular FP activity of adhered cells. The measured FP is representative of the underlying AP.

Information on the characteristics of an AP can be obtained with MEAs. For example, the different segments of the FP during an AP reflect the activity of the different ion currents involved, and the FP rise and duration time is linearly related to the AP rise and duration time respectively **(Figure 1)** (Halbach *et al.*, 2003; Meyer *et al.*, 2004B). MEAs are advantageous over other *in vitro* electrophysiological methods such as patch-clamp or single-microelectrode recording for numerous reasons: it is non-invasive and does not affect the integrity of cell membranes; the cells are kept sterile which allows long-term experiments to be conducted; it is a quick and simple method which allows many samples to be studied in a short period of time; and up to 60 different sites (from 60 microelectrodes) of a culture can be examined simultaneously providing information on conduction velocity. In addition, the cells can be grown and analyzed under physiological or pathophysiological conditions which would mimic *in vivo* conditions (Stett *et al.*, 2003). Neonatal cardiomyocytes are capable of coupling together through gap junctions to form a syncytium, a culture where the cells beat simultaneously to mimic the synchronous contractions in the myocardium (Meyer *et al.*, 2004A). Various parameters can be analyzed with MEAs such as the contraction rate, signal propagation, ion channel currents, and even the prolongation of the QT interval which is generally measured in an electrocardiogram. The diverse functions of MEAs allow them to be very useful for the acute and chronic screening of cardiac electrophysiological effects of xenobiotics, such as detecting the effects of ion channel antagonists E4031, quinidine, verapamil, and nimodipine (Halbach *et al.*, 2003; Meyer *et al.*, 2004A).

1.7 Cytochrome P450s

Cytochrome P450s (CYPs) are a family of heme-containing enzymes that are involved in the metabolism of xenobiotics. A xenobiotic is metabolized to render a metabolite more polar than its parent compound, and is easier to excrete from the body. Their most common metabolizing reaction is oxidation, but other reactions include reduction, epoxidation, methylation, dealkylation, or hydrolysis (Cedarbaum, 2006). The addition or exposure of a functional group (*i.e.,* –OH, $-NH_2$) increases the hydrophilicity of the metabolite (Yan & Caldwell, 2001). From this perspective, CYPs can be viewed as enzymes that are involved in detoxifying foreign substances that have entered the body. In addition, CYPs contribute to the synthesis or metabolism of endogenous substances such as sterols, cholesterol, fat-soluble vitamins, fatty acids, and eicosanoids (Guengerich, 2003).

CYPs are the main enzymes involved in the metabolism of pharmaceuticals, metabolizing approximately 90% of pharmaceuticals on the market, and therefore are a great area of focus in the pharmaceutical industry since CYPs can contribute to the safety and efficacy of a drug by affecting its bioavailability (Lewis, 2004). In fact, information on the specific CYPs involved in the metabolism of new drugs is required by drug regulatory bodies such as Health Canada and the Food and Drug Administration (Furge and Guengerich, 2006).

CYPs are typically found in the endoplasmic reticulum of cells and require the flavoprotein NADPH-P450 reductase to transfer electrons from reduced nictotinamide adenine dinucleotide phosphate (NADPH) during the metabolism reaction. Another protein called cytochrome b_5, can also assist in the transfer of electrons to CYPs. CYPs

are located in the majority of tissues such as the small intestine, pancreas, brain, lung, adrenal gland, kidney, skin, heart, ovaries, and testis, but they are predominantly found in the liver where an extensive amount of xenobiotic metabolism occurs (Chang & Kam, 1999; Furge & Guengerich, 2006). Xenobiotic metabolizing CYPs account for 70% of the total hepatic CYP content (Yan & Caldwell, 2001). The liver is involved in first pass metabolism, where orally ingested xenobiotics are absorbed by the digestive system, enter the hepatic portal vein, and undergo metabolism by hepatic CYPs to reduce the concentration of the drug entering the circulatory system as much as 95% (Flanagan, 2005). CYPs present in the enterocytes of the small intestine also play an important role by metabolizing xenobiotics prior to their entry to the portal vein.

CYPs are divided into families and subfamilies based on their genetic sequence homology. CYPs sharing at least a 40% or a 55% sequence identity are grouped under the same family or subfamily respectively. Each CYP isoform is designated with a numerical number in its subfamily. For example, CYP3A4 belongs to the CYP3 family, CYP3A subfamily, and is the 4th member of the subfamily. In the human genome, 57 genes coding for CYPs have been currently found, with 17 of them coding for CYPs involved in xenobiotic metabolism: 19, 1A1, 1A2, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 3A4, 3A5, and 3A7 (Guengerich, 2008). Genetic polymorphisms can exist with the xenobiotic-metabolizing CYP isoforms, especially of the CYP2D and 2C subfamilies (Anzenbacher & Anzenbacherova, 2001). Xenobiotic metabolism typically occurs with members of CYP1, CYP2, and CYP3 family, because of their low substrate specificity compared to the other isoforms with endogenous roles (Lewis *et al.*, 2006a). Nonetheless, each of these isoforms is involved in metabolizing groups of substrates with

different properties in terms of their size, charge, and lipophilicity. It is very common for a particular CYP to be greatly involved in metabolizing a specific family of drugs such as the sulfonylureas (2C9) or the calcium channel blockers (3A4) (Triplitt, 2006; Siest *et al.*, 2007). However, there are numerous drugs that are metabolized by multiple CYP isoforms such as amitriptyline (an anti-depressant) which is metabolized by CYP2C9, 2D6 and 3A4 (Ghahramani, 1997). Amitriptyline is metabolized through an N-methylation reaction by all three isoforms resulting in the formation of the same metabolite, but with some other drugs, different metabolites are formed by each CYP isoform (Lewis, 2003). CYP3A4 is the main isoform involved in xenobiotic metabolism, metabolizing approximately 50-60% of all marketed pharmaceuticals (Zhou *et al.*, 2005; Burk & Schwab, 2007). The isoforms 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7 were chosen in this study (as they are commercially available) and are described below.

CYP1A2 is a member of the CYP1A subfamily, and is known for its involvement in the metabolism of polycyclic aromatic compounds, and heterocyclic and aromatic amines into toxic compounds (Chang & Kam, 1999). Studies have shown that an up-regulation of CYP1A2 by induction from cigarette smoke and charbroiled meat can increase the production of carcinogens and the likelihood of the development of cancer (Guengerich, 2003). CYP1A2 substrates are typically planar, contain aromatic rings or heterocyclic nitrogen atom rings, and are neutral or weakly basic (Lewis, 2003). CYP1A2 is also involved in metabolizing caffeine by N-demethylation, as well as the bronchial dilator theophylline, the non-steroidal anti-inflammatory drug naproxen, and the analgesic acetaminophen (Cornelis *et al.*, 2006; Chang & Kam, 1999; Anzenbacher &

Anzenbacherova, 2001). It metabolizes approximately 4 to 8% of the drugs on the market (Anzenbacher & Anzenbacherova, 2001; Lewis, 2003).

CYP2B6 has a minor contribution to the metabolism of drugs as it metabolizes less than 3% of the drugs on the market (Anzenbacher & Anzenbacherova, 2001; Lewis, 2003). Many of its substrates are non-planar, either neutral or slightly basic, and lipophilic (Turpeinan *et al.*, 2006). It is involved in the metabolism of the anti-depressant bupropion by oxidation (Lewis 2003); cyclophosphamide (an anticancer agent) and propofol (an anesthetic) by hydroxylation; and ketamine (an anesthetic) by demethylation (Turpeinan *et al.*, 2006).

The CYP2C subfamily accounts for approximately 20% of the total hepatic CYPs (Lin & Lu, 1998). Collectively, the CYP2C subfamily metabolizes approximately 30% of the drugs on the market, and is involved in metabolizing many of the commonly used drugs (Furge & Guengerich, 2006).

CYP2C8 is involved in the metabolism of numerous glucose-lowering families of drugs such as the meglitanides and the thiazolidinediones (Triplitt, 2006; Canadian Diabetes Association, 2007). It is also involved in the hydroxylation of paclitaxel, an anticancer drug (Rahman, *et al.*, 1994). CYP2C8 also metabolizes endogenous substances such as arachidonic acid and retinoic acid (Zeldin *et al.*, 1996; Marill *et al.*, 2000; Totah & Rettie, 2005). CYP2C8 accounts for approximately 35% of the CYP2C subfamily (Lin & Lu, 1998) and its activity accounts for 5% of the metabolism of marketed drugs (Totah & Rettie, 2005). Most substrates of CYP2C8 are relatively large and are mildly acidic, basic or neutral (Totah & Rettie, 2005).

CYP2C9 is the most clinically relevant member of the CYP2C subfamily as it metabolizes more drugs than the other members and many commonly used drugs such as the anti-inflammatory drugs ibuprofen and diclofenac; the anticoagulant *S*-warfarin; and drugs from the glucose-lowering sulfonylurea family including glyburide and glimepiride (Triplitt, 2006; Canadian Diabetes Association, 2007). It is also involved in the metabolism of angiotension-II type 1 receptor blockers losartan and irbesartan, which are used to treat hypertension (Triplitt, 2006). CYP2C9 has substrate specificity toward mildly acidic and lipophilic compounds with an aromatic ring (Totah & Rettie, 2005; Lewis, 2003). CYP2C9 accounts for approximately 60% of the CYP2C subfamily.

CYP2C19 is involved in the metabolism of the proton pump inhibitors (omeprazole and lansoprazole) via hydroxylation, antiepileptics and tricyclic antidepressants (Klotz *et al.*, 2004; Goldstein, 2001; Lewis, 2003). CYP2C19 is only expressed in the liver and duodenum, and its substrates are typically mildly basic and large with an aromatic ring (Totah & Rettie, 2005; Lewis, 2003). Its expression accounts for approximately 1% of the total expression of the CYP2C subfamily (Lin & Lu, 1998) and metabolizes approximately 8% of the drugs on the market (Lewis, 2003).

CYP2D6 is an important drug-metabolizing CYP as it metabolizes approximately 25% of marketed drugs (Ingleman-Sundberg, 2005). Most CYP2D6 substrates are lipophilic bases which contain a protonable nitrogen atom (Ingleman-Sundberg, 2005). It is involved in the metabolism of cardiac drugs such as the β-blockers (carvedilol, metoprolol, and propranolol), tricylic depressants, and antipsychotics (Triplitt, 2006; Anzenbacher & Anzenbacherova, 2001). It has been proposed that CYP2D6 is involved in metabolizing alkaloids from NHPs and food due to its high affinity for these

compounds (Ingleman-Sundberg, 2005). CYP2D6 is highly polymorphic in humans and the metabolism rate of drugs that are substrates for CYP2D6 can vary 1000-fold among individuals (Anzenbacher & Anzenbacherova, 2001). These polymorphisms have been clinically shown to cause adverse events due to an ineffective or toxic plasma level of several drugs such as the anti-depressants desprimine, nortryptiline and paroxetine (Ingleman-Sundberg, 2005).

CYP2E1 is the only member of the CYP2E subfamily in the human. It is involved in the metabolism of numerous volatile anesthetics such as sevoflurane, halothane, diethyl ether and chloroform, as well as ethanol (Chang & Kam, 1999). The activity of CYP2E1 has a potential to be harmful to the body because the metabolites of several of its substrates are toxic (Cedarbaum, 2006). For example, the metabolism of ethanol produces acetaldehyde and the 1-hydroxyethyl radical, which are involved in alcohol-induced liver damage (Cedarbaum, 2006). In addition, the activity of CYP2E1 can generate reactive oxygen species (ROS) such as the superoxide anion radical and hydrogen peroxide (Cedarbaum, 2006). CYP2E1 metabolizes approximately 2 to 4% of the drugs on the market (Anzenbacher & Anzenbacherova, 2001; Lewis, 2003) and its substrates are typically small (Anzenbacher & Anzenbacherova, 2001).

The CYP3A subfamily accounts for approximately 30% of the total hepatic CYPs (Lin & Lu, 1998). High concentrations of CYP3As are found in the enterocytes of the intestine. They account for 80% of all CYPs in this organ (Burk & Schwab, 2007).

As previously mentioned CYP3A4 is the most important xenobiotic metabolizing CYP and metabolizes common drugs such as midalzolam, diazepam, clopidogrel (Plavix®), amitriptyline, cortisol, cyclosporine and erythromycin (Lewis, 2003; Clarke &

Waskell, 2003; Guengerich, 2003). CYP3A4 is involved in the metabolism of numerous drugs used by diabetics such as the Ca^{2+}-channel blockers (verapamil, diltiazem, and felodipine), the HMG-CoA reductase inhibitors (lovastatin and simvastatin), and the angiotension-converting enzyme inhibitors (enalapril, ramipril, perindopril and lisinopril) (Triplitt, 2006; Tracy *et al.*, 1999; Kroemer *et al.*, 1993). The substrates of CYP3A4 are relatively large, are neutral or weakly basic, and contain an aromatic ring system (Lewis, 2003).

CYP3A5 can metabolize many of the same drugs as CYP3A4 with an equal or reduced capability (Andrew Williams *et al.*, 2002). However, CYP3A5 expression is typically one-third to one-quarter of CYP3A4 expression (Lin & Lu, 1998) and therefore, does not contribute as much as CYP3A4 to xenobiotic metabolism. CYP3A5 is expressed in all human kidneys, unlike CYP3A4 which is expressed in only some human kidneys (Haehner *et al.*, 1996).

CYP3A7 is expressed in the liver during the fetal stages of life and has been thought to be absent in the adult (Lin & Lu, 1998). However, several studies have shown that CYP3A7 is expressed in more than half of adult livers but at lower levels relative to CYP3A4 (Tateishi *et al.*, 1999; Schuetz *et al.*, 1994). Its substrates are very similar to CYP3A4; however the activity of CYP3A7 is much lower (Andrew Williams *et al.*, 2002).

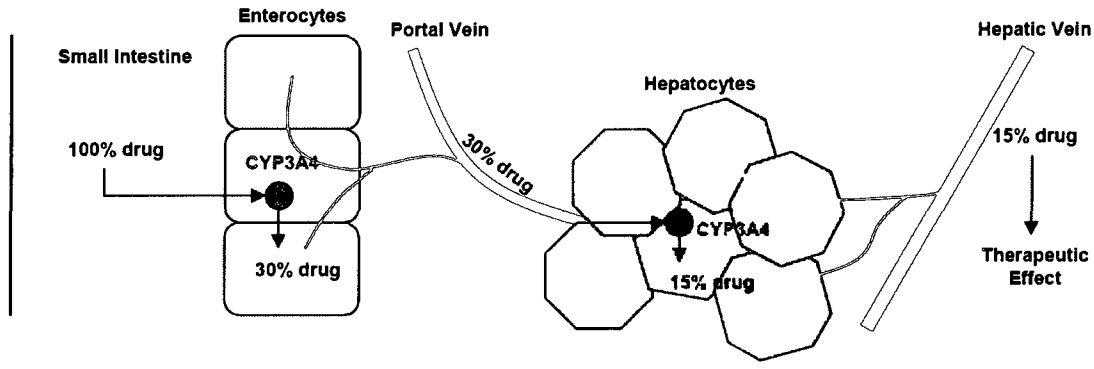
Changes in the expression or activity of CYPs have been known to occur in numerous disease states including diabetes (Cheng & Morgan, 2001). However, changes in the expression of CYPs in diabetic patients have not been extensively studied, especially with the clinically important CYP isoforms 2C9, 2D6, and 3A4 (Cheng &

Morgan, 2001). Several studies have indicated that CYP1A2 and 2E1 activity are increased in diabetic patients (Matzke *et al.*, 2000; Wang *et al.*, 2003). In addition, CYP3A4 activity was shown to decrease with obesity, a common physical state associated with diabetes (Kotlyar & Carson, 1999). Studies conducted with diabetic animals have shown an increase in expression of CYP2E1 and CYP1A and 1B isoforms, and a decrease in CYP2A and 3A activity (Borbas *et al.*, 2006; Cheng & Morgan, 2001). In addition, the activity of flavin-containing monooxygenases (FMOs), another family of drug-metabolizing enzymes, is enhanced in diabetic animals due to the decrease in insulin, a regulator of FMO (Borbas *et al.*, 2006). In the human, FMOs are involved in the monooxygenation of several pharmaceuticals such as cimetidine, ketoconazole, ranitidine, clozapine and tamoxifen (Zhou & Shepard, 2006; Cashman, 2000). These findings suggest that diabetes itself can have an affect on drug metabolism and bioavailability, by altering the activity of drug-metabolizing enzymes.

1.8 Cytochrome P450 Inhibition

The activity of CYPs can be inhibited by xenobiotics, and because CYPs are the main enzymes involved in drug metabolism, CYP inhibition can have a major impact on the bioavailability of a drug by changing the drug's concentration in the blood **(Figure 2)**. Adverse effects can occur when a drug's plasma concentration is not within its therapeutic concentration range. For drugs with narrow therapeutic windows (a small difference between therapeutic, toxic and ineffective concentrations) such as warfarin or cyclophosphamide (Korhonen *et al.*, 2007), interfering with their metabolism can be very harmful or even fatal. In addition, for drugs which are used chronically or for life-sustenance such as antiarrhythmic ß-blockers, interference of their normal metabolism by

A)



B)

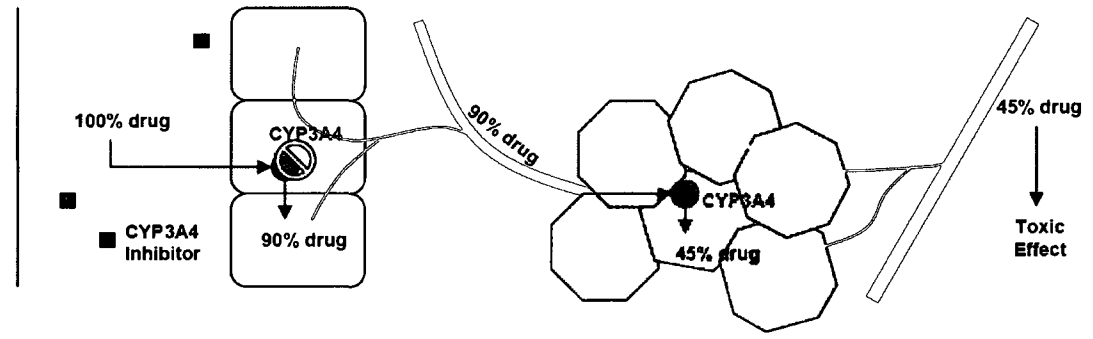


Figure 2: An illustration showing the typical metabolism of an orally administered active drug by CYP3A4 in intestinal enterocytes and liver hepatocytes in the absence and presence of CYP3A4 inhibitors. Typically, the drug is transported from the small intestines to the enterocytes where it can undergo metabolism by CYP3A4. The remaining drug travels through the portal vein to the liver, where it can undergo further metabolism by CYP3A4 in the hepatocytes. The remaining drug then leaves the liver via the hepatic vein and circulates to its target. In the absence of CYP3A4 inhibitors **(A)**, the normal and expected metabolism of a drug occurs and its therapeutic dose is obtained. In the presence of CYP3A4 inhibitors **(B)**, there is a decrease in the drug's metabolism resulting in an overdose of the drug.

CYP inhibition would also have serious effects. Chronic or repeated use of a CYP-inhibiting xenobiotic can have a greater disastrous effect compared to its acute use, as an increasing amount of CYPs would be inactivated.

CYP inhibition can result in either an acute overdose or an underdose of a pharmaceutical. Pharmaceuticals which are pharmacologically active upon its administration (active drugs), require CYP metabolism for clearance into inactive metabolites. Inhibition of the CYP-mediated metabolism would increase the plasma concentration of the active drug and the duration of its pharmacologic efficacy, resulting in a drug overdose, especially with repeated drug administration. Pharmaceuticals can also be administered as pharmacologically inactive drugs (prodrugs) such as the antiplatelet agent Plavix® and the antihypertensive drug enalapril (Clarke & Waskell, 2003; Rautio *et al.*, 2008), and require its metabolism by CYPs to form active metabolites. Inhibition of the CYPs involved in the formation of the active metabolites, would decrease the concentration of the active metabolites in the plasma and hence, an underdose of the drug would occur.

Enzyme inhibition is classified as reversible or irreversible. In reversible inhibition, the inhibitor reduces the effective activity of the enzyme by binding to the active site to prevent a substrate from accessing it (competitive inhibition), or by binding to an allosteric location to alter the catalytic activity without affecting substrate binding (non-competitive inhibition). Inhibition can usually be reversed by increasing the concentration of the substrate to displace the inhibitor; however, very potent inhibitors such as ketoconazole (CYP3A4) and quinidine (CYP2D6) can be difficult to displace and can cause "permanent" inhibition of the enzyme (Lin & Lu, 1998).

Irreversible inhibition differs from reversible inhibition because it involves the metabolic activation of the inhibitor by the CYP, rather than simple binding interactions between the inhibitor and the CYP. It is commonly referred to as mechanism-based inhibition (MBI) or suicide inhibition. In MBI, a substrate is metabolized by a CYP to form a reactive intermediate metabolite which then irreversibly inactivates the same CYP through the formation of covalent bonds, before it can even exit the active site. This substrate is referred to as a mechanism-based inhibitor. MBI is more harmful than reversible inhibition because *de novo* synthesis of enzyme is required to restore basal CYP activity. The average time required to synthesis new CYP is 8 to 12 hours (Lilja *et al.*, 2000; Takanaga *et al.*, 2000; Rogers *et al.*, 1999); however, it has been suggested that it may take one to three days for a substantial recovery from MBI of CYPs based on clinical studies (Flanagan, 2005; Greenblatt *et al.*, 2003). There also exists quasi-irreversible inhibition, where active intermediate metabolites form reversible complexes with the CYP, but these complexes are extremely stable and are irreversible *in vivo* (Lin & Lu, 1998). Mechanism-based inhibitors have been discovered for the majority of the xenobiotic-metabolizing CYP isoforms except for CYP2C18, 3A7, and 19 (Fontana *et al.*, 2005; Zhao *et al.*, 2002; Kartha & Yost, 2008; Lafite *et al.*, 2007). Examples of mechanism-based inhibitors include erythromycin, clarithromycin, tamoxifen, fluoxetine, ritonavir, diltiazem, several furanocoumarins in grapefruit juice, hydrastine in goldenseal (GS), and glabridin in licorice root (Lian-Qing *et al.*, 2000; Naritomi *et al.*, 2004; Zhou *et al.*, 2004; Chatterjee & Franklin, 2003; Sridar *et al.*, 2004). Identifying mechanism-based inhibitors is performed by testing for NADPH-, time-, and concentration-dependence of

the inhibitor. In reversible inhibition, the inhibition is independent of NADPH and time because covalent modifications of the CYPs are not involved.

CYP inhibition is one of the main causes of drug-drug or drug-NHP interactions because of the ability for a single CYP isoform to metabolize many different xenobiotics. Serious adverse effects can occur with drug-drug or drug-NHP interactions including death, and therefore new drug submissions do require information on the drug's ability to inhibit CYPs (Furge and Guengerich, 2006).

1.9 Microtitre Fluorometric Assays as an In Vitro Technique to Screen for Cytochrome P450 Inhibition

Various *in vitro* techniques have been developed to examine CYP inhibition using high pressure liquid chromatography (HPLC), microtitre fluorometric or luminescent assays, radioenzymatic activity assays, and antibody-based assays such as ELISA. The high-throughput and sensitive technique of microtitre fluorometric assays allowing multiple samples to be examined in a short period of time, as well as its extensive documented use in the literature and correlation to *in vivo* and other *in vitro* results, were among the reasons for choosing this method to screen for CYP inhibition for the studies in this thesis (Donato *et al.*, 2004; Miller *et al.*, 2000; Yan & Caldwell, 2001; Yamamoto *et al.,* 2002). For these assays, the activity of the CYPs is determined by the fluorescence conversion of a CYP substrate to a fluorescent product. In the presence of an inhibitor, the CYPs would be less active and less fluorescence would be generated in the assay. The main disadvantage of this method is that the assayed sample may have intrinsic fluorescence and mask the actual inhibition providing false results. On the other hand, the sample may quench the fluorescence and provide false inhibitory results. For the majority

of drugs, this is not an issue as they are typically nonfluorescent. However, many extracts from plants contain coloured pigments such as betalains, carotenoids, and anthocyanins (Grotewold, 2006) and can interfere with the assay results. Zou *et al.* (2000B) reported that several pure constituents found in popular herbal products (isorhammetin, quercetin, vitamin, and yangonin) interfered with these assays by intrinsic fluorescence or quenching.

Various sources of CYPs are available for *in vitro* studies such as isolated human liver microsomes, human hepatocytes, and cDNA-expressed human CYPs (Supersomes). For the studies of this thesis, cDNA-expressed CYPs were used because unlike the other CYP sources, Supersomes express only one isoform of CYP and therefore, screening can be performed for each isoform. However, Supersomes require the co-expression of NADPH-P450 reductase or cytochrome b_5, which are present in concentrations that do not reflect normal biological concentrations (Glue & Clement, 1999). For the purpose of basic screening, the use of cDNA-expressed CYPs is the ideal method.

1.10 Rationale, Hypotheses, and Objectives

1.10.1 Rationale

Traditional healing using traditional medicinal plants is still widely practiced in the CEI and is commonly used by the diabetics either as alternative or complementary medicine to treat their symptoms of T2D. Recently, the extracts of 17 species of plants used by the CEI to treat T2D have been identified and studied to pharmacologically identify their anti-diabetic properties to rank which species have the greatest potential to treat T2D effectively. However, the safety of these plant extracts has not been examined and is important to determine to avoid possible adverse effects upon their use.

Two different types of safety studies were conducted for this thesis. The first study examined the effect the Cree plant extracts had on the heart rate. It is important to examine their chronotropic effects because an elevated resting heart rate has been associated with an increase in risk of mortality in type II diabetics (Stettler *et al.*, 2007; Linnemann & Janka, 2003). In addition, the condition of the heart in diabetics is typically unhealthy due to heart disease, diabetic cardiomyopathy, or other illnesses that affect cardiac function (Canadian Diabetes Association, 2008B; Ren *et al.*, 1999). It is reasonable to expect that diabetic cardiomyocytes would be more sensitive to potential chronotropic effects of NHP compounds due to their unhealthy state. Heart disease is one of the leading causes of death in type II diabetics, contributing to approximately 80% of all deaths (Laakso, 1999; Canadian Diabetes Association, 2008B). It is important to ensure that the NHP compounds do not exacerbate cardiomyopathy.

The second study examined the ability for NHP compounds within the extracts to inhibit the drug-metabolizing enzymes known as CYPs. These enzymes metabolize many of the drugs used by diabetics, and their inhibition can cause adverse drug effects by altering the plasma level of the drug from a therapeutic concentration to a toxic or ineffective concentration. In both safety studies, only the extracts of single NHPs were studied, rather than a combination of multiple NHPs (formulated products).

1.10.2 Hypotheses

1) The Cree anti-diabetic NHP compounds will have a cardiac chronotropic effect.
2) The Cree anti-diabetic NHP compounds will interact with the anti-hyperglycemic drug metformin and affect the contraction rate of cardiomyocytes.
3) The Cree anti-diabetic NHP compounds will inhibit the activity of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7.
4) The Cree anti-diabetic NHP compounds will interact with the anti-hypertensive drug enalapril to change the NHP's CYP3A4 inhibitory potency.

1.10.3 Objectives

1) To determine the toxic concentrations of extracts of the Cree anti-diabetic NHP compounds with neonatal rat cardiomyocytes.
2) To test the chronotropic effects of the extracts using neonatal rat cardiomyocytes by measuring their rate of contraction upon treatment.
3) To determine the chronotropic effect of a combination of an extract with metformin.
4) To test the chronotropic effects of the extracts on diabetic-like neonatal rat cardiomyocytes.
5) To determine the inhibitory potencies of the 17 extracts against 10 CYP isoforms (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7) *in vitro*.
6) To identify if any of the 17 extracts are mechanism-based inhibitors of CYP3A4.
7) To assess whether the inhibitory potencies of several extracts are influenced by enalapril.

2 MATERIALS AND METHODS

2.1 Plant Extract Preparation

2.1.1 Collection and Storage of the Cree Plants

Samples of plant material belonging to the 17 Cree anti-diabetic plant species were collected in the two Cree communities Mistissini and Whapmagoostui by various members of the CIHR Aboriginal Team for Anti-diabetic Medicines. The samples were dried and stored at 4°C until they were used to prepare extractions. The 17 plant species were assigned accession codes based on their source of origin. The eight plant species collected in Mistissini, which were the plant species that underwent the first series of anti-diabetic assessment tests (Spoor *et al.*, 2006) were assigned the accession codes "AD01, AD02, AD03, AD06, AD07, AD08, AD09, and AD11". The AD represents "anti-diabetic". The nine plant species collected in Whapmagoostui, which underwent the second series of anti-diabetic assessment tests (Harbilas *et al.*, in press) were assigned the accession codes "W1, W2, W3, W4, W5, W6, W7, W8, and W9". The W represents "Whapmagoostui". The name of the plants, the plant parts used, and their accession codes are provided in **Table 1**.

2.1.2 Extract Preparation of the Cree Plants

Ethanol extracts of the Cree plants were prepared by members of Dr. John Thor Arnason's lab at the University of Ottawa as described in Spoor *et al.* (2006). These extracts were provided in solidified form and required dissolution in organic solvents. For the cardiotoxicity assays, the extracts were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at a concentration of 1 mg/mL, aliquoted into 0.5 mL microfuge tubes, and stored in -20°C. On experimental days, an aliquot was removed from the freezer,

Table 1: A list of the species names of the 17 Cree anti-diabetic plants. The plant parts used, and their accession numbers are provided.

Plant Species	Plant Part	Accession Number
Rhododendron groenlandicum	Leaves	AD01
Abies balsamea	Bark	AD02
Larix laricina	Bark	AD03
Picea mariana	Cones	AD06
Sorbus decora	Bark	AD07
Alnus incana	Bark	AD08
Sarracenia purpurea	Leaves	AD09
Pinus banksiana	Cones	AD11
Rhododendron tomentosum	Leaves	W1
Kalmia angustifolia	Leaves	W2
Picea glauca	Leaves	W3
Juniperus communis	Fruit	W4
Salix planifolia	Bark	W5
Lycopodium clavatum	Whole Plant	W6
Populus balsamifera	Bark	W7
Gaultheria hispidula	Leaves	W8
Vaccinium vitis-ideae	Fruit	W9

thawed, and added to fresh media or Tyrode's buffer at the required concentration. These extracts were tested within three months of preparation. For the CYP inhibition assays, the extracts were dissolved in 100% methanol (MeOH) (Fisher Scientific) at a stock concentration of 10 mg/mL. A diluted stock solution of 2 mg/mL was prepared from the stock concentration using 100% MeOH. These solutions were stored at -20°C. On experimental days, the diluted stock solution was diluted ten-fold with water to produce a fresh working solution of 0.2 mg/mL. Stock solutions were used within two weeks of preparation, and the working solutions were discarded after its use at the end of the day.

2.1.3 Extract Preparation of Bitter Orange and Goldenseal

BO (*Citrus aurantium*) and GS (*Hydrastis canadensis*) extracts were prepared in a different manner than the Cree plant extracts. These extracts were prepared fresh for each experiment. BO in the form of ground powder from the company Solaray was purchased from a local nutrition store (Nutrition House) in capsule form. In order to obtain a representative sample, six capsules were opened and the ground contents were combined. GS was obtained in ground form from Kentz Farm in Waterloo, ON courtesy of Renée Leduc from Dr. Arnason's lab. Extracts were prepared by measuring an initial amount of ground material in a 1.5 mL microfuge tube. The desired solvent at a volume of 1 mL was then added (water for BO, 55% MeOH for GS) and vortexed at setting eight for one minute. The extracts were then centrifuged for 15 minutes at 12,000 rpm to separate the extract from the undissolved bulk plant material. The mean concentration of BO and GS extract produced (in mg/mL) from each preparation was then determined **(Refer to Appendix A1).**

2.2 Cardiac Chronotropic Effect Studies

2.2.1 Neonatal Rat Cardiomyocyte Cell Culture

Isolated neonatal rat cardiomyocytes (1-2 days old) were obtained from Lonza as cryopreserved vials. The cells were thawed in a 37°C water bath for 2.75 minutes and transferred to a 15 mL centrifuge tube. Dulbecco's modified essential medium combined with Ham's F12 medium at a 1:1 ratio (DMEM/F12) (Invitrogen) containing 7.5% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 7.5% horse serum (Invitrogen), 1% penicillin / streptomycin (Invitrogen), and 5 mM HEPES (Fisher Scientific) was added to the cells. The cells were then plated in sterile MEAs (Multi Channel Systems), 24-well plates, or 96-well plates at densities of 1.5×10^6, 1×10^6, or 5×10^5 cells/mL respectively. The MEAs used were the EcoMEA type (Multi Channel Systems) which consists of 60 gold microelectrodes of 100 μM diameter separated by 700 μM of space located at the bottom of an enclosed culture well **(Figure 3).** White plastic caps were used to cover the wells of the MEAs during incubation. The covered MEAs were placed in sterile glass petri dishes (10 cm in diameter, 2 cm in height) (VWR International). Cells were cultured in a humidified incubator with an atmosphere of 5% CO_2 and 95% air in 37°C. After four hours, 80% of the media was replaced with fresh media. Media changes were performed every 2nd or 3rd day. For experiments using high glucose media, the normal media (glucose concentration of 17.5 mM) was supplemented with extra glucose to obtain a glucose concentration of 25.5 mM. For experiments using low glucose media, the media was prepared in a different manner than the normal and high glucose media because the normal media already exceeded the desired glucose concentration. The low glucose media (5 mM) was prepared by combining DMEM (without glucose) and F12 in a 1:1

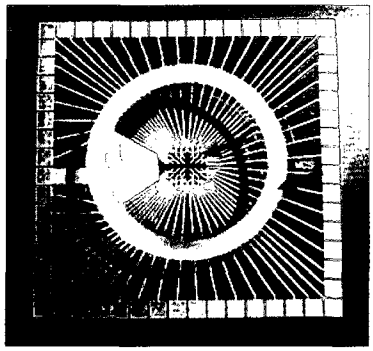


Figure 3: **An image of the microelectrode array (EcoMEA) used for the cardiomyocyte experiments.** The red arrow points to the location of the 60 microelectrodes which is surrounded by an enclosed well indicated by the blue arrow.

ratio, followed by the addition of the other constituents.

2.2.2 Recording of Extracellular Field Potential Activity

The MEA system is a device that allows the *in vitro* recording of the extracellular FP activity of cardiomyocytes that have adhered to the microelectrodes of a MEA. To record this activity, a MEA in its glass culture dish was removed from the incubator, and placed into a sterile biological hood. The MEA was then removed from its glass petri dish and connected to a MEA60 System (Multi Channel Systems) with a MEA heater set at 37°C, and also in the biological hood. Recordings from the MEA were then performed. Signals were recorded at 25 KHz, visualized and stored using the software MCRack (Multi Channel Systems). Spike and contraction information was converted to AxoScope Binary Format using the software MC DataTool (Multi Channel Systems) for visual analysis using the software AxoScope (Molecular Devices). Since there are 60 microelectrodes in each MEA, up to 60 FP activities from different areas of the culture can be examined simultaneously **(Figure 4A).** However, due to the enormous amount of data generated, only select microelectrodes with the highest FP amplitudes and synchronous AP activity were examined in the experiments.

2.2.3 Measuring the Field Potential Duration

The FP duration ($FP_{duration}$) was measured from the highest peak (FP_{start}) of the depolarization phase to the point where the activity of the repolarization phase reaches the baseline (at 0 μV) (FP_{end}) **(Figure 4B).**

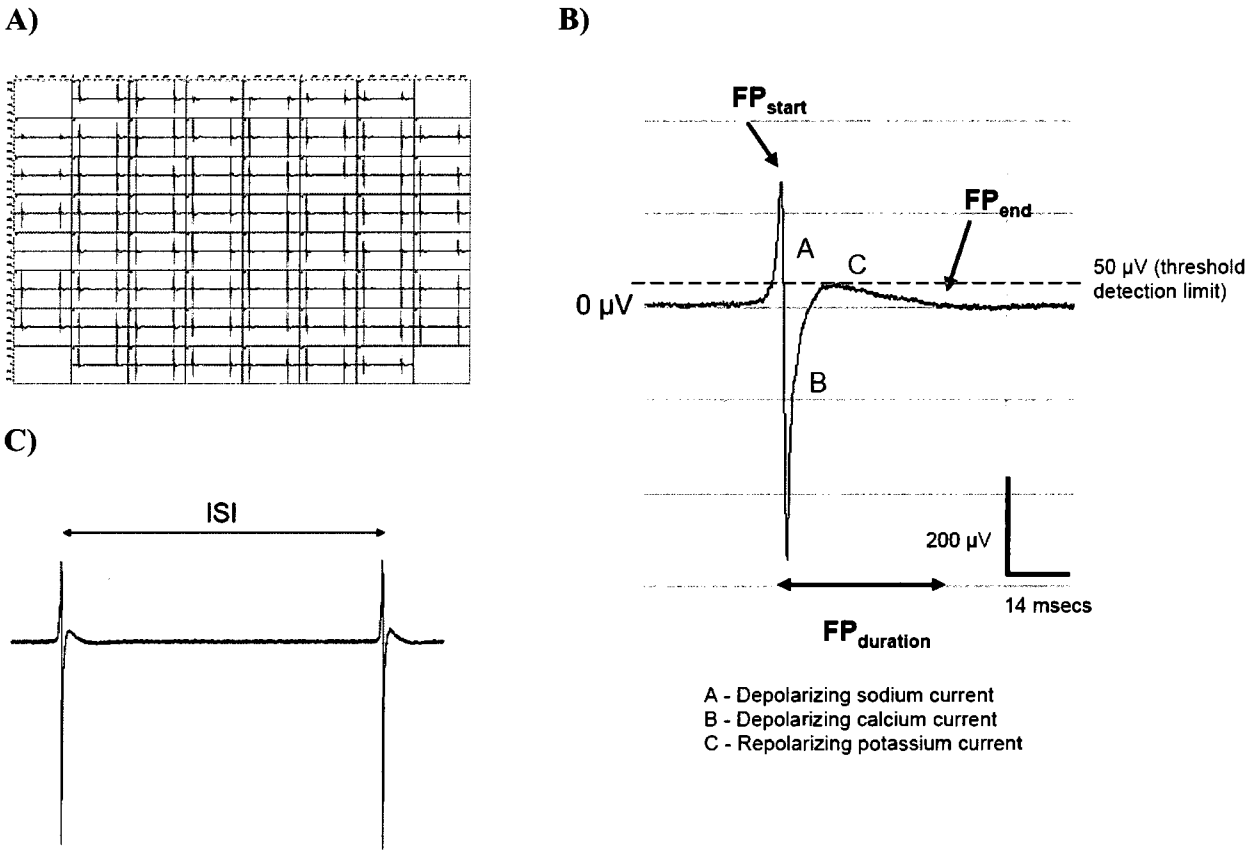


Figure 4: Illustrations of the extracellular field potential activity obtained using the MEA system. A screen view of the FP activity measured from the 60 microelectrodes during one second of a recording is shown in **(A)**. A magnified image and a description of the FP activity changes that occur during an AP are shown in **(B)**. The inter-spike interval (ISI) between two adjacent contractions is shown in **(C)**.

2.2.4 Acute Chronotropic Effects

A MEA with a culture of cardiomyocytes at 5 to 7 days *in vitro* (DIV) was removed from the incubator and connected to the MEA system. A one minute recording of the electrical activity was performed to confirm that the contraction rate of the cardiomyocytes was within the range of 60 to 130 contractions/minute. Only cardiomyocytes which satisfied this criteria were used. The media was then removed and exchanged with 1 mL of pre-warmed Tyrode's buffer (143 mM NaCl, 0.3 mM $NaH_2PO_4 \cdot H_2O$, 5.4 mM KCl, 1.1 mM, $MgCl_2$, 5 mM HEPES, 1.8 mM $CaCl_2$, 5 mM glucose). The media was saved and preserved at 37°C. After one minute of exposure to Tyrode's buffer, a one minute recording was performed and referred to as "Tyrode's Buffer". A volume of 300 µL of the buffer was then removed from the MEA, mixed with the test sample (drug, extract, or vehicle control), and then slowly re-added to the MEA. The volume of the Tyrode's buffer was adjusted before adding the test sample so that the volume re-added was 300 µL. After one minute of treatment, a one minute recording was immediately performed and referred to as the sample. The sample was washed out once by replacing the buffer in the MEA with fresh Tyrode's buffer using 1 mL exchanges. The buffer was then replaced with the initial media using 1 mL exchanges. The MEA was then placed back into its glass petri dish and brought back to the incubator. For one culture, the contraction rate obtained from a treatment with the test sample was normalized to its contraction rate in "Tyrode's buffer". Each culture of cardiomyocytes was typically tested once and then discarded. If the cardiomyocytes were used again, they were used 24 hours later and then discarded. Typically, up to eight cultures of cardiomyocytes in

MEAs were sequentially analyzed. The drugs isoproterenol (Sigma-Aldrich) carbachol (Sigma-Aldrich), and metformin (Sigma-Aldrich) were dissolved in water.

2.2.5 Chronic Chronotropic Effects

A MEA with a culture of cardiomyocytes was removed from the incubator and connected to the MEA system. The media was exchanged with fresh media (same as the culture media) containing the desired concentration of the test sample using 1 mL exchanges. A one minute recording was performed and referred to as "initial". The cardiomyocytes were then placed back in the incubator for an 18 hour chronic treatment with the test sample. The MEA was then removed from the incubator and connected to the MEA system for a one minute recording to determine if there were any chronic effects on the contraction rate from the sample. The sample was then washed out once with fresh media, followed by the addition of fresh media both using 1 mL exchanges. Another one minute recording was performed and referred to as "recovery". For one culture, the contraction rate obtained from a treatment with the test sample was normalized to its contraction rate during "initial". The recovery contraction rate obtained from "recovery" was normalized to the contraction rate during "initial".

2.2.6 Cytotoxic Extract Concentrations

Cardiomyocytes were plated at 5 x 10^5 cells/mL per well in 96-well plates. At 5 DIV a range of extract concentrations (1 – 600 μg/mL) in fresh media was added to the cardiomyocytes. The final DMSO concentration was 1 μL/mL for the extract concentrations that were less than 100 μg/mL. For the higher extract concentrations, the final DMSO concentrations increased by 1 μL/mL for each additional 100 μg/mL of

extract. After 18 hours of treatment, cell viability was assessed by measuring the amount of lactate dehydronase (LDH) released into the media using a LDH kit (Promega). Briefly, supernatants were collected, transferred to 1.5 mL microfuge tubes, and centrifuged for 4 minutes at 18,000 rpm. Supernatants (50 μL) were then transferred to the wells of a 96-well plate with white walls (Corning) in triplicates. The substrate solution provided with the kit was added (50 μL) and allowed to incubate at room temperature in the absence of light. After 30 minutes, the stop solution was added (50 μL) and the absorbance was read at 409 nm in a VERSAmax Microplate Reader. The background absorbance from a media background control was subtracted from the measured absorbance values obtained for each of the extract samples. A maximum LDH release by cell lysis was used as a positive control. The cytotoxicity was calculated by the percent of LDH released in the presence of the extract relative to the DMSO vehicle control:

$$LDH\ \mathrm{Re}lease = \frac{(Absorbance\ in\ the\ presence\ of\ the\ extract - Background\ absorbance\ from\ media)}{(Absorbance\ in\ the\ presence\ of\ DMSO - Background\ absorbance\ from\ media)} \times 100\%$$

2.2.7 Immunohistochemistry

The media was removed from cardiomyocytes plated in 24-well plates, and fixed with 1.6% paraformaldehyde in Lana's fixative for 20 minutes. The cardiomyocytes were then rinsed with phosphate buffer solution (PBS) (8.1 mM $NaHPO_4$, 1.9 mM $NaH_2PO_4 \cdot H_2O$, 136.9 mM NaCl, 2.7 mM KCl) five times. The cardiomyocytes were stained with monoclonal anti-α-actinin (1:1000, mouse) (Sigma-Aldrich) and connexin-43 (1:400, rabbit) (Invitrogen) primary antibodies in 0.3% Triton X-100 PBS with Tween 20 (PBST) overnight at 4°C in the absence of light. The cardiomyocytes were then rinsed as previous with PBS. The cardiomyocytes were then incubated with Alexa 594 labelled donkey anti-

rabbit (1:200) (Invitrogen), Alexa 488 labelled goat anti-mouse (1:100) (Invitrogen) secondary antibodies, and Hoescht solution (1:10000) (Sigma-Aldrich) in 0.3% Triton X-100 PBST for 30 minutes at 37°C in the absence of light. The cardiomyocytes were then rinsed as previous. Images were captured under a fluorescence microscope and a Q image digital camera.

2.2.8 Statistical Analysis

In most cases, statistical analysis were performed using one-way analysis of variance (ANOVA) followed by the Tukey test using the statistical program StatsDirect. A two-way ANOVA followed by the Tukey test was performed for the studies involving the multiple media with different glucose concentrations, using the statistical program StatsDirect. The Student's t-test was performed to determine if metformin had a significant effect on the contraction rate relative to a water vehicle control using SigmaPlot. A p-value ≤ 0.05 would indicate that the null hypothesis is rejected, suggesting a significant difference between the compared values. The null hypotheses tested for the cytotoxicity assays was: no difference in the release of LDH between the extract and the vehicle control. The null hypothesis tested for the chronotropic screening assays was: no difference in the chronotropic effect between the test sample (drug or extract) and the vehicle control. The null hypothesis tested for the production of diabetic-like cardiomyocytes study was: no difference in the FP duration between the different media. The null hypothesis tested for the long-term FP duration study: no difference between the FP duration at 5 DIV and the FP duration at 13, 28 or 35 DIV.

2.3 Cytochrome P450 Inhibition Studies

2.3.1 Assessing the Inhibition of Cytochrome P450s

Microtitre fluorometric assays were used to determine the inhibition of 10 CYP isoforms by the Cree plant extracts. The protocol was adapted from Budzinski *et al.* (2005) and Scott *et al.* (2006). Microsomes expressing cDNA-expressed human CYPs which were obtained from baculovirus infected insect cells (BD Biosciences) were used for the experiments. Also known as Supersomes, these microsomes also express cDNA-expressed human P450 reductase. The CYP isoforms tested were: 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7. The CYP2C8, 2C19, 2E1, and 3A7 Supersomes also expressed cDNA-expressed human cytochrome b_5. The substrates used were 3-cyano-7-ethoxycoumarin (CEC) (BD Biosciences) (1A2, 2C19), 7-methoxy-4-(trifluoromethyl)-coumarin (MFC) (BD Biosciences) (2B6, 2C9, 2E1), dibenzylfluorescein (DBF) (BD Biosciences) (2C8, 3A4, 3A5, 3A7), and AMMC (BD Biosciences) (2D6), and were dissolved in acetonitrile. The positive inhibitors used were furafylline (BD Biosciences) (1A2), tranylcypromine (Sigma-Aldrich) (2B6, 2C19), ketoconazole (Calbiochem) (2C8, 3A4, 3A5, 3A7), sulphaphenazole (Sigma-Aldrich) (2C9), quinidine (Sigma-Aldrich) (2D6), and diethyldithiocarbamate (Sigma-Aldrich) (2E1), and were dissolved in 100% MeOH. The concentrations of the positive inhibitors tested were based on the concentrations used in similar *in vitro* studies (Totah & Rettie, 2005; Ghosal *et al.*, 2003; Donato *et al.*, 2004). Assays were performed using 96-well plates with clear, flat bottoms and white walls (Corning). All test samples were diluted ten-fold with water before the assays were initiated. In these assays, three separate solutions were prepared and added to the wells. Wells were designated as "control,"

"control blank," "sample," or "sample blank." The control represented the vehicle control (10% MeOH), whereas the sample represented the extract or positive control drug. Solution A contained 1.08 mM NADPH (Sigma-Aldrich), and the substrate in 0.25 M potassium phosphate buffer solution (buffer, pH 7.4). Solution B contained the enzyme in 0.13 M buffer solution. Solution C was identical to Solution B but instead contained denatured enzyme rather than active enzyme ("blank"). A volume of 100 µL of Solution A was added to each well. The diluted sample was then added into each well at a volume of 10 µL and hence, the final volume of 100% MeOH added to each well was only 1 µL. 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 µL. The plate was inserted into a Cytofluor4000 Plate Reader (Applied Biosystems) mechanically, shaken for three seconds and then the initial fluorescence ($T_{initial}$) was measured at various excitation and emission wavelengths depending on the substrate used. The plate was then incubated at 37°C for 20 to 60 minutes depending on the isoform tested and then final fluorescence (T_{final}) was measured. The following formula was used to determine % inhibition:

$$\left[1-\left(\frac{(Sample_{Tfinal}-Sample_{Tinitial})-(Sample\ Blank_{Tfinal}-Sample\ Blank_{Tinitial})}{(Control_{Tfinal}-Control_{Tinitial})-(Control\ Blank_{Tfinal}-Control\ Blank_{Tinitial})}\right)\right]\times 100\%$$

The total volume of solution per well was 200 µL and was performed in triplicate. The final volume of extract tested per well was 10 µg/mL. All experiments were performed in the absence of light. For a complete description of experimental conditions for each isoform, refer to **Table 2**.

Table 2: Description of the experimental condition for the CYP inhibition studies (CEC: 3-cyano-7-ethoxycoumarin; MFC: 7-methoxy-4-(trifluoromethyl)-coumarin; DBF: dibenzylfluorescein).

CYP isoform	CYP Concentration (nM)	Substrate	Substrate Concentration (μM)	Positive Inhibitor (μM)	Positive Inhibitor Concentration (μM)	Excitation (bandwidth) (nm)	Emission (bandwidth) (nm)	Gain	Incubation Time (min)
1A2	5	CEC	25	Furafylline	50	409	460	50	40
2B6	25	MFC	100	Tranylcypromine	1000	409	530	85	40
2C8	15	DBF	2	Ketoconazole	10	485	530	60	60
2C9[a]	70	MFC	100	Sulphaphenazole	100	409	530	80	60
2C19	20	CEC	25	Tranylcypromine	100	409	460	60	60
2D6[b]	10	AMMC	0.12	Quinidine	2	409	460	85	40
2E1	30	MFC	100	Diethyldithiocarbamate	100	409	530	80	60
3A4	10	DBF	1	Ketoconazole	1.9	485	530	50	20
3A5	10	DBF	1	Ketoconazole	1.9	485	530	50	20
3A7	10	DBF	1	Ketoconazole	1.9	485	530	50	40

[a] 0.35 M and 0.05 M Tris buffer (pH 7.5) were used instead of potassium phosphate buffer in Solutions A and B respectively

[b] 0.54 mM NADPH was used in Solution A

2.3.2 Mechanism-based Inhibition of CYP3A4

A similar method to the CYP3A4 inhibition assay previously mentioned was used, but with modifications to test for NADPH- and time-dependence (two requirements for MBI). Two types of solutions were prepared: a pre-incubation solution containing 0.25 μM EDTA, the sample, and 100 nM CYP3A4 in 0.13 M buffer; and an incubation solution containing 1.08 mM NADPH and 1 μM DBF in 0.14 M buffer. For each experiment, a single drug or extract was tested and compared to a vehicle control. Two identical pre-incubation solutions were prepared for both the vehicle control and the sample for a total of four pre-incubation solutions per experiment. A volume of 180 μL of incubation solution was aliquoted into wells of a 96-well plate with clear, flat bottoms and white walls and allowed to warm to 37°C for five minutes. The pre-incubation solutions were also warmed to 37°C but did not contain the vehicle control, sample and enzyme during this period. After five minutes, the vehicle control, sample and enzymes were quickly added to the pre-incubation solutions. NADPH (1.08 mM) was added to one pre-incubation solution for the vehicle control and one pre-incubation solution for the sample, to test for NADPH-dependence. Aliquots of 20 μL from each pre-incubation solution were then immediately transferred to three wells containing the warmed incubation solution. These samples were referred to as samples that have undergone a one minute pre-incubation time. The remaining pre-incubation solutions were then each aliquoted into empty wells of the assay plate and allowed to pre-incubate for a longer period of time at 37°C. At five and ten minutes, 20 μL from each pre-incubation solution was added to another three wells containing the incubation solution to test for time-dependence. Hence, both the NADPH- and time-dependence assays were performed

simultaneously. After 30 minutes of incubation, fluorescence released from metabolized DBF (fluorescein) was measured to determine the activity of the remaining active enzymes. The values of the samples were normalized to the values of its vehicle control. For each extract, the activity of the enzymes was compared between the absence and presence of NADPH in the pre-incubation solutions, or between the one, five and ten minute pre-incubation times, to determine if there were differences in activity between the different NADPH or pre-incubation time conditions. For the Cree plant extract samples exhibiting NADPH- and time-dependence, other concentrations were tested. GS extract (11.5 to 48.5 μg/mL) and azamulin (0.1 to 5 μM) were used as positive controls. Ketoconazole (0.2 to 1.9 μM) was used as a negative control. Azamulin was dissolved in acetonitrile.

2.3.3 Median Inhibitory Concentrations

The method used to test for CYP3A4 inhibition was used to determine the median inhibitory concentrations (IC_{50}s) of the Cree plant extracts using a range of extract concentrations (0.25 to 160 μg/mL). All samples were standardized for 100% MeOH concentration by the addition of 100% MeOH when required. The log concentrations were plotted as a function of CYP3A4 inhibition to obtain a sigmoidal curve for each assayed extract. The concentrations within the linear portion of these curves were used for linear regression. The IC_{50} values were obtained using the linear equations of the linear regressions.

2.3.4 Drug Interaction Study with Enalapril

This method was used to determine if the combination of 0.3 μM enalapril and a Cree plant extract can affect the activity of CYP3A4 compared to the solitarily extract. Only the extracts AD01, AD08, and AD09 were analyzed. A similar method to the assay for CYP3A4 inhibition was used, but was modified to accommodate the testing of enalapril and the extract rather than just the extract. In the first experiment, there was no modification from the original assay except the enalapril and the extract were added together after Solution A, and before Solution B/C. In the second experiment, enalapril and the extract were allowed to incubate at 37°C for 15 minutes in the absence of the experiment reagents before the addition of Solutions A, B and C. For the third experiment, enalapril and the extract were allowed to incubate at 37°C for 15 minutes in Solution A without the CYP3A4 substrate DBF, before the addition of Solution B/C. Instead, DBF was added to Solution B/C. After 30 minutes, the fluorescence released from metabolized DBF (fluorescein) was measured to determine the activity of the enzyme.

2.3.5 Statistical Analysis

Statistical analysis for the CYP inhibition studies were performed using one-way ANOVA followed by the Tukey test using the statistical program StatsDirect. A p-value ≤ 0.05 would indicate that the null hypothesis is rejected, suggesting a significant difference between the compared values. For the CYP inhibition studies of the 10 isoforms, the null hypotheses tested for each CYP isoform were: 1) no difference between the inhibition by the extract and the inhibition by the vehicle control; and/or 2) no difference between the inhibition by the extract and the inhibition by the positive control. For the CYP3A4 MBI studies, the null hypotheses tested were: 1) no difference

between the inhibition by the extract in the absence of NADPH and in the presence of NADPH in the pre-incubation solution; and 2) no difference between the inhibition by the extract with a one minute of pre-incubation time and five or ten minutes of pre-incubation times. For the drug interaction study with enalapril, the null hypothesis tested was: no difference between the inhibition by the extract and the inhibition by the extract with enalapril.

3 RESULTS

3.1 Cardiac Chronotropic Effects

3.1.1 Chronotrope Controls

To confirm that neonatal rat cardiomyocytes plated in MEAs can be used to detect the chronotropic effects of the Cree plant extracts, experiments were conducted with known chronotropes to determine if the expected changes in cardiac contraction rates were observed when the cardiomyocytes were treated with these substances. The drugs or extracts used were: isoproterenol, an adrenergic-ß receptor agonist which increases the contraction rate; carbachol, a muscarinic acetylcholine receptor agonist which decreases the contraction rate, and BO a NHP which is reported to activate adrenergic α- and ß-receptors to increase the contraction rate (Haaz *et al.*, 2006). The drugs were dissolved in water and the BO extract was extracted in water. In the context of these experiments and the experiments to follow, the acute chronotropic effects refer to the contraction rate following one minute of treatment, whereas the chronic chronotropic effects refer to the contraction rate following 18 hours of treatment. The chronotropic effects of the drugs and extract were compared to the chronotropic effect of the water vehicle control. The water vehicle control had an insignificant decrease in the contraction rate relative to Tyrode's buffer **(Figure 5)**. The expected, acute, dose-dependent results were observed for isoproterenol, carbachol and the BO extract **(Figure 6)**. A significant effect was observed with 400 µM carbachol ($p \leq 0.05$) **(Figure 6A)**. The mean contraction rate of cardiomyocytes treated with 400 µM carbachol was 36.6% ± 14.2% of the mean contraction rate of cardiomyocytes treated with the water vehicle control. Significant effects were observed with 5 and 10 µM isoproterenol ($p \leq 0.05$) **(Figure 6B)**. The mean

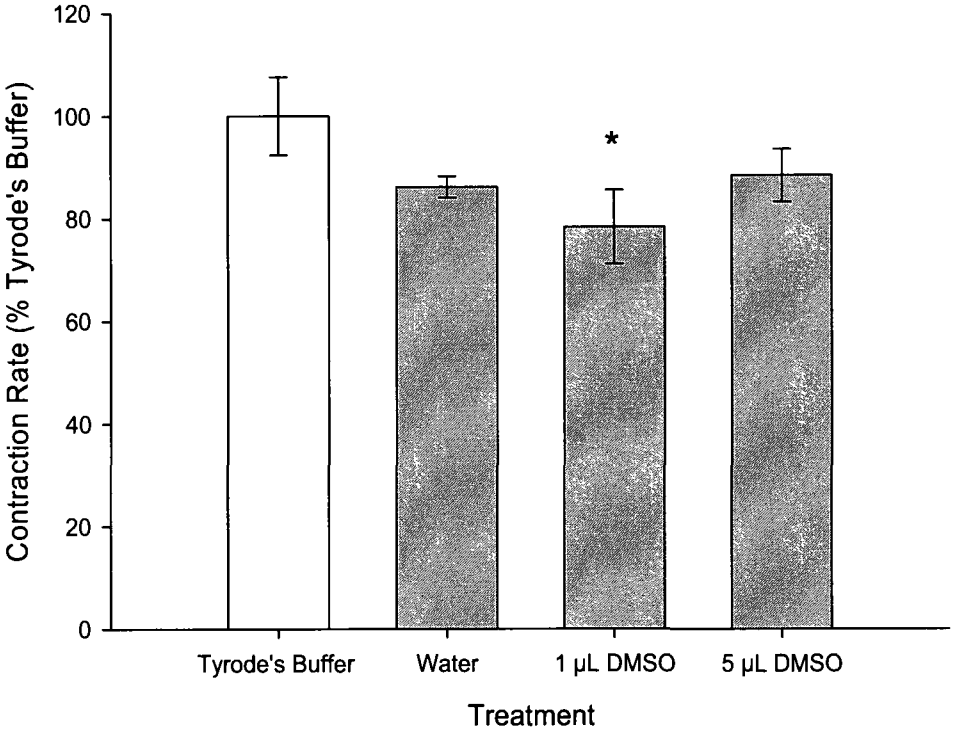


Figure 5: The acute chronotropic effects of the vehicle controls with neonatal rat cardiomyocytes. Cardiomyocytes were treated with the vehicle controls (1 µL water, 1 µL DMSO, and 5 µL DMSO) for one minute and the resultant contraction rates were measured using the MEA system and counted using AxoScope software. The contraction rates of treated cardiomyocytes were expressed relative to their contraction rates observed in Tyrode's buffer, as the mean contraction rate ± SEM. Pooled data ($n = 5$) is presented. $^*p \leq 0.05$ with respect to the mean contraction rate in Tyrode's buffer, using one-way ANOVA followed by the Tukey test.

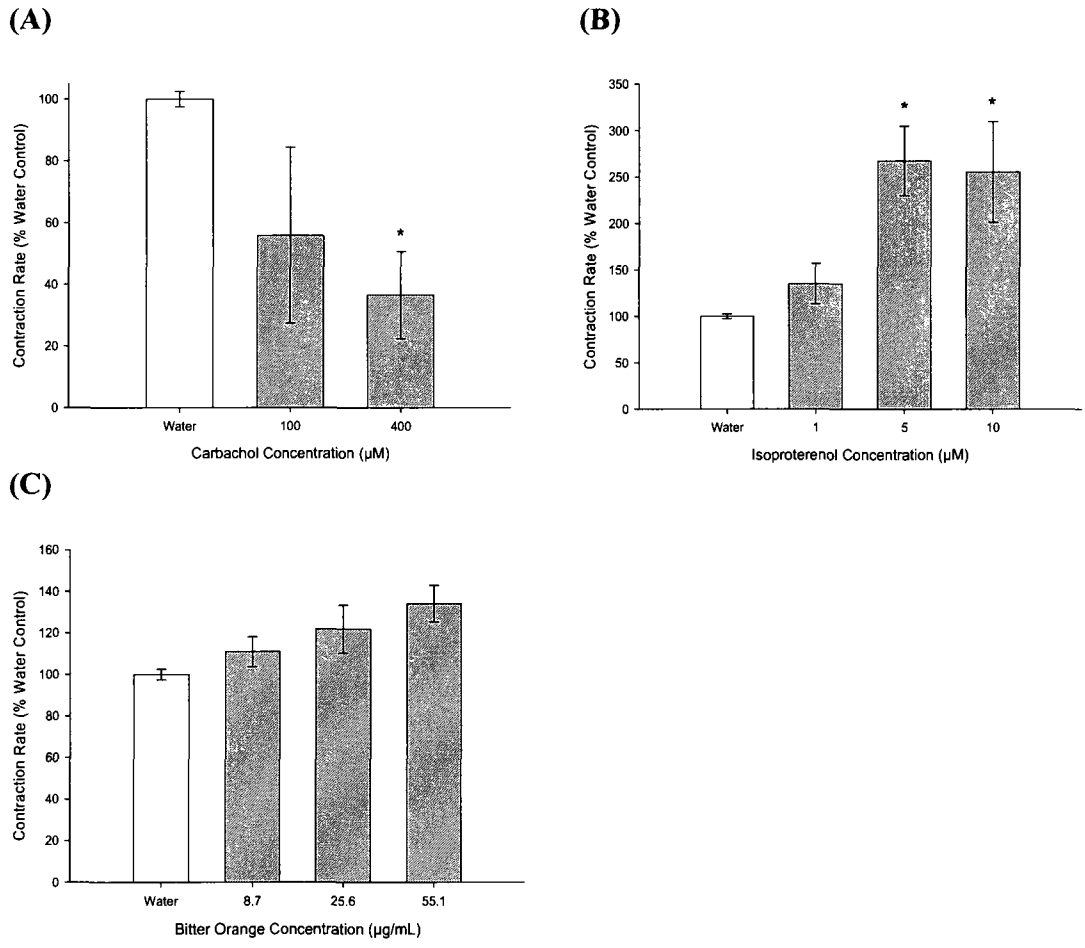


Figure 6: The acute chronotropic effects of carbachol, isoproterenol and bitter orange extract with neonatal rat cardiomyocytes. The cardiomyocytes were treated with different concentrations of the cardioactive substances **(A)** carbachol, **(B)** isoproterenol, and **(C)** BO extract for one minute, and the resultant contraction rates were measured using the MEA system and counted using the software AxoScope. The contraction rates were expressed relative to the contraction rate of the water vehicle control, as the mean contraction rate ± SEM. Pooled data (n = 4-6) is presented. *$p \leq 0.05$ with respect to the mean contraction rate in the water vehicle control, using one-way ANOVA followed by the Tukey test.

contraction rates of cardiomyocytes treated with 5 and 10 μM isoproterenol were 267.5% ± 37.4% and 255.4% ± 54.4% of the mean contraction rate of cardiomyocytes treated with the water vehicle control respectively. Increases in the contraction rate were observed with increasing BO concentration; however these increases were not significantly different from the water vehicle control using ANOVA **(Figure 6C)**.

Overall, these results indicate that the chronotropic effects of the Cree plant extracts can be detected using neonatal rat cardiomyocytes plated in MEAs, as the expected chronotropic effects were observed with known chronotropes.

3.1.2 Dose Ranging of Cree Plant Extracts

DMSO was used to solubilize the Cree plant extracts for the cardiac experiments, as this was the chosen solvent for all *in vitro* cell work for the CIHR project. To confirm that the DMSO was not toxic to the cardiomyocytes, the cells were treated with different concentrations of DMSO (1 to 6 μL/mL) in media for 18 hours, and then screened for cytotoxicity by measuring the amount of LDH released in the media from the cardiomyocytes. Cardiomyocytes which have been damaged or killed by necrosis will release LDH into their extracellular environment by the weakening of their cell membranes. These results show that concentrations up to 6 μL/mL DMSO did not significantly effect cell viability ($p > 0.05$) and therefore, the concentrations of DMSO used for the experiments were not toxic **(Figure 7)**.

Due to time limitations (from limited access to the MEA system), only extracts from four plant species were chosen to be studied: W2, W4, W5, and W9. As well, only their highest non-toxic concentrations were examined for chronotropic effects. The highest non-toxic concentrations of these extracts were identified by measuring the

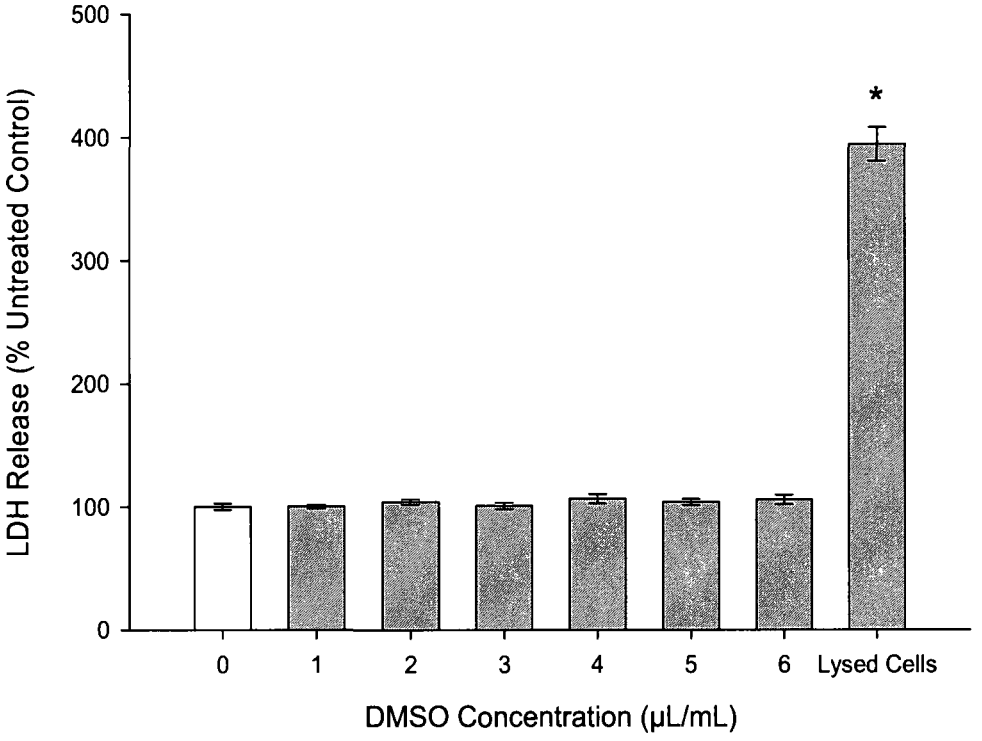


Figure 7: The cytotoxic effects of various concentrations of DMSO with neonatal rat cardiomyocytes. Cardiomyocytes were treated with various concentrations of DMSO (1 to 6 µL/mL) for 18 hours, and their cytotoxic effects were determined by the measurement of the resultant LDH released. Lysed cells were used as a positive control. The results with treated cardiomyocytes were expressed relative to untreated cardiomyocytes, as the mean LDH release ± SEM. Pooled data (n = 3-14) is presented. *$p \leq 0.05$ with respect to the untreated cardiomyocytes, using one-way ANOVA followed by the Tukey test.

amount of LDH released in the media from cardiomyocytes that were treated with various extract concentrations. Different toxic concentrations were observed for the four extracts **(Figure 8)**. The lowest significant toxic concentrations for W2, W5 and W9 extracts were 200, 50, and 10 μg/mL respectively ($p \leq 0.05$). For W4 extract, toxic concentrations were not observed up to concentrations of 600 μg/mL and did not approach significance. It is physiologically unattainable to obtain such high extract plasma concentrations and therefore, further studies to determine the lowest toxic concentration for W4 was not conducted. Dose-dependent release of LDH was observed with increasing extract concentrations for W2, W5, and W9 extracts. Overall, the highest non-toxic concentrations to be tested on the cardiomyocytes were 100, 500, 25, and 5 μg/mL for the extracts of W2, W4, W5, and W9 respectively.

3.1.3 Cree Plant Extracts

The DMSO vehicle controls (1 μL/mL for W2, W5 and W9, and 5 μL/mL for W4) were first tested to determine if they had an acute effect on the contraction rate of cardiomyocytes. A significant decrease ($p \leq 0.05$) in the contraction rate was observed with 1 μL/mL of DMSO (78.4% ± 7.2% of the contraction rate in Tyrode's buffer), but not with 5 μL/mL DMSO (88.4% ± 5.2% of the contraction rate in Tyrode's buffer) **(Figure 5)**. As the chronotropic effects of the Cree plant extracts will be assessed relative to its vehicle control, the significant negative chronotropic effect of 1 μL/mL of DMSO should not impede the results. The extract concentrations that were tested were 100, 500, 25, and 5 μg/mL for W2, W4, W5, and W9 respectively, which corresponded to their highest non-toxic concentrations. At these concentrations, there were no significant changes ($p > 0.05$) in acute chronotropy observed **(Figure 9)**. W2 had a minor

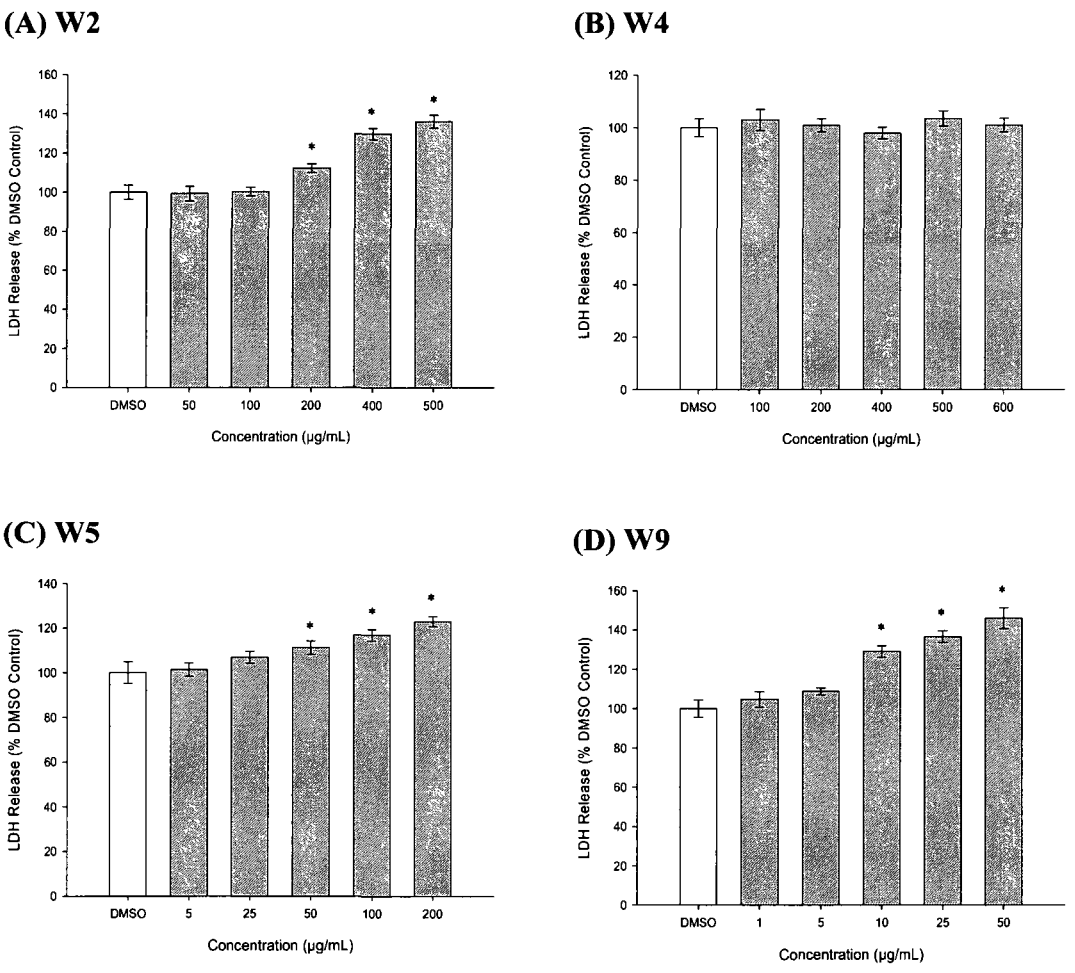


Figure 8: The cytotoxic effects of various concentrations of W2, W4, W5, and W9 extracts with neonatal rat cardiomyocytes. Cardiomyocytes were treated with various concentrations of **(A)** W2, **(B)** W4, **(C)** W5, and **(D)** W9 extracts for 18 hours, and their cytotoxic effects were determined by the measurement of the resultant LDH released. The results with the extracts were expressed relative to the DMSO vehicle control, as the mean LDH release ± SEM. Pooled data (n = 7-9) is presented. *$p \leq 0.05$ with respect to the DMSO vehicle control, using one-way ANOVA followed by the Tukey test.

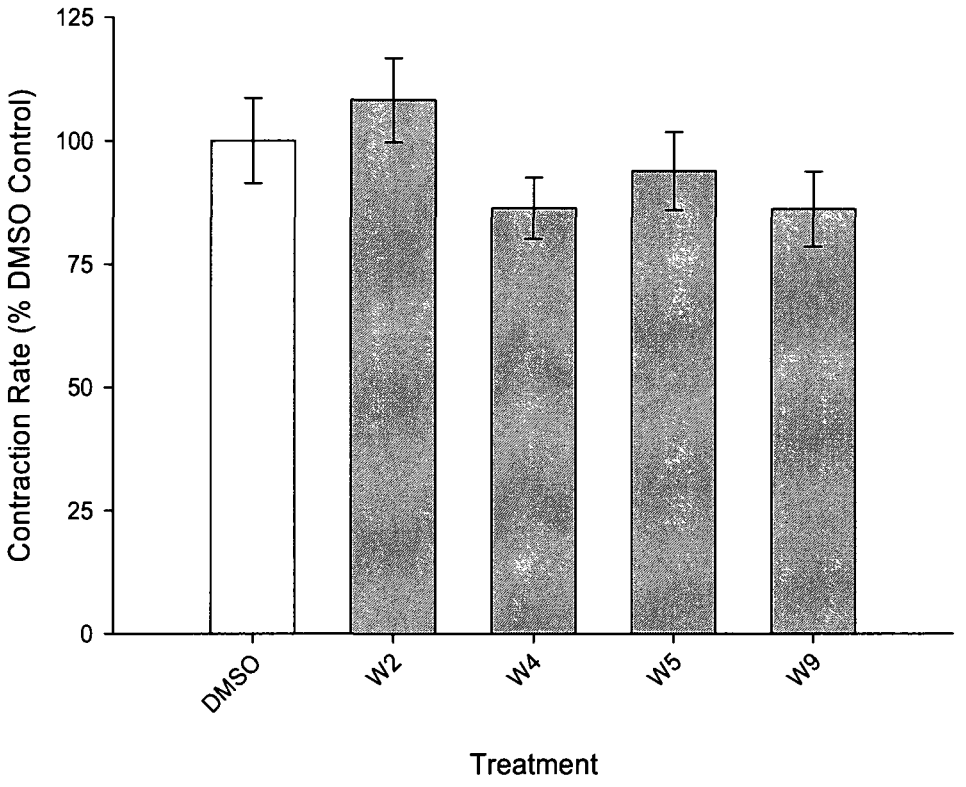


Figure 9: The acute chronotropic effects of W2, W4, W5 and W9 extracts with neonatal rat cardiomyocytes. The cardiomyocytes were treated with the highest non-toxic concentrations of W2, W4, W5 and W9 extracts for one minute, and the resultant contraction rates were measured using the MEA system and counted using AxoScope software. The contraction rates were expressed relative to the contraction rate of the DMSO vehicle control, as the mean contraction rate ± SEM. Pooled data (n = 5-7) is presented. *$p \leq 0.05$ with respect to the contraction rate in the DMSO vehicle control, using one-way ANOVA followed by the Tukey test.

positive chronotropic effect (108.3% ± 8.5% of vehicle control), while W4, W5, and W9 had minor negative chronotropic effects (86.4% ± 6.2%, 93.9% ± 7.9%, and 86.2% ± 7.6% respectively of vehicle control).

W9 was chosen for additional analysis because it had the greatest effect on the contraction rate, and had a non-toxic concentration (5 μg/mL) that can reasonably be within biological plasma levels. W4 had a similar extent of a chronotropic effect, but since the highest non-toxic concentration of W4 was not determined, it was not chosen for additional analysis. Even though toxicity was observed with concentrations greater than 10 μg/mL of W9, the concentrations of 25 and 100 μg/mL were tested for acute chronotropic effects to determine if higher doses had any significant effects. Neither of these concentrations significantly affected the contraction rate relative to the DMSO vehicle control ($p > 0.05$) **(Figure 10)**. The contraction rates of cardiomyocytes treated with 25 and 100 μg/mL were 95.0% ± 4.8% and 97.2% ± 8.4% of the vehicle control respectively.

The chronic chronotropic effects of the extracts were also studied. For these experiments, the cardiomyocytes were treated for 18 hours with the extracts in the media. The chronic chronotropic effects of the vehicle controls were first examined. A significant decrease ($p \leq 0.05$) in the contraction rate was observed with 5 μL/mL of DMSO (62.1% ± 5.4% of the contraction rate after initial exposure), but not 1 μL/mL (84.6% ± 6.7% of the contraction rate after initial exposure) **(Figure 11)**. As the chronotropic effects of the Cree plant extracts will be assessed relative to its vehicle control, the significant negative chronotropic effect of 5 μL/mL of DMSO should not impede the results. At the highest non-toxic concentrations of the extracts, there were no

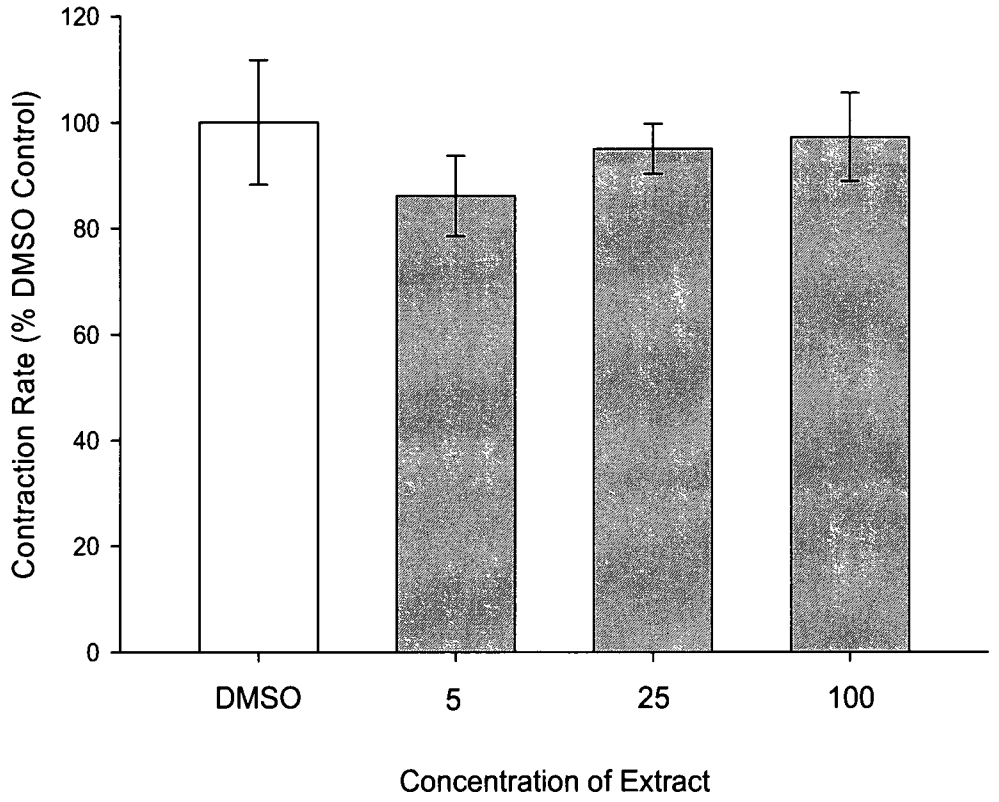


Figure 10: The acute chronotropic effects of various concentrations of W9 extract with neonatal rat cardiomyocytes. The cardiomyocytes were treated with 5, 25 or 100 μg/mL of W9 extract for one minute, and the resultant contraction rates were measured using the MEA system and counted using AxoScope software. The contraction rates were expressed relative to the contraction rate of the DMSO vehicle control, as the mean contraction rate ± SEM. Pooled data (n = 4-7) is presented. *$p \leq 0.05$ with respect to the contraction rate in the DMSO vehicle control, using one-way ANOVA followed by the Tukey test.

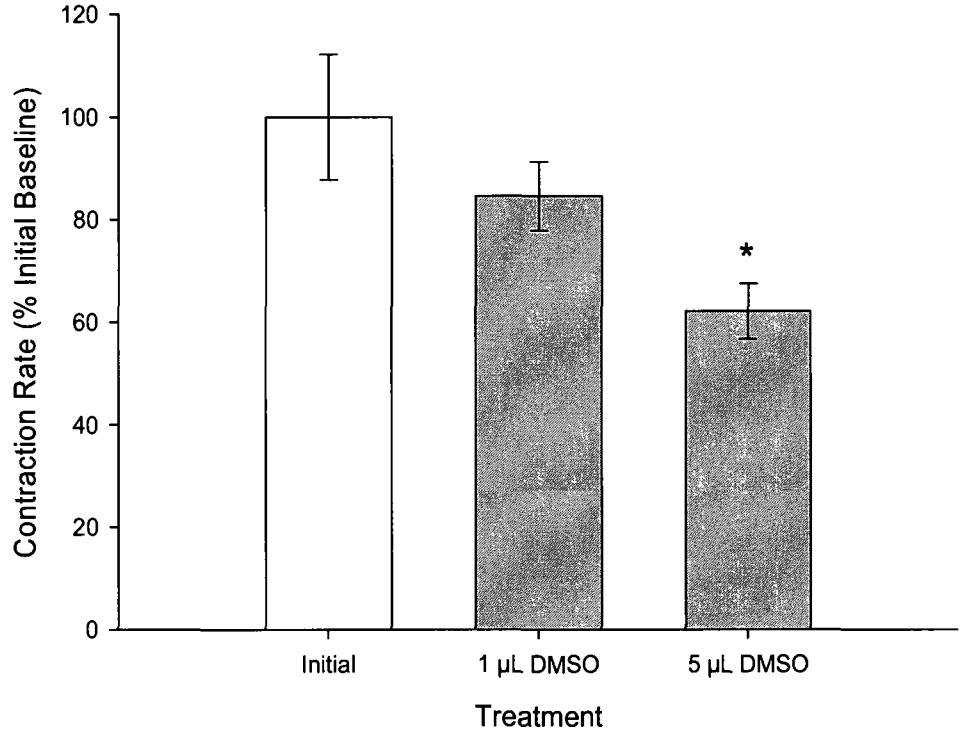


Figure 11: The chronic chronotropic effects of the vehicle controls with neonatal rat cardiomyocytes. The cardiomyocytes were treated with the vehicle controls (1 µL DMSO, and 5 µL DMSO) for 18 hours and the resultant contraction rates were measured using the MEA system and counted using AxoScope software. The contraction rates of treated cardiomyocytes were expressed relative to their contraction rates after their initial treatment, as the mean contraction rate ± SEM. Pooled data (n = 4-5) is presented.*$p \leq$ 0.05 with respect to the mean contraction rate in media, using one-way ANOVA followed by the Tukey test.

significant changes in the contraction rates observed ($p > 0.05$) **(Figure 12)**. W4 had a positive chronotropic effect (129.9% ± 15.8% of vehicle control), while W2, W5, and W9 had minor negative chronotropic effects (89.4% ± 11.8%, 87.5% ± 19.8%, and 79.2% ± 8.9% respectively of vehicle control).

Overall, from the results of **Figure 9 to 12**, W2, W4, W5, and W9 extracts do not significantly affect the contraction rate of cardiomyocytes at their non-toxic concentrations with both acute (1 minute) and chronic (18 hour) treatments.

3.2 Drug Interaction Study with Metformin

A study was conducted to determine if the combination of the anti-hyperglycemic drug metformin, and W9 extract had an effect on the contraction rate of the cardiomyocytes. Metformin was first tested to determine if it had an effect on the contraction rate. Metformin did not have a significant chronotropic effect relative to its water vehicle control (94.5% ± 8.2% of vehicle control) ($p > 0.05$) **(Figure 13)**. To determine if the combination of metformin and W9 extract had a significant effect on the contraction rate, 50 μM metformin was first added to the Tyrode's buffer before the Tyrode's buffer was added to the cardiomyocytes. Different concentrations of W9 extract (5 or 100 μg/mL) were then added to the Tyrode's buffer. After one minute of exposure to both metformin and W9, the resulting contraction rate of the cardiomyocytes was measured. The combination of 50 μM metformin and 100 μg/mL W9 significantly decreased the contraction rate relative to the combination of 50 μM metformin and the DMSO vehicle control (contraction rate was 67.4% ± 7.7% of metformin and the vehicle control) ($p \leq 0.05$) **(Figure 14)**. However, there was not a significant difference between 100 μg/mL W9, and 50 μM metformin with 100 μg/mL W9 (67.4% ± 7.7% vs. 97.2% ±

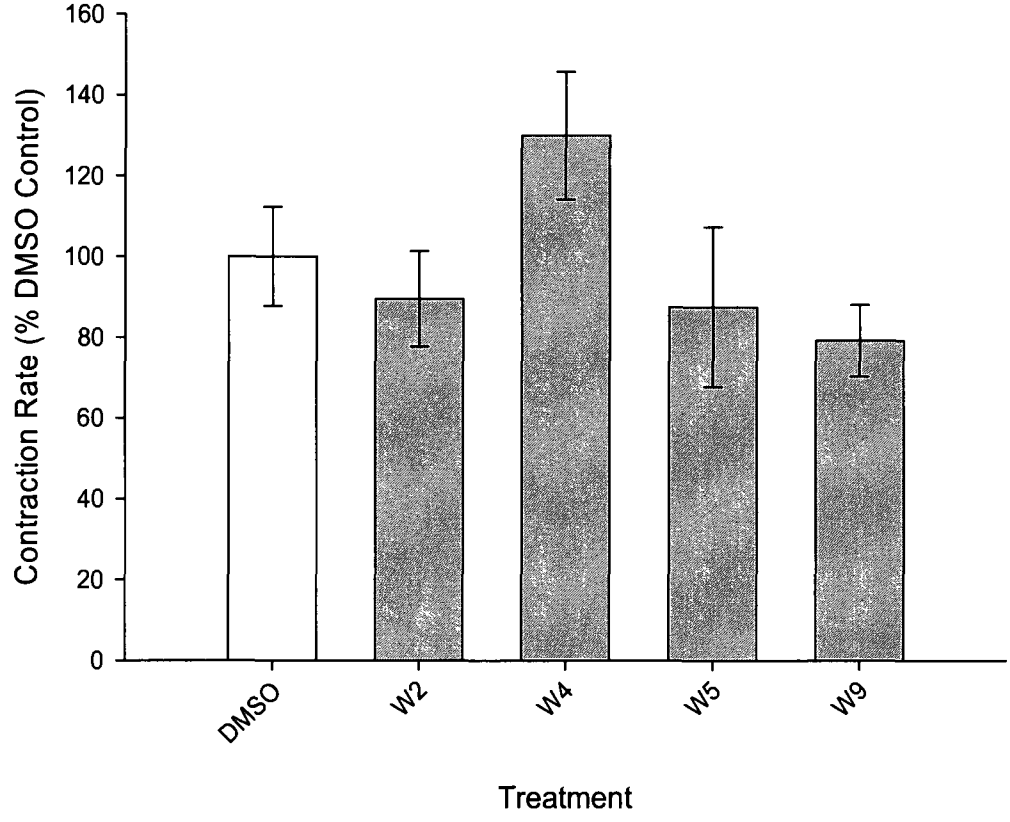


Figure 12: The chronic chronotropic effects of W2, W4, W5 and W9 extracts with neonatal rat cardiomyocytes. The cardiomyocytes were treated with the highest non-toxic concentrations of W2, W4, W5 and W9 extracts for 18 hours, and the resultant contraction rates were measured using the MEA system and counted using AxoScope software. The contraction rates were expressed relative to the contraction rate of the DMSO vehicle control, as the mean contraction rate ± SEM. Pooled data (n = 3-4) is presented. *$p \leq 0.05$ with respect to the contraction rate in the DMSO vehicle control, using one-way ANOVA followed by the Tukey test.

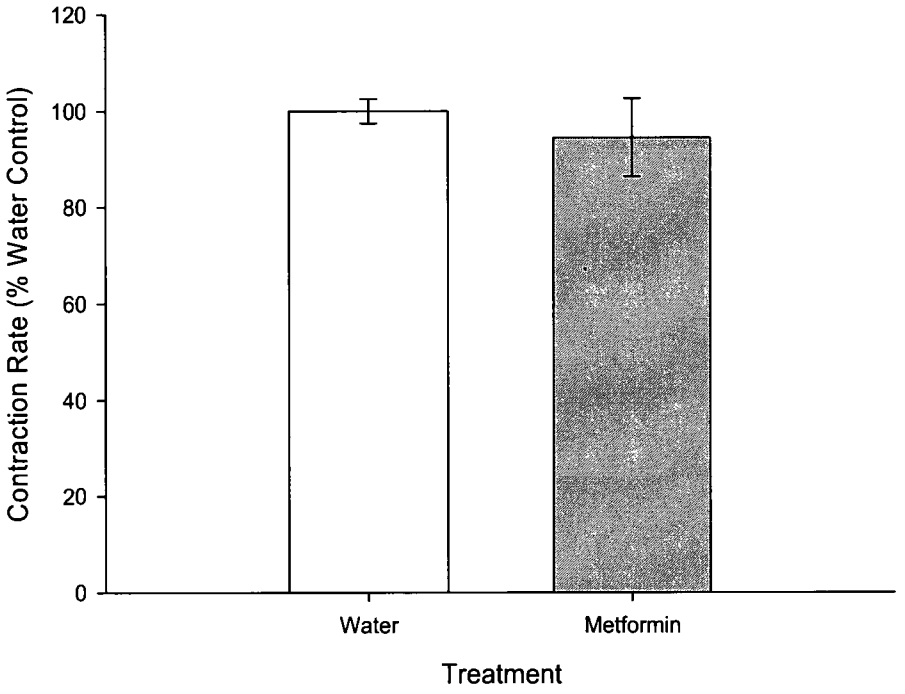


Figure 13: The acute chronotropic effect of 50 μM metformin with neonatal rat cardiomyocytes. The cardiomyocytes were treated with 50 μM of metformin for one minute, and the resultant contraction rates were measured using the MEA system and counted using AxoScope software. The contraction rates were expressed relative to the contraction rate of the water vehicle control as the mean contraction rate ± SEM. Pooled data ($n = 7$) is presented. $*p \leq 0.05$ with respect to the contraction rate in the water vehicle control, using the Student's t-test.

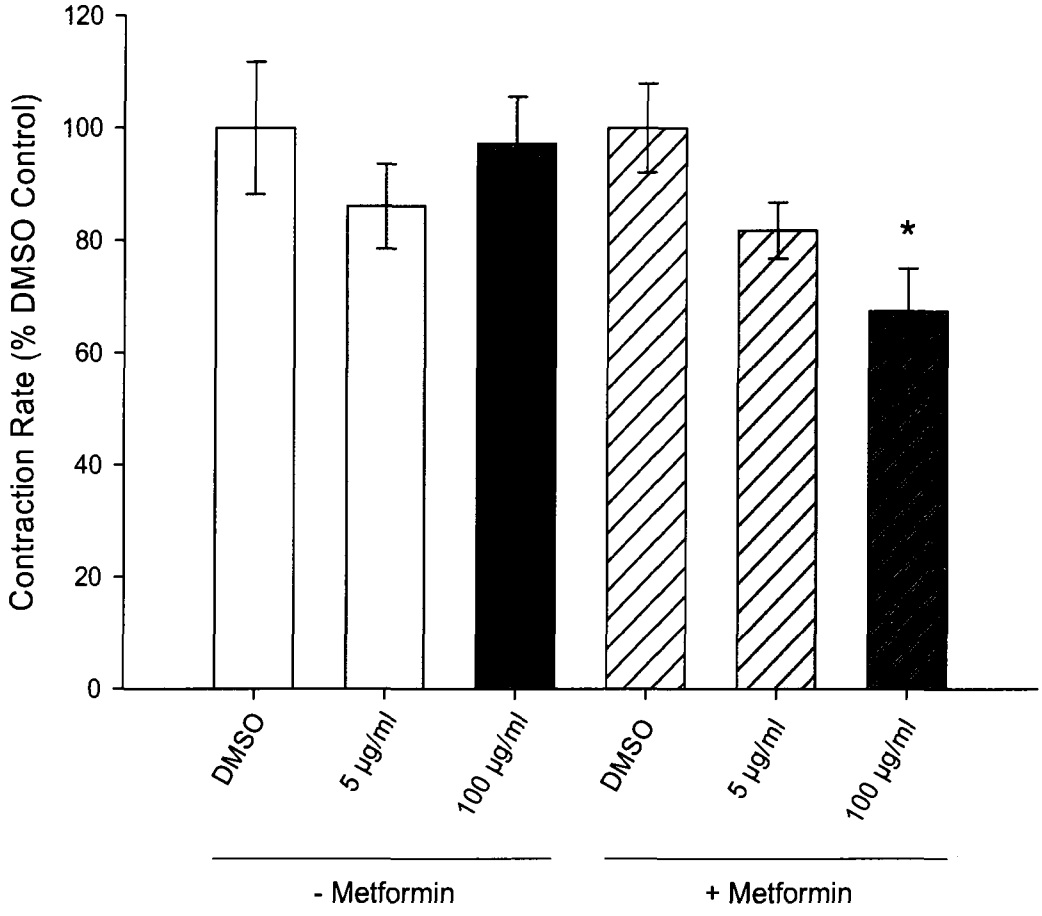


Figure 14: The acute chronotropic effect of the combination of metformin and W9 extract with neonatal rat cardiomyocytes. The cardiomyocytes were treated with 50 µM of metformin and 5 or 100 µg/mL of W9 extract for one minute, and the resultant contraction rates were measured using the MEA system and counted using AxoScope software. The contraction rates were expressed relative to the contraction rate of the DMSO vehicle control as the mean contraction rate ± SEM. Pooled data (n = 4-7) is presented. *$p \leq 0.05$ with respect to the DMSO vehicle control with metformin, using one-way ANOVA followed by the Tukey test. Note that a significant difference was not observed between 100 µg/mL of W9 without metformin and 100 µg/mL of W9 with metformin as determined by a one-way ANOVA followed by the Tukey test ($p \leq 0.05$).

8.4% respectively) ($p > 0.05$).

The mechanism of the negative chronotropic effect observed with 50 μM metformin and 100 μg/mL W9 was unknown. It may be possible that the combination of 50 μM metformin and 100 μg/mL W9 was toxic to the cardiomyocytes and affected their ability to depolarize and undergo contraction, or that there was a synergetic interaction between the two substances that affected cellular signaling pathways. The concentration of 100 μg/mL W9 was previously shown to be toxic to the cells after an 18 hour treatment **(Figure 8)**, but had no effect on the contraction rate after one minute of treatment **(Figure 10)**. In addition, 50 μM metformin had no effect on the contraction rate after one minute of treatment **(Figure 13)**. To determine if the combination of 50 μM metformin and 100 μg/mL W9 was toxic, the recovery contraction rate of treated cardiomyocytes was studied. After the cardiomyocytes were treated with metformin and W9, the drug and extract were washed out with Tyrode's buffer, and the media was re-added to the cardiomyocytes. If there was a toxic effect and not a cellular signaling effect from metformin and W9, a recovery would be expected in the presence of media. Upon the washout of the drug and extract, and the re-addition of the media, there was a full recovery from the cardiomyocytes treated with metformin and 5 μg/mL W9 (98.3% ± 9.5% of vehicle control), and a minor recovery from those treated with metformin and 100 μg/mL W9 (76.1% ± 10.8% of vehicle control) **(Figure 15)**. The recovery observed with metformin and 100 μg/mL W9 was not significantly different than the recovery from metformin and the DMSO vehicle control, indicating the possibility that the combination of metformin and W9 was toxic.

Overall, the combination of 50 μM metformin and 100 μg/mL W9 may have a negative chronotropic effect from toxicity.

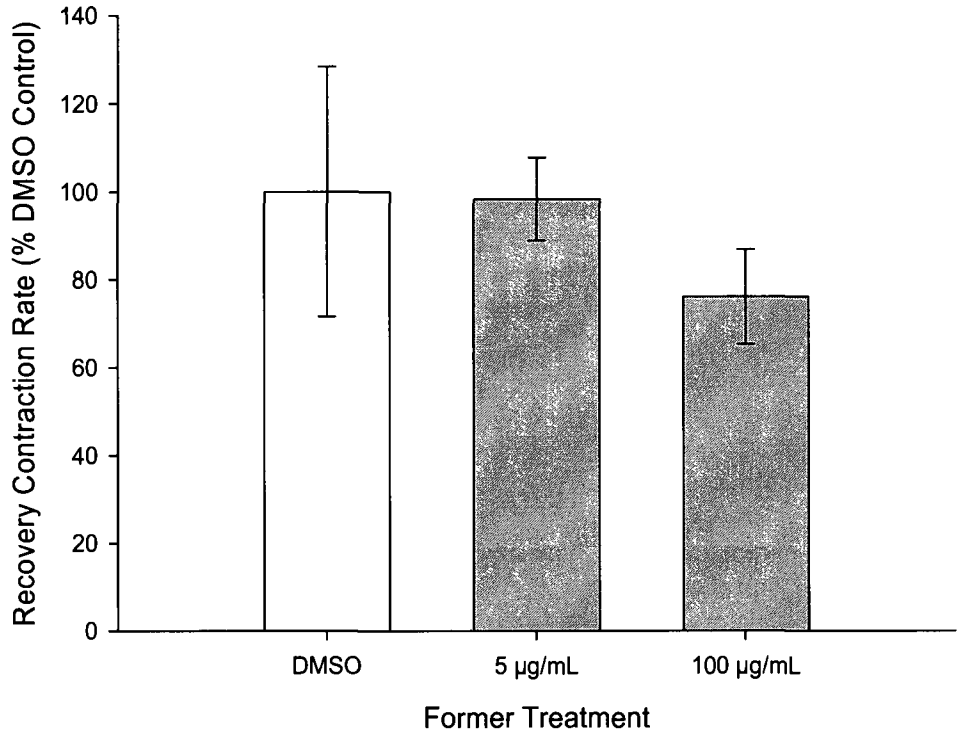


Figure 15: The acute recovery contraction rate of neonatal rat cardiomyocytes in media following the washout of metformin and W9 extract. Metformin and W9 extract (5 or 100 µg/mL) were washed out with Tyrode's buffer after a one minute treatment with the cardiomyocytes. The buffer was then replaced with media and the resultant recovery contraction rates were measured using the MEA system and counted using AxoScope software. The recovery contraction rates were expressed relative to the recovery contraction rate of the DMSO vehicle control as the mean contraction rate ± SEM. Pooled data (n = 5-6) is presented.*$p \leq 0.05$ with respect to the recovery DMSO vehicle control, using one-way ANOVA followed by the Tukey test.

3.3 Diabetic-like Cardiomyocytes

3.3.1 Confirming Their Presence

The Cree plant extracts should also be tested on diabetic cardiomyocytes since diabetic patients will be using these plants to treat the disease. The current experiments showed that the extracts did not have an affect on the contraction rate of healthy cardiomyocytes, but diabetic cardiomyocytes may be more sensitive and influenced by the extracts. Ren *et al.* (1997) produced diabetic-like cardiomyocytes by culturing adult rat cardiomyocytes in high glucose (25.5 mM) defined media (Ren *et al.*, 1996). This technique was applied to the neonatal rat cardiomyocytes using the same media that was used for the previous studies but supplementing the media with extra glucose to obtain a glucose concentration of 25.5 mM. Confirmation of the production of diabetic-like cardiomyocytes was performed by measuring their FP duration as a measure of AP duration. AP durations are longer in diabetic cardiomyocytes (Pacher *et al.*, 1999; Ren *et al.*, 1997; D'Amico *et al.*, 2001). The FP durations were compared to the FP durations of cardiomyocytes cultured in the normal media which had a glucose concentration of 17.5 mM. In addition, low glucose media (5 mM) was used for several cultures of cardiomyocytes to determine if a dose-dependent effect could be observed. These cardiomyocytes were expected to have the shortest FP durations. The FP durations were measured at 5, 7, 9, and 11 DIV **(Figure 16)**. Overall, there were no significant differences in FP durations between the cardiomyocytes cultured in different media for each day. In addition, the cardiomyocytes cultured in the low glucose media had the longest FP durations overall, compared to the cardiomyocytes cultured in the normal or high glucose media. In general, the cardiomyocytes cultured in the high glucose media

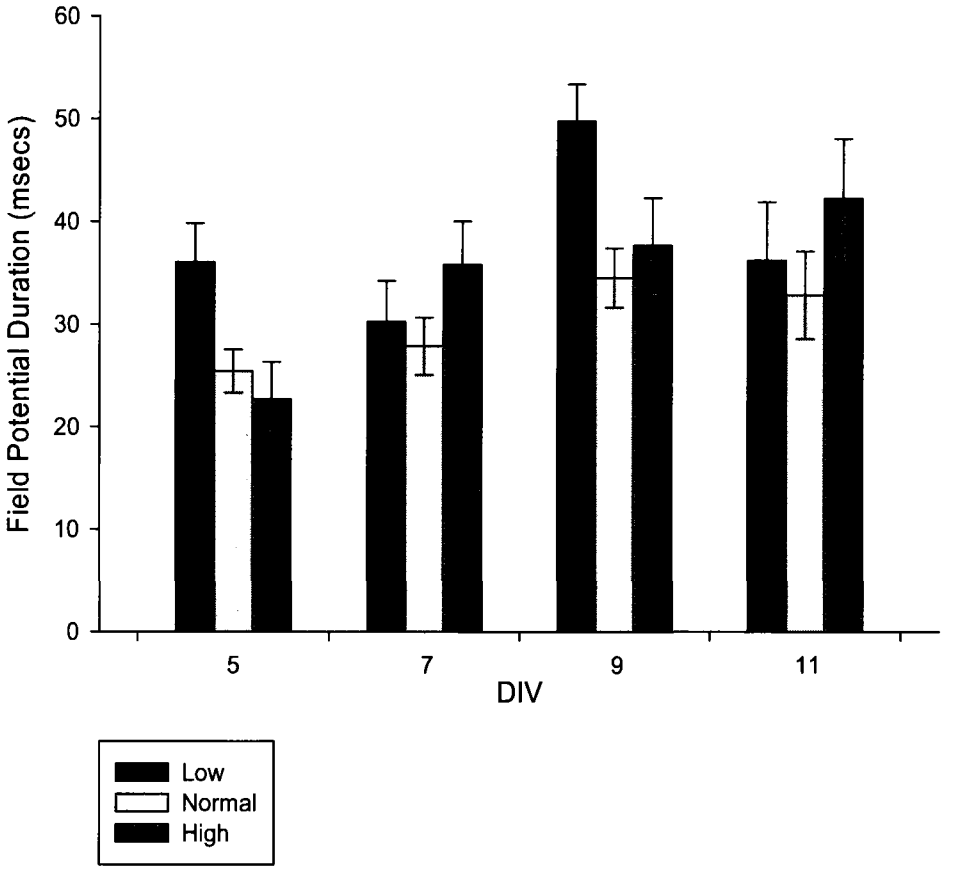


Figure 16: The measured field potential durations of neonatal rat cardiomyocytes cultured in media with various concentrations of glucose from 5 to 11 DIV. Cultures of cardiomyocytes were grown in low (5 mM), normal (17.5 mM), or high (25.5 mM) glucose media, and FP activity recordings were performed at 5, 7, 9, and 11 DIV using the MEA system. The FP durations were measured using AxoScope software. For each culture, the FP durations were measured from the same three microelectrodes and averaged. The results were expressed as the mean FP duration ± SEM. Pooled data (n = 8-12) is presented. $^{*}p \leq 0.05$ with respect to the FP duration of cells cultured in normal glucose media at the same DIV; $^{\#}p \leq 0.05$ with respect to the FP duration of cells cultured in the same glucose media at 5 DIV, using two-way ANOVA followed by the Tukey test.

had a longer FP duration than those cultured in the normal glucose media, but it was not significant ($p > 0.05$). There was a general trend of an increasing FP duration the longer the cardiomyocytes were in culture for those cultured in normal and high glucose media.

Since the FP durations were not extensively prolonged in cardiomyocytes cultured in high glucose media, it cannot be concluded that diabetic-like cardiomyocytes were produced. The extracts were not tested on these cardiomyocytes.

3.3.2 Viability

The viability of the cardiomyocytes cultured in the high glucose media was observed to be lower than those cultured in normal glucose by visual analysis **(Figure 17)**. The greater amount of detached, rounded up cardiomyocytes observed in these cultures indicated that fewer cardiomyocytes were adhered and functional. These cells were not fibroblasts or endothelial because they were positive for α-actinin (stained green), a protein of the contractile apparatus of cardiomyocytes. The cultures did contain non-cardiac cells such as fibroblasts or endothelial cells indicated by the numerous stained nuclei of cells that did not express α-actinin. A similar decrease in viability was observed with the cardiomyocytes cultured in the low glucose media; however no data was collected to verify this observation.

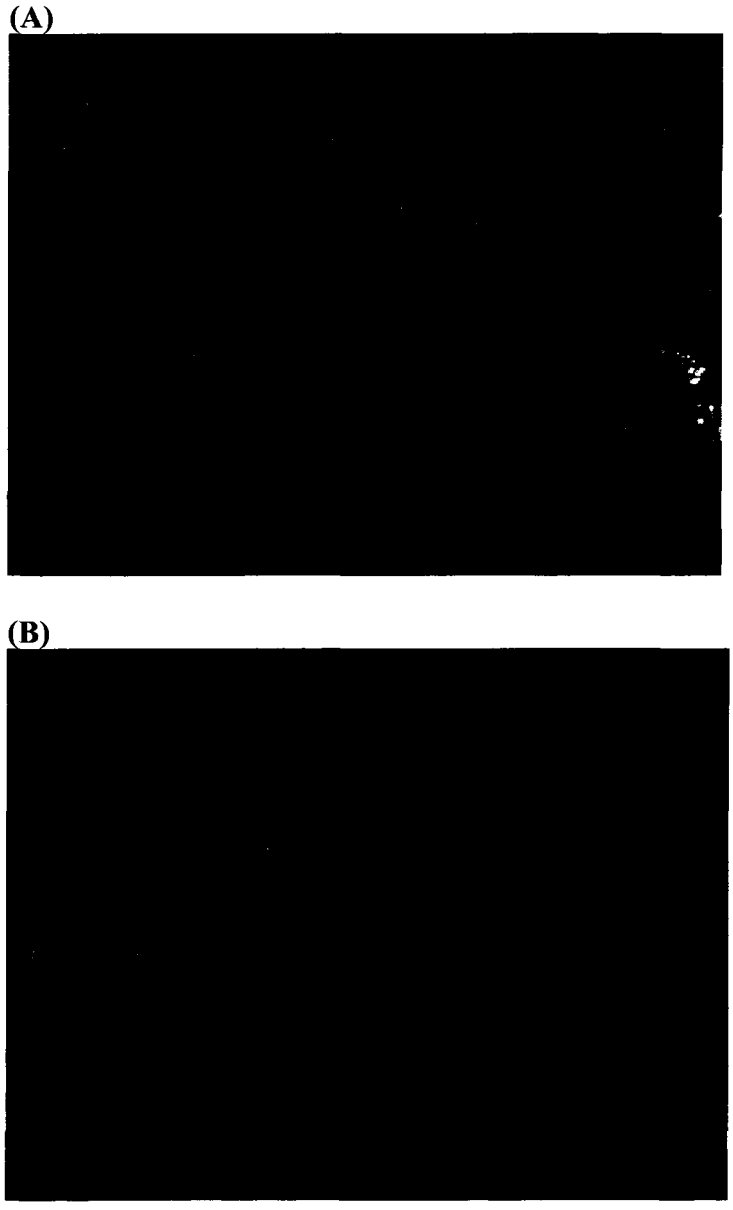


Figure 17: Images of rat neonatal rat cardiomyocytes at 7 DIV cultured in normal and high glucose media. Cardiomyocytes were cultured in **(A)** normal glucose media (17.5 mM), and **(B)** high glucose media (25.5 mM) for 7 DIV, fixed with Lana's fixative, and stained for α-actinin (green), connexin43 (red) a gap junction protein, and DNA (blue). The images were captured at 320X magnification under a fluorescence microscope.

3.4 Changes of the Field Potential Duration

A long-term experiment was conducted with one culture of cardiomyocytes plated on a MEA where the FP durations were measured from eight separate microelectrodes on various days. Eight microelectrodes were analyzed because the extracellular electrical activity from different clusters of cardiomyocytes of the culture can be simultaneously examined. Measurements were made at 5, 13, 28 and 35 DIV to determine if changes in the FP duration occurred at different stages of development of the cardiomyocytes **(Figure 18)**. A progressive increase in the FP duration was observed when the cardiomyocytes were kept in culture, similar to the results observed in **Figure 16**. The mean FP duration at 35 DIV (25.5 msecs) was significantly longer ($p \leq 0.05$) than the mean FP duration measured at 5 DIV (6.1 msecs).

3.5 Inhibition of Cytochrome P450 Isoforms

3.5.1 The Inhibitory Potency of the Cree Plant Extracts

The 17 Cree plant extracts were analyzed for their inhibitory potency against 10 different CYP isoforms: 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7. A 10 μg/mL concentration of each extract was tested, and their inhibitory effects relative to a 100% MeOH vehicle control were used for comparison analysis. The inhibitory potencies were organized into three categories: weak potency (< 30% inhibition); moderate potency (31-74% inhibition); and strong potency (> 75% inhibition). The results were organized into four figures based on their subfamilies (1A2 – **Figure 19**; 2B6, 2D6, 2E1 – **Figure 20**; 2C8, 2C9, 2C19 – **Figure 21**; 3A4, 3A5, 3A7 – **Figure 22**).

Due to the extensive amount of repetition for the inhibitory results for each CYP isoform, an organized summary of the results is provided in **Table 3**.

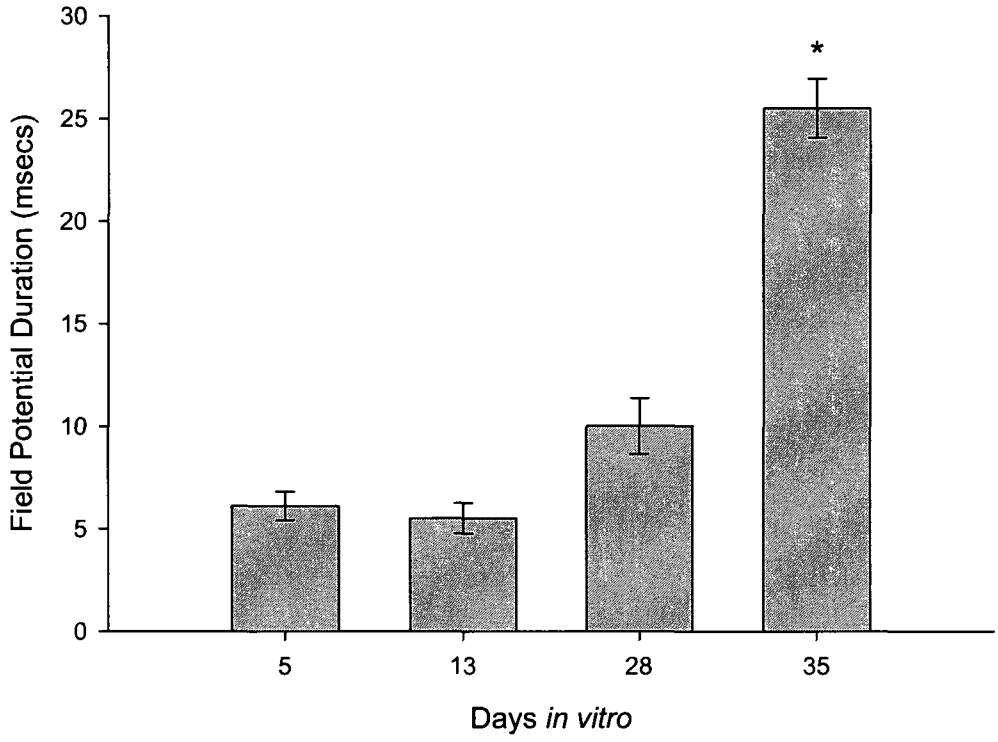


Figure 18: The field potential durations of a long-term culture of neonatal rat cardiomyocytes up to 35 DIV. A culture was kept in culture for 35 DIV, and FP activity recordings were performed at 5, 13, 28, and 35 DIV using the MEA system. The FP durations were measured using the program AxoScope. The results were expressed as the mean FP duration measured from 8 microelectrodes ± SEM ($n = 8$). *$p \leq 0.05$ with respect to the mean FP duration at 5 DIV, using one-way ANOVA followed by the Tukey test.

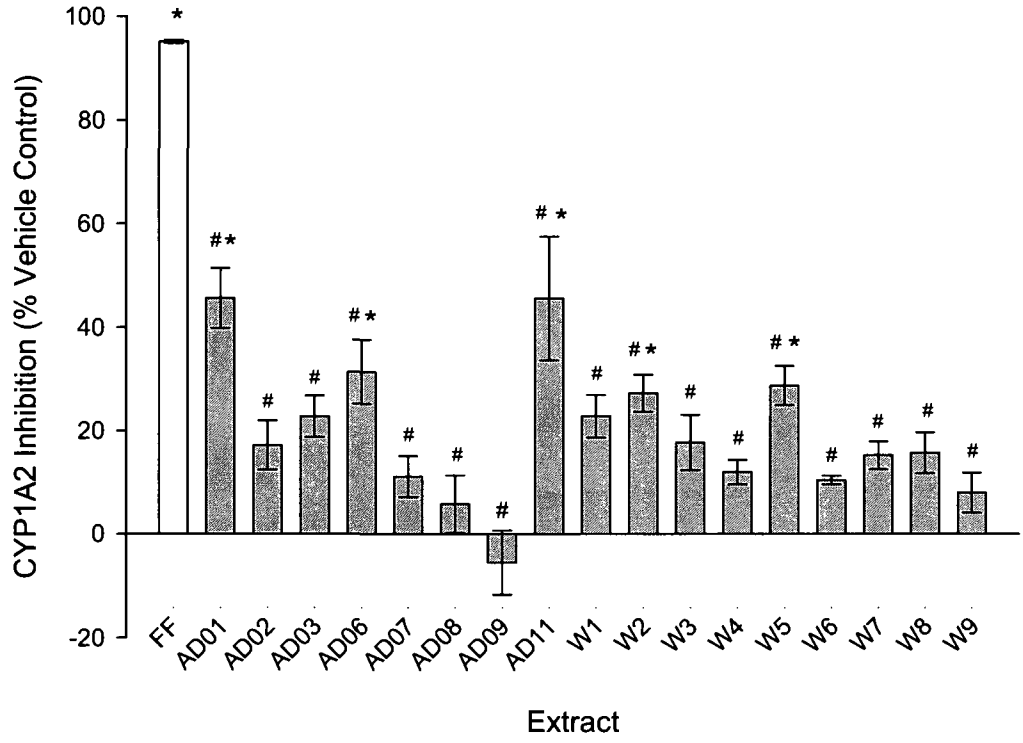
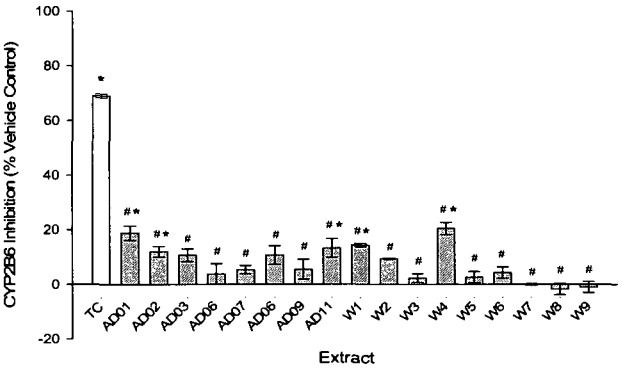
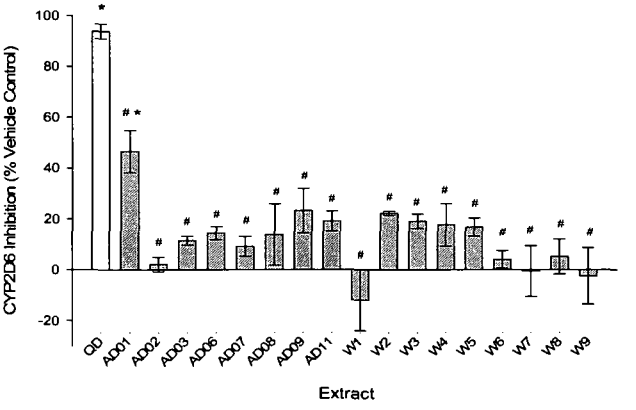


Figure 19: The inhibition of CYP1A2 by the 17 Cree plant extracts. The Cree plant extracts were tested for their potency to inhibit CYP1A2 by measuring the metabolism of the CYP1A2 substrate CEC in a microtitre fluorometric assay. A single concentration of 10 μg/mL was tested for each extract. Furafylline (FF) at a concentration of 50 μM was used as a positive control. The results were expressed relative to a 100% MeOH vehicle control, as the mean inhibition ± SEM. Pooled data ($n = 3$) is presented. $^{*}p \leq 0.05$ with respect to the 100% MeOH vehicle control, using one-way ANOVA followed by the Tukey test. $^{\#}p \leq 0.05$ with respect to FF, using one-way ANOVA followed by the Tukey test.

(A) CYP2B6



(B) CYP2D6



(C) CYP2E1

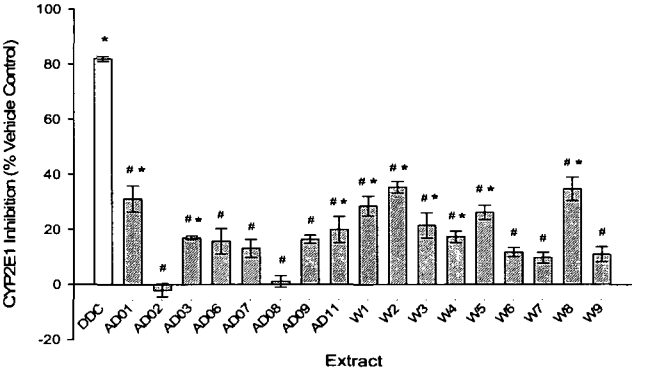
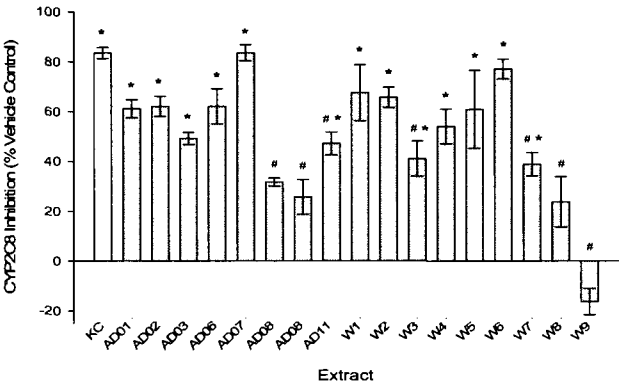
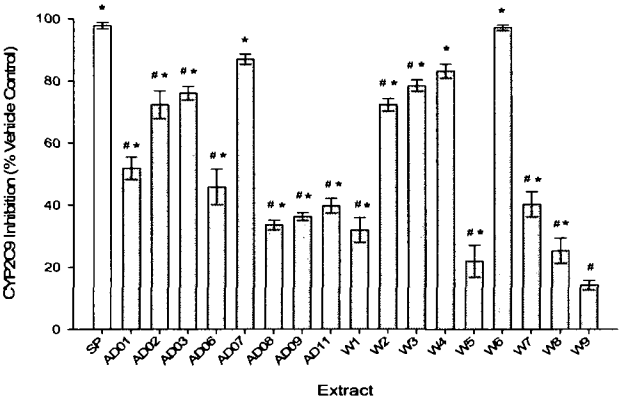


Figure 20: The inhibition of CYP2B6, 2D6, and 2E1 by the 17 Cree plant extracts. The Cree plant extracts were tested for their potency to inhibit **(A)** CYP2B6, **(B)** CYP2D6, and **(C)** CYP2E1 by measuring the metabolism of the substrates MFC (2B6 and 2E1) or AMMC (2D6) in a microtitre fluorometric assay. A single concentration of 10 μg/mL was tested for each extract. The positive controls tranylcypromine (TC) (1 mM), quinidine (QD) (2 μM), and diethyldithiocarbamate (DDC) (100 μM) were used for CYP2B6, 2D6 and 2E1 respectively. The results were expressed relative to a 100% MeOH vehicle control, as the mean inhibition ± SEM. Pooled data ($n = 3$) is presented. $^{*}p \leq 0.05$ with respect to the MeOH vehicle control, using one-way ANOVA followed by the Tukey test. $^{\#}p \leq 0.05$ with respect to the positive control, using one-way ANOVA followed by the Tukey test.

(A) CYP2C8



(B) CYP2C9



(C) CYP2C19

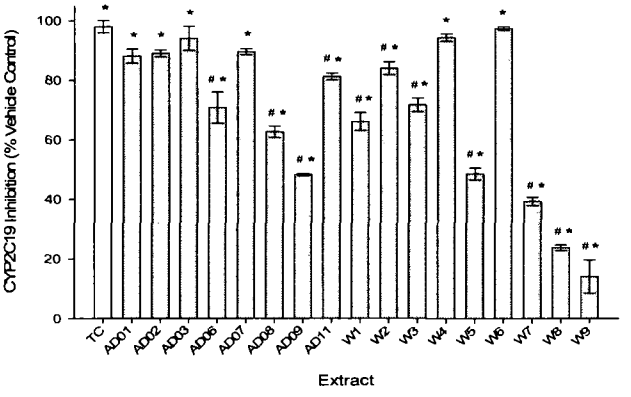
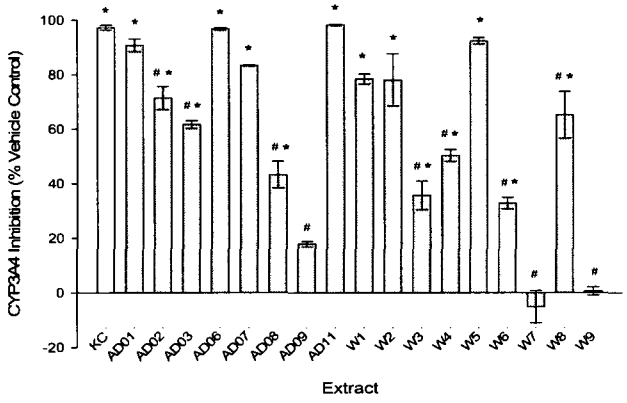
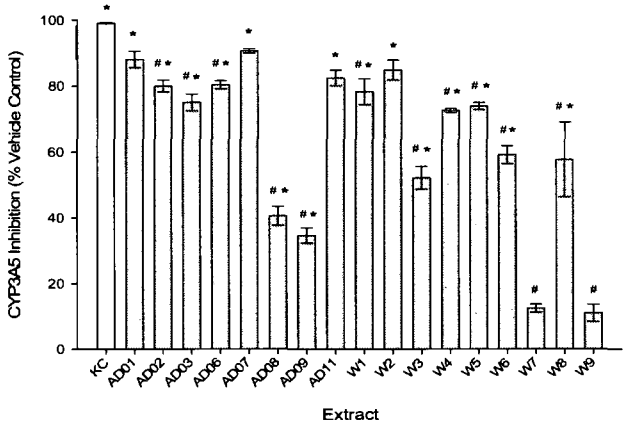


Figure 21: The inhibition of CYP2C8, 2C9, and 2C19 by the 17 Cree plant extracts. The Cree plant extracts were tested for their potency to inhibit **(A)** CYP2C8, **(B)** CYP2C9, and **(C)** CYP2C19 by measuring the metabolism of the substrates DBF, MFC and CEC respectively, in a microtitre fluorometric assay. A single concentration of 10 μg/mL was tested for each extract. The positive controls ketoconazole (KC) (10 μM), sulphaphenazole (SP) (100 μM), and TC (100 μM) were used for CYP2C8, 2C9 and 2C19 respectively. The results were expressed relative to a 100% MeOH vehicle control, as the mean inhibition ± SEM. Pooled data ($n = 3$) is presented. $^{*}p \leq 0.05$ with respect to the MeOH vehicle control, using one-way ANOVA followed by the Tukey test. $^{\#}p \leq 0.05$ with respect to the positive control, using one-way ANOVA followed by the Tukey test.

(A) CYP3A4



(B) CYP3A5



(C) CYP3A7

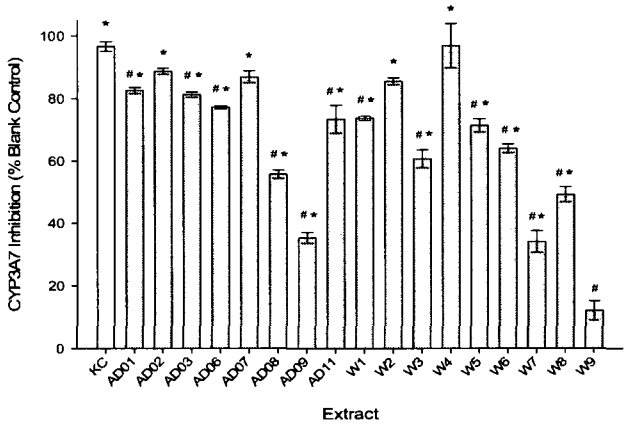


Figure 22: The inhibition of CYP3A4, 3A5, and 3A7 by the 17 Cree plant extracts. The Cree plant extracts were tested for their potency to inhibit **(A)** CYP3A4, **(B)** CYP3A5, and **(C)** CYP3A7 by measuring the metabolism of the substrate DBF in a microtitre fluorometric assay. A single concentration of 10 μg/mL was tested for each extract. The positive control KC (1.9 μM) was used for all three isoforms. The results were expressed relative to a 100% MeOH vehicle control, as the mean inhibition ± SEM. Pooled data ($n = 3$) is presented. $^{*}p \leq 0.05$ with respect to the MeOH vehicle control, using one-way ANOVA followed by the Tukey test. $^{\#}p \leq 0.05$ with respect to KC, using one-way ANOVA followed by the Tukey test.

Table 3. A summary of the inhibition results for each CYP isofom. The overall mean inhibition of the 17 extracts, the categorization of the extract potencies, and the significance of the extracts against the MeOH vehicle and positive control for each CYP isoform are provided.

			Inhibitory Potency			$p \leq 0.05$	
CYP Isoform	**Figure Number**	**Mean Inhibition (% ± SEM)**	**Weak**	**Moderate**	**Strong**	**MeOH Vehicle Control**	**Positive Control**
1A2	19	19.50 ± 3.24	AD02, AD03, AD07, AD08, AD09, W1, W2, W3, W4, W5, W6, W7, W8, W9	AD01, AD06, AD11	FF	AD01, AD06, AD11, W2, W5, FF	All
2B6	20A	7.66 ± 1.62	All	None	TC	AD01, AD02, AD11, W1, W4, TC	All
2C8	21A	49.03 ± 5.81	AD09, W8, W9	AD01, AD02, AD03, AD06, AD08, AD11, W1, W2, W3, W4, W5, W7	AD07, W6, KC	All except for AD08, AD09, W8, W9	AD08, AD09, AD11, W3, W7, W8, W9
2C9	21B	53.32 ± 6.26	W5, W8, W9	AD01, AD02, AD06, AD08, AD09, AD11, W1, W2, W7	AD03, AD07, W3, W4, W6, SP	All except for W9	All except for AD07, W4, W6
2C19	21C	68.40 ± 6.21	W8, W9	AD06, AD08, AD09, W1, W3, W5, W7	AD01, AD02, AD03, AD07, AD11, W2, W4, W6, TC	All	All except for AD01, AD02, AD03, AD07, W4, W6
2D6	20B	12.34 ± 3.17	All except for AD01	AD01	QD	AD01	All
2E1	20C	18.09 ± 2.59	All except for AD01, W2, W8	AD01, W2, W8	DDC	AD01, AD03, AD11, W1, W2, W3, W4, W5, W8, DDC	All
3A4	22A	58.25 ± 7.99	AD09, W7, W9	AD02, AD03, AD08, W3, W4, W6, W8	AD01, AD06, AD07, AD11, W1, W2, W5, KC	All except for AD09, W7, W9	AD02, AD03, AD08, AD09, W3, W4, W6, W7, W8, W9
3A5	22B	63.12 ± 6.11	W7, W9	AD08, AD09, W3, W4, W5, W6, W8	AD01, AD02, AD03, AD06, AD07, AD11, W1, W2, KC	All except for W7, W9	All except for AD01, AD07, AD11, W2
3A7	22C	66.38 ± 5.54	W9	AD08, AD09, AD11, W1, W3, W5, W6, W7, W8	AD01, AD02, AD03, AD06, AD07, W2, W4, KC	All except for W9	All except for AD02, AD07, W2, W4

3.5.2 The Inhibition Trends of the 10 Cytochrome P450 Isoforms

The mean of the inhibitory values against each CYP isoform by the plant extracts ranged from 7.7% ± 1.6% to 68.4% ± 6.2% inhibition **(Table 3)**. The inhibitory ranking from the most inhibited to the least inhibited CYP isoform was: 2C19 > 3A7 > 3A5 > 3A4 > 2C9 > 2C8 > 1A2 > 2E1 > 2D6 > 2B6. There were several extracts that were common strong inhibitors for several CYP isoforms: AD01, AD03, AD07, AD11, W2, W4, and W6 **(Table 3)**. There were also two extracts that were exclusively a strong inhibitor for only one isoform: W3 – CYP2C9; W5 – CYP3A4. Similarly, there were several extracts that were common weak inhibitors of the different CYP isoforms such as AD09, W5, W7, W8, and W9.

Similar inhibition trends were observed among members of the same subfamily. For example, W6 and AD07 were common extracts with strong inhibitory potency towards all members of the CYP2C subfamily (2C8, 2C9, and 2C19). W8 and W9 were common extracts with weak inhibitory potency towards all members of the CYP2C subfamily. Similar observations were observed with the CYP3A subfamily. Differences in the inhibitory potency of the extracts between the CYP2C and 3A subfamily were observed. The differences were difficult to observe with the isoforms CYP1A2, 2B6, 2D6, and 2E1 because of their overall low inhibitions. For a more visual comparison of the differences in inhibitory potencies of the extracts against CYP2C and 3A, a representative isoform from each subfamily was chosen, and their inhibitory values from the extracts were compared **(Figure 23)**. CYP2C9 and 3A4 were chosen because they are the most clinically relevant, and they also had similar overall mean inhibitions by the extracts (53.3% ± 6.3% and 58.3% ± 8.0% mean inhibition respectively).

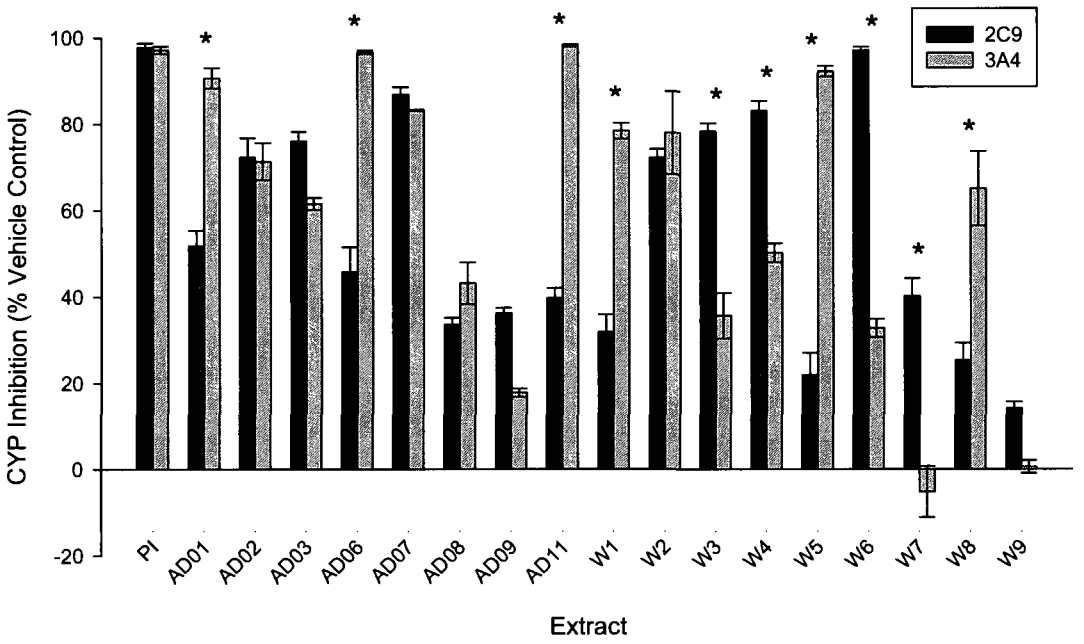


Figure 23: A comparison of the inhibition of CYP2C9 and 3A4 by the 17 Cree extracts. The inhibitory values of the 17 extracts for CYP2C9 and 3A4 were graphed together for a comparison analysis. The positive inhibitors (PI) used were SP (100 μM) and KC (1.9 μM) for CYP2C9 and 3A4 respectively. The results were expressed relative to a 100% MeOH vehicle control, as the mean inhibition ± SEM. Pooled data ($n = 3$) is presented. Significant differences in inhibition ($p \leq 0.05$) observed between CYP2C9 and 3A4 for the same extract using one-way ANOVA followed by the Tukey test, are marked with an asterisk (*).

Approximately half of the extracts inhibited both isoforms with similar potencies: AD02, AD03, AD07, AD08, AD09, W2, and W9. All of the other extracts (AD01, AD06, AD11, W1, W3, W4, W5, W6, W7, and W8) had significant differences in inhibitory potencies between CYP2C9 and 3A4 ($p \leq 0.05$). The extract W5 showed the greatest inhibitory difference as it was a weak inhibitor for CYP2C9, but a strong inhibitor for CYP3A4. Six of the extracts were more potent towards CYP3A4: AD01, AD06, AD11, W1, W5, and W8. The other four extracts, W3, W4, W6, and W7, were more potent to CYP2C9.

The mean of the CYP inhibitory values obtained for each plant extract ranged from 5.1% ± 3.1% to 60.4% ± 8.2% mean inhibition **(Table 4)**. The five overall top inhibitory plant extracts were AD01 > W2 > AD07 > AD11 > W4, and the five overall least inhibitory plant extracts were W9 < W7 < AD09 < W8 < AD08.

3.6 Mechanism-Based Inhibition of CYP3A4

3.6.1 Mechanism-Based Inhibitors

The 17 Cree plants extracts were screened for MBI of CYP3A4 using two screening assays which screened for NADPH- and time-dependence. Two positive controls were used which were GS (11.5 μg/mL) and azamulin (0.1 μM). Ketoconazole (0.2 μM) was used as a negative control. A single extract concentration of 10 μg/mL was tested for both the NADPH- and time-dependence assays.

Mechanism-based inhibitors require NADPH for the formation of covalent bonds with the enzyme. In the absence of NADPH, the formation of these covalent bonds cannot occur and the enzyme cannot be inhibited through MBI. CYP3A4 was pre-incubated with the extracts or the positive/negative control in the presence or absence of NADPH. Decreases in activity were observed with the samples pre-incubated with

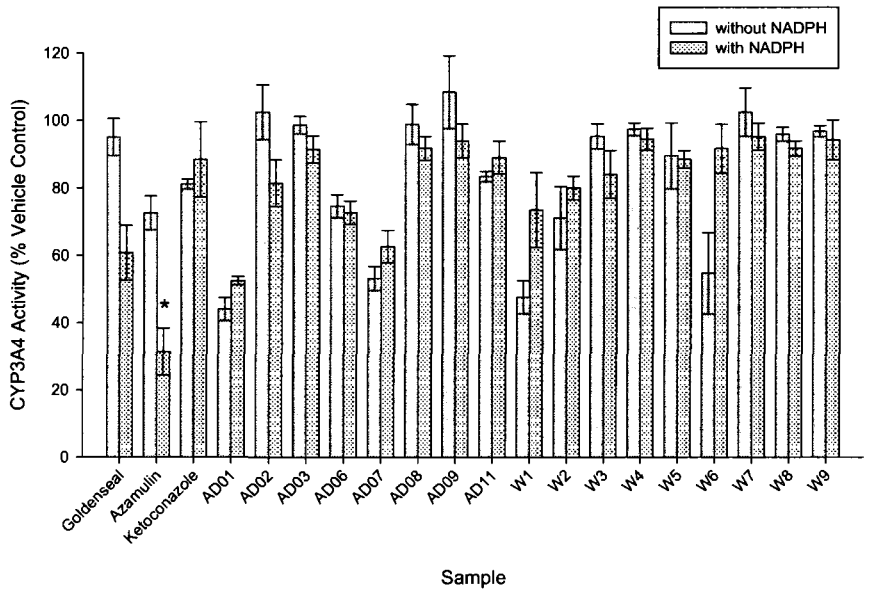
Table 4. The mean of the CYP inhibitory values obtained for each extract. The extracts were ranked according to their inhibitory capability ($n = 3$).

Extract	Mean Inhibition (% ± SEM)	Ranking
AD01	60.4 ± 8.2	1
AD02	49.3 ± 11.8	8
AD03	49.9 ± 10.1	7
AD06	49.8 ± 10.2	6
AD07	55.9 ± 12.6	3
AD08	29.9 ± 6.7	13
AD09	23.7 ± 5.1	15
AD11	52.1 ± 9.6	4
W1	44.9 ± 10.1	10
W2	56.4 ± 9.4	2
W3	40.0 ± 8.0	12
W4	51.8 ± 10.6	5
W5	44.3 ± 9.3	11
W6	45.8 ± 11.9	9
W7	18.4 ± 5.7	16
W8	29.8 ± 6.9	14
W9	5.1 ± 3.1	17

NADPH with GS (95.0% ± 5.6% without NADPH vs. 60.8% ± 8.1% with NADPH), and NADPH with azamulin (72.5% ± 5.0% without NADPH vs. 31.4% ± 7.0% with NADPH) as expected **(Figure 24A)**. However, the only significant decrease was observed with azamulin ($p \leq 0.05$). A significant difference in activities between the two pre-incubation conditions was absent for ketoconazole as expected (81.1% ± 1.5% without NADPH vs. 88.3% ± 11.2% with NADPH). The same was true for the Cree plant extracts ($p > 0.05$) and hence under these test conditions, mechanism-based inhibitors were not detected among the extracts in the NADPH-dependence assay. Several of the plant extracts showed modest differences in their inhibition of CYP3A4 activity under the two conditions (at least a 15% difference) (AD02, AD09, W1, and W6) but were not significant. All of the other plant extracts had comparable inhibitory effects on CYP3A4 activity with the two pre-incubation conditions, similar to the negative control ketoconazole.

Mechanism-based inhibitors cause greater CYP inhibition the longer they are allowed to react with the enzyme and NADPH. Mechanism-based inhibitors can be identified by the decrease in CYP activity the longer the inhibitor is pre-incubated with the CYPs compared to a shorter pre-incubation time. CYP3A4 was pre-incubated with the extracts or the positive/negative controls in the presence of NADPH for either one or ten minutes. Decreases in activity were observed with the samples pre-incubated for ten minutes in GS (81.0% ± 4.6% with one minute pre-incubation vs. 60.8% ± 8.1% with ten minutes pre-incubation) and azamulin (62.9% ± 11.3% with one minute pre-incubation vs. 31.4% ± 7.0% with ten minutes pre-incubation) as expected, but they were not significant **(Figure 24B)** ($p > 0.05$). However, azamulin did approach significance ($p = 0.13$). There

(A) NADPH-dependence



(B) Time-dependence

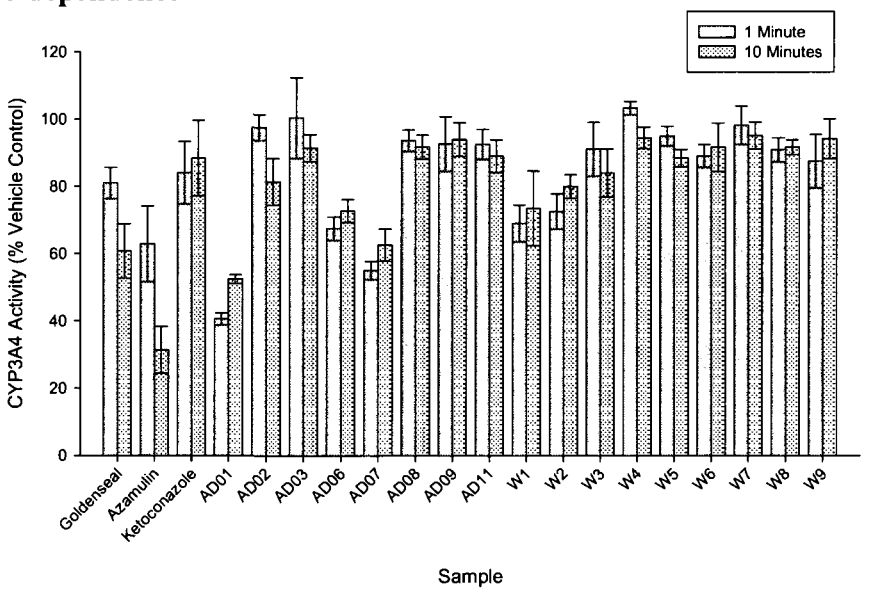


Figure 24: Identifying mechanism-based inhibitors of CYP3A4 of the 17 Cree extracts. The activity of CYP3A4 after a pre-incubation with each extract (10 μg/mL) **(A)** in the absence or presence of NADPH for ten minutes, and **(B)** in the presence of NADPH for one or ten minute(s). The results were expressed relative to a 100% MeOH vehicle control, as the mean CYP3A4 activity ± SEM. GS (11.5 μg/mL) and azamulin (0.1 μM) were used as positive controls. Ketoconazole (0.2 μM) was used as a negative control. Pooled data ($n = 3$) is presented. Significant differences in activity ($p \leq 0.05$) observed between the two conditions tested for the same sample using one-way ANOVA followed by the Tukey test, are marked with an asterisk (*).

were no significant differences in inhibition by ketoconazole under the two pre-incubation times as expected (84.0% ± 9.3% with one minute pre-incubation vs. 88.3% ± 11.2% with ten minutes pre-incubation). Similarly for the Cree plant extracts, significant differences in CYP3A4 activity were absent between the two pre-incubation times and hence under these test conditions, mechanism-based inhibition was not detected by the time-dependent assay. The AD02 extract showed a moderate difference in its ability to inhibit CYP3A4 activity between the two conditions (at least a 15% difference) but it was not significant ($p > 0.05$).

Overall, none of the 17 Cree plants extracts were significant mechanism-based inhibitors of CYP3A4 using DBF as a probe substrate.

3.6.2 Confirmation of Mechanism-Based Inhibitors

It is possible that mechanism-based inhibitors of CYP3A4 are among the Cree plant extracts, but the concentration initially tested in the previous experiment (10 μg/mL) was too low for inhibition. In **Figures 24A and 24B**, many of the extracts had low CYP3A4 inhibition. Further MBI tests were performed with three of the extracts that showed signs of mechanism-based inhibition in **Figures 24A and/or 24B**, using a higher concentration of 50 μg/mL: AD02, AD09, and W3. These additional tests also included another pre-incubation time of five minutes, and two additional concentrations of the controls were tested: 18.5 and 48.5 μg/mL of GS; 1 and 10 μM of azamulin; and 0.9 and 1.9 μM of ketoconazole. Decreases in CYP3A4 activity was observed with all concentrations of GS and azamulin with the pre-incubation with NADPH, and with a five or ten minute pre-incubation time; however the decreases in activity were only significant with 2.3 μg/mL GS (95.0% ± 3.3% without NADPH vs. 52.5% ± 8.1% with NADPH)

and 0.1 μM azamulin (72.5% ± 5.0% without NADPH vs. 31.4% ± 7.0% with NADPH) in the NADPH-dependence assay ($p \leq 0.05$) **(Figures 25)**. There were no differences observed with ketoconazole as expected in both the NADPH- and time-dependence assays. Significant differences in CYP3A4 activity were also not observed with 50 μg/mL of AD02, AD09 and W3 in both the NADPH- and time-dependence assays. Interestingly, at both tested AD02 concentrations, there was a decrease in CYP3A4 activity with NADPH in the pre-incubation solution (10 μg/mL - 102.5% ± 8.1% without NADPH vs. 81.4% ± 7.0% with NADPH; 50 μg/mL – 44.0% ± 6.6% without NADPH vs. 31.5% ± 3.3% with NADPH). In addition, a decrease in CYP3A4 activity was observed with increasing pre-incubation times at both tested concentrations indicating that AD02 may contain compounds which are CYP3A4 mechanism-based inhibitors.

3.7 Drug Interaction Study with Enalapril

3.7.1 Identifying the Median Inhibitory Concentrations

A study was performed to determine if the extracts and the hypertensive drug enalapril interacted with each other to affect CYP3A4 activity. The results were assessed by comparing the CYP3A4 activity in the presence of the extract, to the CYP3A4 activity in the presence of the extract and enalapril. The extracts which showed the greatest anti-diabetic potential in previous studies from collaborators were chosen: AD01, AD08, AD09, AD11, W1, W3, and W5 (Spoor *et al.*, 2006; Harbilas *et al.*, in press). The median inhibitory concentration (IC_{50}) was chosen to be tested to maximize the ability to observe deviations between the CYP activity with the extract, and the CYP activity with the extract and enalapril. The IC_{50} value of an extract was determined by testing a range of

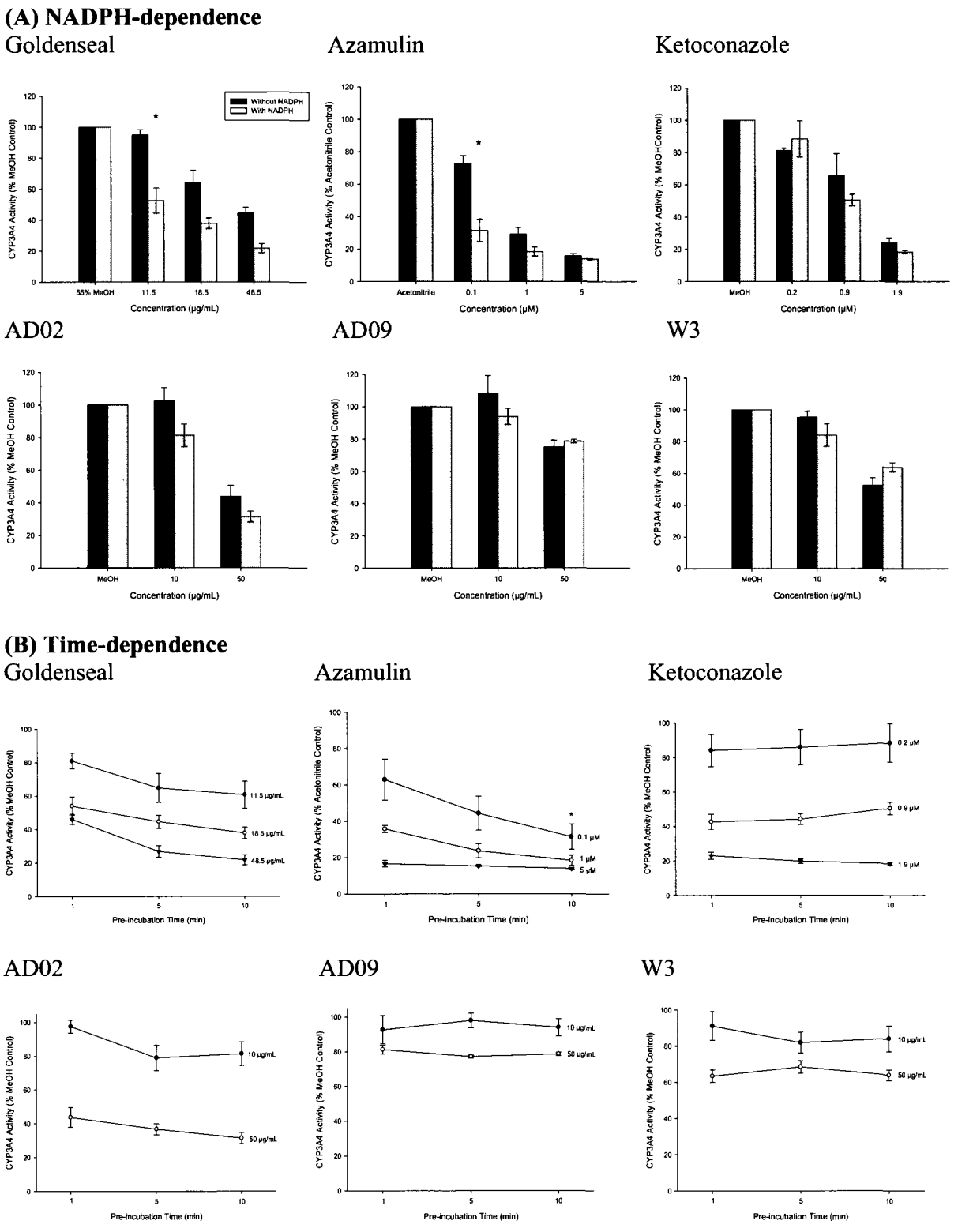


Figure 25: Identifying mechanism-based inhibition of CYP3A4 from AD02, AD09, and W3 extracts using multiple concentrations. CYP3A4 activity after a pre-incubation with different concentrations (10 and 50 µg/mL) of AD02, AD09 and W3 **(A)** for 10 minutes in the absence or presence of NADPH, and, **(B)** in the presence of NADPH for 1, 5, or 10 minute(s). The results were expressed relative to a vehicle control as the mean CYP3A4 activity ± SEM. Pooled data ($n = 3$) is presented. Significant differences in activity ($p \leq 0.05$) observed between the different conditions tested for a concentration of a sample using one-way ANOVA followed by the Tukey test, are marked with an asterisk (*).

concentrations in the CYP3A4 assay to obtain a sigmoidal curve by plotting the log concentrations as a function of CYP3A4 inhibition. The data points of the linear portion of the curve were then plotted to obtain a linear regression line with 95% confidence intervals **(Figures 26)**. The IC_{50} value was obtained using the linear equations of the lines **(Table 5)**. The IC_{50} values obtained were: 1.51, 10.96, 20.89, 3.63, 2.00, 12.88, and 9.12 μg/mL for AD01, AD08, AD09, AD11, W1, W3, and W5 respectively. High 95% confidence intervals were obtained for AD01, AD08, AD09, and W1. The extracts AD11, W3, and W5 had fairly low 95% confidence intervals.

3.7.2 Identifying Changes of CYP3A4 Activity with Enalapril

Extracts of AD01, AD08, and AD09 were selected for the drug interaction studies with enalapril rather than all of the assayed extracts because of their high 95% confidence intervals and their broad range of IC_{50} values. The concentrations tested were: 1.51 μg/mL – AD01; 10.96 μg/mL – AD08; and 20.89 μg/mL – AD09. A single concentration of 0.3 μM of enalapril was tested. Three different versions of the basic CYP3A4 assay as mentioned in Chapter 2 (Materials and Methods) were used to unequivocally test for potential *in vitro* interactions. In all three assays, 0.3 μM enalapril did not affect the metabolism of DBF because a significant difference in activity was not observed between the MeOH vehicle control and enalapril ($p > 0.05$) **(Figure 27)**. In addition, a significant difference in CYP3A4 activity was not observed when comparing the extract, and the extract with enalapril, indicating interactions between the extract and enalapril were absent ($p > 0.05$). In the first assay, there were significant decreases in CYP3A4 activity in the presence of extract, and the extract with enalapril, compared to the 100% MeOH vehicle control ($p \leq 0.05$). In the second and third assay, there were significant decreases

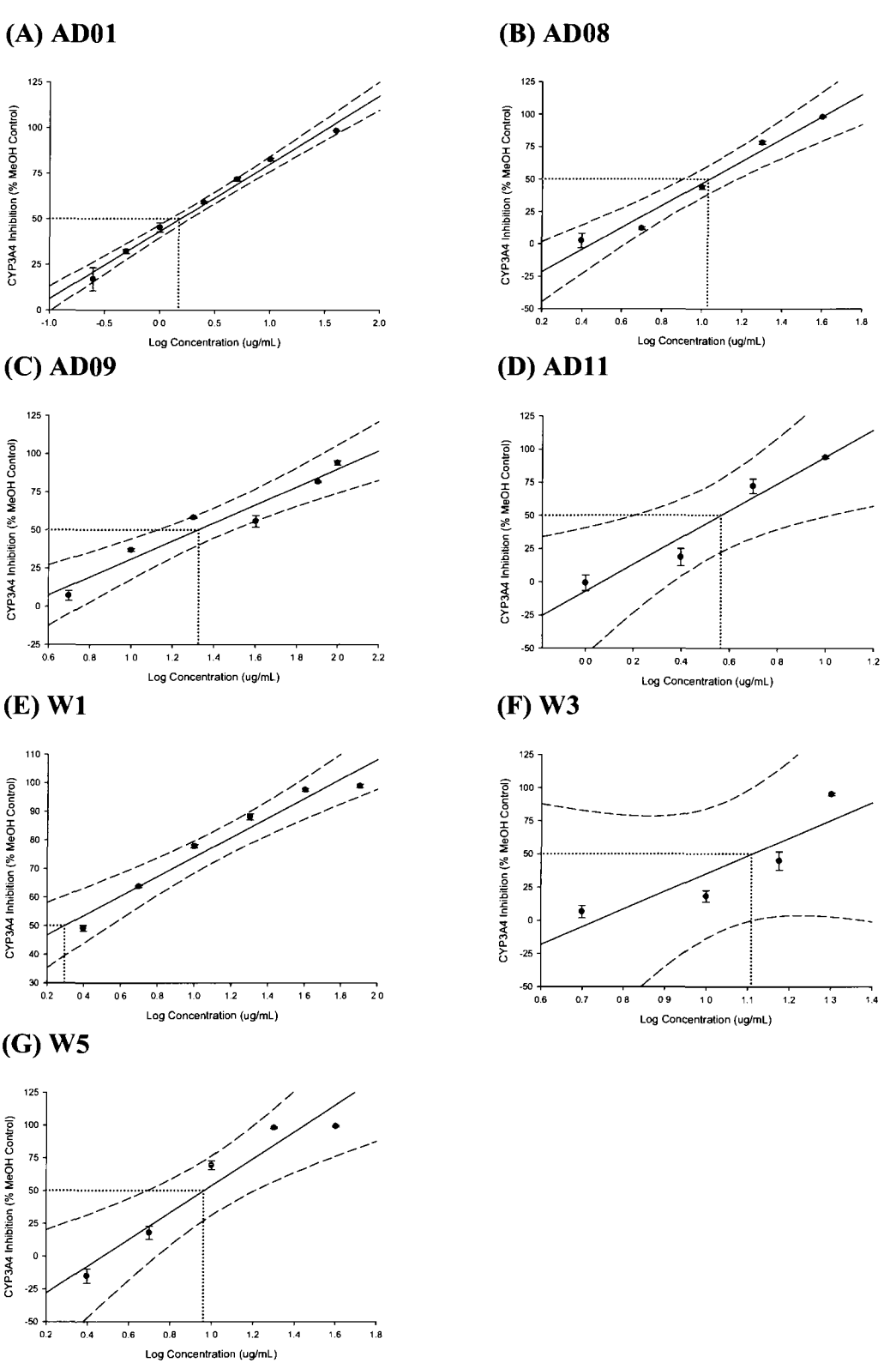


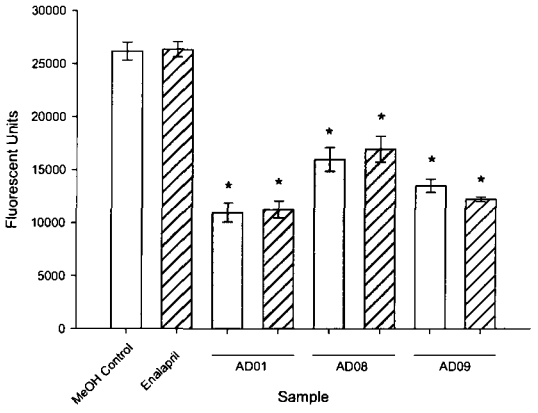
Figure 26: Linear regression lines with the 95% confidence intervals used in determining the median inhibitory concentration of seven of the Cree plant extracts. Various concentrations (0.25 to 160 µg/mL) of the extracts (A) AD01, (B) AD08, (C) AD09, (D) AD11, (E) W1, (F) W3, and (G) W5 were tested for their ability to inhibit CYP3A4. The concentrations were transformed into log form and the results were expressed relative to a 100% MeOH vehicle control, as the mean CYP3A4 activity ± SEM. Pooled data (n = 3) is presented.

Table 5. The median inhibitory concentration (IC_{50}) values for the extracts AD01, AD08, AD09, AD11, W1, W3, and W5 to affect the metabolism of the substrate DBF by CYP3A4. The extracts were ranked according to their inhibitory capability based on their IC_{50} values, and their inhibitory values from the original inhibition assay for comparison ($n = 3$).

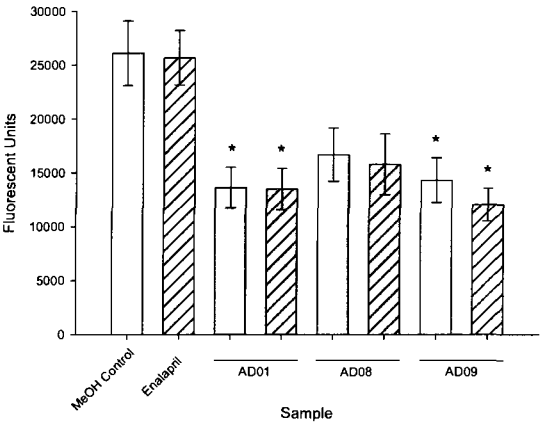
Extract	IC_{50} Concentration (µg/mL)[a]	Equation	R^2	Rank from IC_{50} Results	Rank from Original Inhibition Assay
AD01	**1.51** (1.23, 1.86)	y = 37.08x + 43.18	0.989	1	3
AD08	**10.96** (7.94, 14.79)	y = 54.44x - 38.59	0.974	5	5
AD09	**20.89** (13.18, 30.20)	y = 58.96x - 28.04	0.938	7	7
AD11	**3.63** (1.62, 10.72)	y = 101.02x – 7.05	0.944	3	1
W1	**2.00** (0.76, 3.47)	y = 34.12x + 39.88	0.953	2	4
W3	**12.88** (NA, NA)	y = 133.43x -98.25	0.784	6	6
W5	**9.12** (4.90, 15.85)	y = 102.48x – 48.67	0.926	4	2

[a] Values in brackets correspond to the lower and upper 95% confidence limits respectively. Note that the confidence limit values could not be obtained with W3

(A) No incubation



(B) 15 minutes of incubation



(C) 15 minutes of incubation with buffer and NADPH

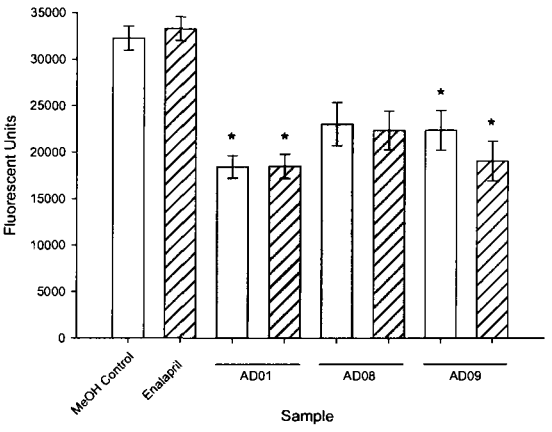


Figure 27: Identifying the effect of enalapril on the inhibitory effects of the Cree plant extracts AD01, AD08, and AD09 against CYP3A4. Enalapril (0.3 μM) was tested for its interaction with the extracts AD01, AD08, and AD09 to determine if the drug affected the inhibitory potencies of the extracts against CYP3A4. The IC_{50} values of the extracts were tested. Three different experiment conditions were used: **(A)** the extract and enalapril were not incubated together before the addition of the CYP3A4; **(B)** the extract and enalapril were incubated together for 15 minutes before the addition of the experimental reagents and CYP3A4; and **(C)** the extract and enalapril were incubated together for 15 minutes with buffer and NADPH before the addition of CYP3A4. The results were expressed relative to a 100% MeOH vehicle control, as the mean CYP3A4 activity in fluorescent units ± SEM. Pooled data ($n = 3$) is presented. *$p \leq 0.05$ with respect to the MeOH vehicle control, using one-way ANOVA followed by the Tukey test. Note that a significant difference was not observed between the extracts and the combination of enalapril and the extracts as determined by a one-way ANOVA followed by the Tukey test ($p \leq 0.05$). (solid bars – without enalapril; striped bars – with enalapril).

in CYP3A4 activity in the presence of extract, and the extract with enalapril except for the extract AD08, compared to the 100% MeOH vehicle control ($p \leq 0.05$). However, AD08 did approach significance in these assays ($p = 0.09$ to 0.11 and $p = 0.11$ to 0.15 respectively).

4 DISCUSSION

The studies in this thesis were conducted to assess the safety of 17 Cree anti-diabetic plants by looking at the capacity for their ethanolic extracts to affect the contraction rate of cardiomyocytes, and to inhibit drug-metabolizing CYPs. The cardiac and CYP studies will be discussed separately, as these two studies are independent of each other.

4.1 Cardiac Chronotropic Effects

The first study investigated the effects of the Cree plant extracts on the contraction rate of neonatal rat cardiomyocytes *in vitro* as a representation of the heart rate in humans. As an elevated heart rate has been associated with an increase risk of mortality in type II diabetics (Stettler *et al.*, 2007; Linnemann & Janka, 2003), it was important to determine if the extracts had the capacity to influence the heart rate. In particular, it was important to identify positive chronotropic extracts as their chronic use may contribute to an elevated resting heart rate.

4.1.1 Chronotrope Controls

The expected, acute, chronotropic effects of carbachol, isoproterenol, and BO were observed after one minute of treatment, validating the use of MEAs to detect the chronotropic effects of the Cree plant extracts. However, the concentrations required to elicit a significant response were higher than those stated in published studies using the same cells. In the studies for this thesis, a significant negative chronotropic effect was observed with 400 μM carbachol which is much higher than the concentrations used to elicit a negative chronotropic effect in other studies (1-100 μM) (Kohashi *et al.*, 2003).

Similarly, the concentrations of isoproterenol which had a significant positive chonotropic effect (greater than 5 μM) was much higher than the concentrations used to elicit a positive chronotropic effect in other studies (10^{-10} to 10^{-11} M) (Simpson & Savion, 1982). Decreases in the pharmacological activity of a drug can occur when serum binds to the drug, restricting its access to its target receptors. Serum is absent in the Tyrode's buffer; however it may be possible that a minute amount of serum remained in the MEA during the media and Tyrode's buffer exchange especially since serum has a "sticky" nature and can bind to proteins. In addition, the presence of non-cardiac cells may have hindered the response of the chronotropes (Simpson & Savion, 1982). The chronotrope wash-in time of one minute may not have been sufficient to induce a great chronotropic effect. The wash-in times used in similar studies ranged as low as two to three minutes (Meyer *et al.*, 2004A; Simpson & Savion, 1982; Kohasi *et al.*, 2003). A short wash-in time was chosen to reduce the time that the cardiomyocytes were left out of the incubator which had been shown to have a negative chronotropic effect **(Figure A5).**

BO was chosen as a positive control, because it is a NHP that possesses sympathomimetic activity. BO is closely related to ephedra and contains synephrine alkaloids which act as α- and ß-adrenergic receptor agonists (Haaz *et al.*, 2006). BO has been found to increase the blood pressure and the heart rate in several clinical and case studies (Bui *et al.*, 2006; Haaz *et al.*, 2006; Firenzuoli *et al.*, 2005). There are currently no published *in vitro* studies that test the chronotropic effect of BO extracts in isolated cardiomyocytes. The results obtained with the BO extract, correspond to the chronotropic effects observed in humans. The typical blood plasma level of BO after a typical dose and

its rate of its metabolism are unknown. However, based on the dose-dependent result obtained, multiple dosing or chronic use of BO can result in positive chronotropic effects.

4.1.2 Dose Ranging of Cree Plant Extracts

Only four plant extracts were chosen to be tested for chronotropic effects due to time limitations with the MEA system. The extracts were chosen based on their greater potential to affect cardiac function, or to interact with anti-hyperglycemic pharmaceuticals relative to the other extracts: W2 (*Kalmia angustifolia*) contains a diterpenoid called kalmanol a compound found to have cardiotoxic properties (Burke *et al.*, 1989); W4 (*Juniperus communis*) is a related species of *Juniper oxycedrus* whose oil was found to cause tachycardia (Koruk *et al.*, 2005); W5 (*Salix planifolia*) is found in natural weight loss supplements containing ephedra and it was important to confirm that the cardiotoxic effect of the supplements were caused by ephedra rather than W5 (Coffey *et al.*, 2004); and W9 (*Vaccinium vitis-ideae*) has hypoglycemic properties and may exacerbate the effects of anti-hyperglycemic pharmaceuticals (Cignarella *et al.*, 1996).

Similar to the studies performed by collaborators in the Cree project, the highest non-toxic concentrations of each extract was tested for their pharmacological effect (in this case, the chronotropic effect) (Spoor *et al.*, 2006; Harbilas *et al.*, in press). At its therapeutic dose, adverse effects of an extract may not be observed, but they may become apparent at a higher dose. The highest non-toxic concentrations were chosen to maximize the chances of observing these adverse effects. In addition, this concentration was chosen because it may represent a dose which may occur from chronic use or an overdose of the plant, or the inhibition of CYPs involved in its metabolism.

The toxicity of the DMSO vehicle control and the extracts was assessed by measuring the LDH released from cardiomyocytes after an 18 hour treatment. Concentrations of DMSO up to 6 μL/mL had no effect on cell viability. The highest non-toxic concentrations for W2, W5, and W9 were 100, 25 and 5 μg/mL respectively. W4 had extremely low toxicity because the highest tested concentration of 600 μg/mL was non-toxic. Typical plasma levels of phytochemicals such as polyphenols can range from the nanogram to low microgram per millilitre region. For example, the plasma level of quercetin after the ingestion of apples can range as low as 0.1 μg/mL, and the plasma level of naringenin after drinking grapefruit juice can average around 1.6 μg/mL (Manach *et al.*, 2003). Even with a single oral administration of artichoke leaf extract (used for the treatment of dyspeptic and hepatic disorders), plasma concentrations of chlorogenic acids range from 6 to 40 ng/mL (Wittemer *et al.*, 2005). Since it is not biologically possible for extract levels to reach concentrations of 600 μg/mL, further studies to determine the toxic concentrations of W4 were not pursued.

The plasma levels of the Cree plant compounds after a typical dose are unknown due to the present lack of clinical studies with these plant species. W9 was the most potent in terms of cardiotoxicty and its toxic concentrations (>10 μg/mL) can possibly occur in the plasma under the appropriate conditions. W5 was also relatively potent in terms of toxicity. The toxic concentrations of W5 (>50 μg/mL) may be reached in the plasma but would be extremely rare. However, this would depend on the dose and frequency of W5 administration, which is unknown due to confidentiality. W2 and especially W4 have low toxicity, as their toxic concentrations would never be obtained in the body physiologically. Interestingly, W2 had low toxicity even though kalmanol, a

known cardiotoxin is present in this species (Burke *et al.*, 1989). It is possible the kalmanol was present in very low amounts in the extract, or possibly that kalmanol was not present in the extract. For example, extracts can be produced using water, ethanol, methanol, hexane, or ethyl acetate, and extract different phytochemicals based on their polarity. Most extractions are performed with ethanol or methanol because many of the active phytochemicals are extracted in these solvents. Growing conditions such as the season of collection, the altitude, location, and environmental influences can also affect the phytochemical profiles of plants from the same species (Adams, 1987; Williamson, 2006; Foster *et al.*, 2005). Overall, the results indicate that W9 should be used with caution because of its potential for cardiotoxicity at concentrations plausibly obtained in the plasma. In addition, diabetics typically suffer from cardiac dysfunction as a result of heart disease or diabetic cardiomyopathy (Canadian Diabetes Association, 2008B; Ren *et al.*, 1999) and may be more susceptible to cardiotoxicity of the plants even at concentrations deemed to be non-toxic in the present study. Further studies using diabetic cardiomyocytes should be performed to validate the aforementioned postulation. It is important to note that the toxicity results were obtained *in vitro* using neonatal rat cardiomyocytes, and may not equate to *in vivo* toxicity in humans.

4.1.3 Cree Plant Extracts

The extracts were solubilized in DMSO and the final DMSO concentration during the screening tests was 1 μL/mL for the majority of the extracts except for the W4 extract which had a DMSO concentration of 5 μL/mL. DMSO has been reported to have a chronotropic effect of the myocardium, and therefore, the 5 μL/mL DMSO would be expected to have more of a chronotropic effect than 1 μL/mL DMSO (Matheny *et al.*,

1976). A significant, acute, negative chronotropic effect was only observed with the 1 µL DMSO vehicle control, relative to the initial contraction rate in Tyrode's buffer. It is unknown why the lower concentration of 1 µL/mL, but not 5 µL/mL had a significant, acute, negative chronotropic effect, and it may be possible that with an increase in the number of cultures tested (n greater than 5) a significant effect would not be observed. In addition, the treatment time of one minute, may have been too short to observe a significant chronotropic effect with 5 µL/mL DMSO. Indeed, after an 18 hour treatment, 5 µL/mL DMSO had a significant negative chronotropic effect. An 18 hour treatment was sufficient to allow DMSO to affect cellular signaling pathways or cellular processes within the cardiomyocytes, which may account for the negative chronotropic effect observed. Since the chronotropic effect of an extract was measured relative to its vehicle control, the significant negative chronotropic effect of 1 µL/mL DMSO with acute treatment, and 5 µL/mL DMSO with chronic treatment should not affect the results.

The highest non-toxic concentration for each extract had no significant, acute, chronotropic effect relative to its DMSO vehicle control. W2 extract had a minor positive chronotropic effect, whereas the other three extracts had minor negative chronotropic effects. Oil from a related species to W4 has been associated with a positive chronotropic effect (Koruk *et al.*, 2005). This is opposite to the effect observed in this study. It is possible that constituents present in the oil are absent in the ethanolic extract, or that the active constituents differ among the species.

The W9 extract was chosen for further analysis as it had the greatest negative chronotropic effect, and potentially the highest non-toxic concentration that can be reasonably obtained in the plasma. In addition, if W9 had a dose-dependent negative

chronotropic effect, W9 would be a suitable plant to treat T2D as an alternative medicine. Recent results from two separate follow-up studies in Switzerland and Germany, have shown that a high resting heart rate in type II diabetics is associated with an increase in mortality (Stettler *et al.*, 2007; Linnemann & Janka, 2003). If W9 does have the ability to influence the lowering of the heart rate, this plant material can be beneficial for diabetics because it also has hypoglycemic properties (Cignarella *et al.*, 1996). However, if used concomitantly with anti-hyperglycemic pharmaceuticals as a complementary medicine, a drastic drop in glucose levels may ensue which could be harmful to the patient.

The two additional concentrations of W9 extract tested for acute chronotropic effects (25 and 100 μg/mL) did not affect the contraction rate and therefore, and it is unlikely that W9 would not have an impact on the heart rate even at high doses.

The four extracts of W2, W4, W5, and W9 had no significant chronic chronotropic effects. However, these results are not conclusive. The contraction rate of a single culture changed on a daily basis **(Figure A1)** which increased the difficulty of observing chronic chronotropic effects. In addition, a small number of samples were tested for this study (n of 3).

Overall, the results indicate that the ethanolic extracts of W2, W4, W5 and W9 do not have an effect on the *in vitro* cardiac contraction rate at their highest non-toxic concentrations after acute or chronic use, and use of these plants would not be expected to affect the heart rate in humans, disproving the hypothesis (1, page 25) that the Cree plants will have a chronotropic effect. These plants do not have a beneficial or harmful effect with respect to the heart rate and its associated risk of mortality upon heart rate elevation. In addition, the order of toxic potencies from the lowest to the greatest was W4

< W2 < W5 < W9. Toxic cardiac concentrations of W9 (> 10 μg/ml) can plausibly be obtained in the plasma and should not be used frequently in high doses. W2 and W4 would not have any toxic effects on cardiomyocytes, but W5 may have a toxic effect in very rare cases of extreme overdose. However, because of the common cardiac dysfunction observed in diabetics, the plants may be toxic at lower concentrations. In addition, if multiple Cree plants are used concomitantly the risk of cardiac toxicity may increase. The toxicity and the chronotropic effects of the remaining 13 Cree plant extracts have not been determined, and should be studied to assess their safety with respect to cardiac function.

4.2 Drug Interaction Study with Metformin

It is possible that the Cree plants will be taken as a complementary medicine and used concomitantly with conventional drugs for the treatment of T2D. Interactions may occur between the drug and the plant compounds to affect the heart rate. One of the most common pharmaceuticals used by diabetics is metformin (Glucophage®), an anti-hyperglycemic medication. Metformin does not undergo metabolism by CYPs because of its hydrophilic nature and typically is excreted unchanged from the kidney (Kirpichnikov *et al.*, 2002). The typical plasma concentration of metformin can reach as high as 40 μM (Sum *et al.*, 1992); however, concentrations up to 50 μM can be obtained in the presence of renal dysfunction (Kazory *et al.*, 2007), a common complication suffered by approximately half of CEI diabetics (Kuzmina & Dannenbaum, 2004). The single concentration of 50 μM metformin was chosen for these reasons and was tested on cardiomyocytes in combination with W9 extract to determine if the combination of the

two had an affect on the contraction rate. A metformin concentration of 50 μM, was found to be non-toxic in adult rat cardiomyocytes (Ren *et al.*, 1999).

There have been no reports of metformin affecting the heart rate in humans, and in our study, metformin did not have an effect on the contraction rate of the cardiomyocytes. The combination of 50 μM metformin and 100 μg/mL of W9 had a significant negative chronotropic effect relative to the DMSO control; whereas, 100 μg/mL of W9 and 50 μM metformin individually did not have this significant effect. The results from the recovery experiment indicate that the negative chronotropic effect observed was due to toxicity, rather than a cellular signaling mechanism. Significant toxicity was observed with 100 μg/mL of W9 after an 18 hour treatment (determined using the LDH assay), and this concentration of W9 along with 50 μM metformin may have been too overwhelming for the cardiomyocytes. A full recovery was observed upon the washout of metformin and 5 μg/mL of W9; however only a minor recovery was observed with metformin and 100 μg/mL of W9. The recovery contraction rates were measured immediately after the washout of the xenobiotics. If the recovery contraction rates were measured an hour after, a full recovery with the cardiomyocytes treated with 50 μM metformin and 100 μg/mL of W9 may have been observed.

A concentration of 50 μM metformin can be obtained in the plasma, not 100 μg/mL of W9. Therefore, the combination of metformin and W9 should not have an effect on the heart rate of diabetics in the CEI. In addition, since W9 itself did not have a chronotropic effect, the results signify that any extract at an extremely high dose with 50 μM metformin may have a toxic effect on cardiomyocytes and affect their contraction rate. These results suggest that the hypothesis (2, page 25) that the Cree plants will

interact with metformin to cause an adverse effect is false, as the chronotropic effect was due to toxicity rather than a direct interaction between the plant extract and metformin.

It is important to note that extracts were tested on healthy cardiomyocytes and not diabetic cardiomyocytes. Diabetic cardiomyocytes are typically in a weakened and dysfunctional state and hence, may be more sensitive to the extract effects. It is plausible to hypothesize that toxicity can occur with lower concentrations of the extracts using diabetic cardiomyocytes. It would be important to test for chronotropic effects of the extracts, as well as the combination of drugs and extracts using diabetic cardiomyocytes as these conditions would be closer to physiological conditions. On the other hand, the extracts may have a favourable effect on the diabetic cardiomyocytes similar to metformin, by reversing the contractile dysfunction commonly observed in diabetic cardiomyocytes such as a prolonged AP duration, or slowed clearing of Ca^{2+} ions during depolarization (Ren *et al.*, 1999).

4.3 Diabetic-Like Cardiomyocytes

4.3.1 Presence and Viability

Diabetic-like cardiomyocytes have been used as a model of diabetic cardiomyocytes, as they exhibit the typical properties of diabetic cardiomyocytes including a prolonged AP duration and slowed Ca^{2+} clearance (Ren *et al.*, 1997; Shimoni *et al.*, 1994). High glucose levels has been associated with the abnormal glycosylation of proteins involved in cardiac contraction (*i.e.* ion channels or the regulatory protein, protein kinase C) (Laakso, 1999; Ren *et al.*, 1997), and an increase in cytosolic Ca^{2+} levels (D'Amico *et al.*, 2001; Ren *et al.*, 1997). A similar protocol by Ren *et al.* (1997) was used to produce diabetic-like cardiomyocytes from the same source of

cardiomyocytes used for the other cardiac experiments, using high glucose media. Confirming the presence of diabetic-like cardiomyocytes was assessed by measuring their FP duration (as a measure of AP duration). The results indicated that diabetic-like cardiomyocytes were not produced because the FP duration of these cells were not prolonged compared to the normal cardiomyocytes cultured in the normal glucose media. However, the difference in the glucose concentration between the normal (17.5 mM) and high (25.5 mM) glucose media was minor, which may account for the small increase in FP duration observed with the cardiomyocytes cultured in high glucose media. A patient is diagnosed with diabetes when their fasting plasma glucose level is over 7 mM. The normal glucose media had more than twice the glucose concentration of this and therefore, long FP durations may already have occurred with the normal glucose media.

Ren *et al.* (1997) used low glucose media with a glucose concentration of 5 mM. Interestingly in the studies for this thesis, the cardiomyocytes cultured in the 5 mM glucose media had unexpectedly longer FP durations than the cardiomyocytes cultured in the normal glucose media even though the media constituents were identical expect for the glucose concentration. It appeared that the cardiomyocytes did not thrive as well in the low glucose media because the viability of these cardiomyocytes was much lower as observed under the microscope. Unfortunately, no viability studies such as the LDH assay were performed to confirm this. The cardiomyocytes cultured in the high glucose media also had lower viability which was observed visually, but their viability was not as low as the cardiomyocytes cultured in the low glucose media. Again, viability studies were not performed to confirm this. A lower viability of cardiomyocytes can be expected

in high glucose media since high glucose is detrimental to cardiomyocytes and is the pathological basis of diabetes.

Since the cultured cardiomyocytes did not have the diabetic-like characteristics to those of Ren *et al.* (1997), the Cree plant extracts were not tested for their chronotropic effects with these cells. It is unknown how differently the Cree plant extracts would act on diabetic-like cardiomyocytes compared to healthy cardiomyocytes.

4.4 Changes in the Field Potential Duration

An interesting observation of a progressive prolonged FP duration was observed with the cultured cardiomyocytes the longer the cells were kept in culture. (These findings were an observation, and are not relevant to the chronotropic and toxic effects of the Cree plant extracts.) The prolongation observed was inconsistent with literature results which demonstrate a shortening of the FP duration during cardiomyocyte maturity measured using the patch-clamp technique (Kilborn & Fedida, 1990; Guo *et al.*, 1996; Meiry *et al.*, 2001). The AP durations of fetal or neonatal cardiomyocytes are very long compared to adult cardiomyocytes (Kilborn & Fedida, 1990; Guo *et al.*, 1996). For example, the AP duration of neonatal rat cardiomyocytes can last approximately 700 msecs, whereas in adult cells the AP duration may last 100 to 200 msecs (Kilborn & Fedida, 1990). By 7 DIV, the AP duration of cultured neonatal rat cardiomyocytes can be extremely short like that in the adult (Meiry *et al.*, 2001; Kilborn & Fedida, 1990). In a study using neonatal rat cardiomyocytes cultured for 3 to 8 DIV in MEAs, the FP durations of the cardiomyocytes progressively decreased and ranged from 200 to 300 msecs (Meiry *et al.*, 2001). As it has been shown that there is a direct relationship between the AP and the FP duration observed using MEAs, it is uncertain why the AP

durations measured in these experiments were extremely short (Halbach *et al.*, 2001). A possible explanation is that the culture media used during the FP duration recordings did not contain sufficient Na^+, Ca^{2+}, or K^+ ions to sustain a long AP duration. The ion concentrations of the media were lower than the ion concentrations in the Tyrode's buffer, a typical electrophysiological buffer. A decrease in the flow of the Ca^{2+} ions through Ca^{2+} channels (such as during Ca^{2+} channel blockage) has been shown to shorten the FP duration (Halbach *et al.*, 2003). However, the FP durations of the cardiomyocytes in Tyrode's buffer showed the same FP duration shortening (data not shown). The ion content of the media and buffer may have an effect on the shortened FP durations observed in these studies and should be verified with additional future experiments. It may be possible that diabetic-like cardiomyocytes were produced in the previous experiments, but were not detected due to the unexpected, shortened FP durations observed under the experimental conditions.

The progressive increase in the FP duration indicates that the cardiomyocytes were undergoing hypertrophy and dedifferentiation which occurs when the cardiomyocytes revert back to a more fetal phenotype in the presence of stress or growth factors (Gaughan *et al.*, 1998). Hypertrophy can be induced from the presence of serum in the culture media, and from factors secreted from fibroblasts such as vascular endothelial growth factor, leptin, macrophage inflammatory protein-1α, IL-6, IL-10 and tumor necrosis factor (LaFromboise *et al.*, 2007; Fredj *et al.*, 2005). The media used for the experiments had a high content of serum (15%) and did not contain any anti-proliferating agents such as bromodeoxyuridine or cytosine 1-ß-D arabinofuranoside, which favoured the growth of fibroblasts. There were many non-cardiac cells observed in

the cultures, most likely fibroblasts. Fibroblasts also influence the contraction rate and the membrane potential of cardiomyocytes (Simpson & Savion, 1981; LaFromboise *et al.*, 2007).

4.5 Inhibition of Cytochrome P450 Isoforms

Diabetes is a disease in which high glucose levels are present in the blood because of an insufficiency of its uptake into cells. The high glucose levels are detrimental to the health and function of various organs and systems in the body. The kidneys, neurons, cardiomyocytes, smooth muscle, and retina cells are commonly affected. Diabetics can take a range of medications depending on what physiological systems are affected **(Table 6)**. The first-line of medication used by diabetics is the anti-hyperglycemic drugs which include various classes: bigunides, sulfonylureas, meglitanides, thiazolidinediones, and α-glucosidase inhibitors. Reducing glucose levels in the blood can help alleviate symptoms of diabetes. Hypertension or renal dysfunctions are treated by angiotensin-converting enzymes inhibitors, angiotensin-II type 1 receptor blockers, Ca^{2+} channel blockers, and diuretics. Cardiac complications are treated by adrenergic ß-blockers, anti-arrhythmic drugs, angiotensin-converting enzymes inhibitors, Ca^{2+} channel blockers, and anti-coagulent drugs. Painful neuropathies are treated with analgesics, tricyclic antidepressants, and anticonvulsants. Lipid-lowering medications such as HMG-CoA reductase inhibitors (statins) and fibric acids are commonly used by diabetics to help lower their cholesterol levels to manage cardiovascular disease. As the Cree medicinal plants may be used as complementary medicine to treat T2D, it was important to study the inhibitory potencies of the Cree plant extracts against the drug-metabolizing CYPs. Inhibition of the CYPs can prevent the normal metabolism of the drugs used by diabetics

Table 6: A list of the drugs commonly used by type II diabetics and the major CYP isoforms involved in their metabolism.

Drug	CYP	References
Anti-hyperglycemic		
Biguinide		
metformin	none	1
Sulfonylureas		
glyburide	2C9	1 2
glimeperide	2C9	1 2 3
gliclazide	2C9, 2C19	4 5
Meglitanides		
nateglinide	2C9	2 3
repaglinide	2C8, 3A4	2 3
Thiazolidinediones		
pioglitazone	2C8, 3A4	1 2
rosiglitazone	2C8, 2C9	1 2 3 6
α-glucosidase inhibitors		
acarbose	none	1
Hypertension or Nephropathies		
Angiotensin-converting enzyme inhibitors		
captopril	2D6	1 3
enalapril	3A4	1 3
ramipril	3A4	1
perindopril	3A4	1
lisinopril	3A4	1
Angiotensin-II type 1 receptor blocker		
losartan	2C9	1 3 9
irbesartan	2C9, 3A4	1 3 10
Calcium channel blockers		
verapamil	3A4, 3A5, 2C8, 1A2	1 3 11
diltiazem	3A4	1 3
felodipine	3A4	1 3
nifedipine	3A4	1 3
amlodipine	3A4	1 3
Diuretics		
hydrochlorothiazide	None	1
torsemide	2C9	3 12
Lipid-lowering		
HMG-CoA reductase inhibitors (statins)		
atorvastatin	3A4, 2C8	1 3 7
lovastatin	3A4	1 3 7
simvastatin	3A4, 2C8	1 3 7
fluvastatin	2C9, 2C8	1 3 7 22
Fibric Acids		
gemfibrozil	none	8
fenofibrate	none	8
Heart Complications		
β-Blocker		
carvedilol	2C8, 2D6	1 3
metoprolol	2D6	1 3
propranolol	2D6	3 13
Anti-arrhythmic		
flecainide	2D6	3
Anti-coagulent		
warfarin	2C9	1 3 14
Calcium channel blockers		
Angiotension-converting enzyme inhibitors		
Neuropathies		
Analgesics		
ibuprofen + sulindic	2C8, 2C9	15 16
Tricyclic antidepressants		
amitriptyline + fluphenazine	3A4, 2C9, 2D6	17 18
nortriptyline + fluphenazine	2D6, 2C19	18 19
desipramine	2D6	20
Anticonvulsants		
carbamazepine	2C8	18
gabapentin	none	21

References: 1) Triplitt, 2006; 2) Kirchheiner *et al.*, 2005; 3) Siest *et al.*, 2007; 4) Park *et al.*, 2003; 5) Zhang *et al.*, 2007; 6) Baldwin *et al.*, 1999; 7) Neuvonen *et al.*, 2006; 8) Spence, 1998; 9) Yasar *et al.*, 2001; 10) Bourrie *et al.*, 1999; 11) Tracy *et al.*, 1999; 12) Miners *et al.*, 1995; 13) Masabuchi *et al.*, 1994; 14) Juurlink, 2007; 15) Martinez *et al.*, 2005; 16) Garcia-Martin *et al.*, 2004; 17) Ghahramani *et al.*, 1997; 18) Castberg *et al.*, 2005; 19) Olesen & Linnet, 1997; 20) Ball *et al.*, 1997; 21) Riva *et al.*, 1996; 22) Totah & Rettie, 2005.

resulting in a drug dosage which is toxic or inefficient to elicit its pharmacological activity, both having adverse effects. Cases of interactions between anti-diabetic drugs and other drugs or NHPs have already been documented. For example, changes in the bioavailability of the meglitanide repaglinide have been observed during coadministration of several antibiotics (ketoconazole, clarithromycin, or telithromycin) which are inhibitors of CYP3A4 (Scheen, 2007A). Similar interactions have also been observed with thiazolidinediones (pioglitazone and rosiglitazone) and CYP2C8 inhibitors such as rifampicin, gemfibrozil, and trimethoprim (Scheen, 2007B). The NHP St. John's wort used for treating depression and anxiety, has also been shown to increase the clearance of gliclazide due to its inductive effect on CYP2C9 expression (Xu *et al.*, 2008). As shunting of substrates from saturated isoforms is possible, a wide range of isoforms were examined to provide a broad perspective of potential interactions. The CYP isoforms studied were: 1A2, 2B6, 2C8, 2C9, 2C19, 2D5, 2E1, 3A4, 3A5, and 3A7. As these plant species are not common NHPs in Western society, there have been no studies on their inhibitory potency to CYPs.

4.5.1 The Inhibitory Potency of the Cree Plant Extracts

The majority of the extracts had weak inhibitory potencies towards CYP1A2 activity and would not be expected to affect the metabolism of CYP1A2 substrates. The extracts of AD01, AD06, and AD11 have the greatest probability of interacting with CYP1A2 substrates because they had moderate inhibitory potencies. None of the medications commonly used by diabetics are extensively metabolized by CYP1A2 except for several pain killers such as naproxen and acetaminophen. Both of these drugs are associated with adverse effects. With chronic use, naproxen can damage the stomach

lining and increase the risk of cardiac events (Adelman, 2001) and acetaminophen can cause liver toxicity (Watelet *et al.*, 2007). Interactions that would inhibit their metabolism, would increase their risk of toxicity of these drugs.

All Cree plant extracts had weak inhibitory potencies towards CYP2B6 activity and would not be expected to affect the metabolism of CYP2B6 substrates. None of the commonly used drugs for T2D are known to be metabolized by CYP2B6 and therefore, it is not likely for significant effects in the context of drug-NHP interactions to occur in this patient population.

All extracts had weak inhibitory potencies towards CYP2D6 activity except for AD01 which had moderate inhibitory potency. CYP2D6 metabolizes many drugs used to treat cardiac complications such as carvedilol, metoprolol, propranolol, flecainide, and warfarin. It is also involved in metabolizing anti-depressants which are used by diabetics to treat pain from neuropathies (Vinik, 1999). The extract of AD01 would have the greatest probability of interacting with the metabolism of these drugs. Inhibiting the breakdown of drugs used to regulate cardiac function such as the ß-blockers can have adverse and even fatal effects in diabetics, and therefore AD01 should be used with caution with diabetics taking drugs to treat cardiac complications.

The majority of the extracts had weak inhibitory potencies towards CYP2E1 activity and would not be expected to affect the metabolism of CYP2E1 substrates. Extracts of AD01, W2, and W8 had moderate inhibitory potencies and thus, the greatest probability of interacting with CYP2E1 substrates. None of the commonly used drugs for T2D are metabolized by CYP2E1 and as with CYP2B6, it is not likely for significant drug-NHP interactions to occur in this patient population. However, CYP2E1 activity can

generate toxic ROS (Cedarbaum, 2006) that can oxidize DNA, protein, carbohydrates, and lipids (Cai & Harrison, 2000). In addition, ROS can inactivate nitric oxide which is involved in vasodilation and decreasing hypertension (Cai & Harrison, 2000). Diabetics are more prone to producing ROS created during glycation reactions because of their high levels of glucose, and has been proposed to play a role in the pathology of the disease (Kaneto *et al.*, 1999). In addition, CYP2E1 has been found to be induced in diabetics (Wang *et al.*, 2003). In the case of CYP2E1, its inhibition by the Cree plant extracts can be viewed as a positive attribute and a potential anti-diabetic mechanism by reducing the production of ROS. Only extracts of AD01, W2, and W8 had the capacity to moderately inhibit CYP2E1.

CYP2C8 plays a major role in the metabolism of various drugs used by diabetics. CYP2C8 is the major metabolizer of the anti-hyperglycemic thiazolidinediones and the lipid-lowering statins. It also metabolizes the anti-hyperglyemic drug repaglinide and the ß-blocker carvedilol. CYP2C8 is involved in the formation of epoxyeicosatrienoic acids (EETs) by the metabolism of arachidonic acid (Zeldin *et al.*, 1996; Marill *et al.*, 2000). EETs play a role in diabetes because they affect both renal function and vascular smooth muscle (Totah & Rettie, 2005), two common systems affected in diabetes. EETs help alleviate hypertension by acting as vasodilators in the vascular system, and also by inhibiting the reabsorption of Na^+ in the kidney to decrease the blood volume (Sarkis *et al.*, 2004). Hypertension is a complication suffered by diabetics, and inhibition of EET production through the inhibition of CYP2C8, can increase its severity. There was a broad range of inhibition against CYP2C8 by the 17 Cree plant extracts. The extracts of AD09, W8, and W9 had weak inhibitory potencies towards CYP2C8 activity and would

not be expected to affect metabolism of CYP2C8 substrates. The extracts of AD07 and W6 had a strong inhibitory potency, comparable to the positive control ketoconazole (10 μM), and hence may have the greatest probability of affecting the metabolism of CYP2C8 substrates. The other extracts (AD01, AD02, AD03, AD06, AD08, AD11, W1, W2, W3, W4, W5, and W7) were moderately potent inhibitors and have a lower probability of affecting the metabolism of CYP2C8 substrates. The anti-hyperglycemic drugs are important in diabetics as they assist in lowering glucose levels, and inhibition of their metabolism can result in the unstable maintenance of blood glucose levels which can result in adverse events. AD07 and W6 should be used with caution with drugs metabolized by CYP2C8, and also by diabetics suffering from hypertension.

CYP2C9 is extremely important in the metabolism of drugs commonly used by diabetics. It is the major metabolizer of the anti-hyperglycemic sulfonlyureas, nateglinide, and rosiglitazone; the angiotenson-II type 1 receptor blockers which are used to treat hypertension; the diuretic torsemide; the lipid-lowering statin fluvastatin; the anti-coagulant warfarin; and several drugs used to treat neuropathies. The 17 Cree plant extracts had a broad range of inhibition against CYP2C9. W5, W8, and W9 extracts had weak inhibitory potencies towards CYP2C9 activity and would not be expected to affect metabolism of CYP2C9 substrates. Extracts of AD03, AD07, W3, W4, and W6 had strong inhibitory potencies. The other extracts (AD01, AD02, AD06, AD08, AD09, AD11, W1, W2, and W7) were moderately potent inhibitors and have a lower probability of affecting the metabolism of CYP2C9 substrates. The five extracts with strong potent inhibitory effects have a great potential to interact with these drugs and should be used with caution. Several of the moderately potent inhibitors such as AD01 and AD02 should

be used cautiously with any of the five potent CYP2C9 inhibitors or the aforementioned drugs.

CYP2C19 was the most inhibited CYP isoform studied. Only extracts of W8 and W9 had weak inhibitory potencies towards CYP2C19 activity and would not be expected to interact with drugs. The extracts of AD01, AD02, AD03, AD07, AD11, W2, W4, and W6 had strong inhibitory potencies, whereas those of AD06, AD08, AD09, W1, W3, W5, and W7 had moderate inhibitory potencies. However, CYP2C19 is not a metabolizer of drugs used by diabetics except for the anti-depressant nortriptyline used for treating pain. Therefore, even though CYP2C19 was the most inhibited CYP isoform by the Cree plant extracts, the clinical significance of this high inhibition may be low.

CYP3A4 is an important CYP isoform in the context of drug metabolism because it is involved in the metabolism of over 50% of the drugs on the market (Zhou *et al.*, 2005; Burk & Schwab, 2007). In addition, it metabolizes many drugs used by diabetics such as the angiotensin-converting enzyme inhibitors used to treat hypertension and cardiac complications; the calcium channel blockers used to treat hypertension and cardiac complications; and several of the statins and anti-hyperglycemic drugs. Therefore, similar to CYP2C9, CYP3A4 is an important CYP isoform for diabetics. Kenworthy *et al.* (1999) have suggested that CYP3A4 probe substrates can generally be categorized into four distinct groups based on their activity patterns in the presence of various CYP3A4 inhibitors indicating the possibility of the existence of multiple active sites in CYP3A4: the benzodiazepine group that includes midazolam, diazepam, and triazolam; the large molecular weight group that includes cyclosporine, erythromycin, and testosterone; nifedipine; and benzyloxyresorufin. DBF was chosen as a probe substrate for the CYP3A

isoforms as it correlated well with activities observed from members of the benzodiazepine group and the large molecular weight group (Stresser *et al.*, 2000). The extracts with strong inhibitory potency (AD01, AD06, AD07, AD11, W1, W2, and W5) should be used with caution with these drugs. The seven moderate inhibitors (AD02, AD03, AD08, W3, W4, W6 and W8) have a lower potential to interact with the metabolism of these drugs; whereas, the three weak inhibitors AD09, W7 and W9, would not be expected to have an interaction.

CYP3A5 has similar substrate specificity and metabolizes the same drugs as CYP3A4 with the same activity or less (Andrew Williams *et al.*, 2002). However, CYP3A5 contributes less to drug metabolism because of its lower content in most individuals in the liver and enterocytes (Lin & Lu, 1998), and therefore its inhibition may not be as clinically relevant as CYP3A4 inhibition. Unlike CYP3A4, CYP3A5 is present in all kidneys (Haehner *et al.*, 1996), and hence, may play a greater role in the metabolism of the drugs that treat nephropathies such as the angiotension-converting enzyme inhibitors compared to CYP3A4. There was a broad range of inhibition against CYP3A5 by the 17 Cree plant extracts. Similar inhibitory trends as CYP3A4 were observed except AD02 andAD03 were strong potent inhibitors, and W5 was a moderately potent inhibitor. As with CYP3A4, the strongly potent inhibitors AD01, AD02, AD03, AD06, AD07, AD11, W1 and W2, would have the greatest probability of affecting the metabolism of CYP3A4 substrates; whereas, the two weak inhibitors W7 and W9, would not be expected to have an affect.

CYP3A7 metabolizes the same drugs as CYP3A4 but at a much lower degree (Andrew Williams *et al.*, 2002). CYP3A7 is typically expressed only during the fetal

stages of life; however there are current conflicting reports indicating its expression in some adult livers (Lin & Lu, 1998). The inhibition of CYP3A7 would be more relevant in cases of gestational diabetes, when a woman develops T2D during pregnancy. If a woman with gestational diabetes was taking anti-hyperglycemic medication, the fetus may be exposed to the drug. The inhibitory trends were more similar to CYP3A5 than CYP3A4. W9 was the only weak inhibitor and is not expected to have an affect on metabolism of CYP3A4 substrates. The extracts of AD01, AD02, AD03, AD06, AD07, W2 and W4 were strong potent inhibitors and would have the greatest probability of affecting the metabolism of CYP3A4 substrates which may cause harmful effects in the fetus.

Overall, the CYP inhibition results support the hypothesis (3, page 25) that the Cree plants will inhibit the activity of the CYP isoforms. However, the extent of inhibition varied among the isoforms and depended on the plant species.

4.5.2 The Inhibition Trends of the 10 Cytochrome P450 Isoforms

The most contributory isoforms in the metabolism of drugs used by diabetics are CYP2C8, 2C9, 2D6, and 3A4. These isoforms except for CYP2D6 were moderately or strong inhibited by many of the extracts and therefore, there is a great potential for drug interactions to occur from these plants. Each CYP isoform had different strong inhibitors and therefore, a drug-NHP interaction would depend on both the drug and the plant species. For example, W5 would potentially interact with a CYP3A4 substrate such as diltiazem but not with a CYP2C9 substrate such as glyburide.

Common weak and potent inhibitors were observed among several of the CYP isoforms examined. The extracts which were commonly potent inhibitors (AD01, AD03,

AD07, AD11, W2, W4, W6), may be highly potent because they are rich in phytochemicals which may cause non-specific inhibition, they contain common inhibitory constituents, or they contain lipophilic constituents that have a greater capability in accessing the CYP active sites (Lewis *et al.*, 2006b). Lipophilicity has been shown to play a role in inhibitory potency in CYP2C9 and 2C19 (Lewis *et al.*, 2006b). The common weak potent inhibitors (AD09, W5, W7, W8, W9) may be scarce in phytochemical content, or contain constituents which are non-inhibitory towards CYPs or hydrophilic in nature. In addition, they may contain constituents that are not bioavailable because they are unstable and undergo decomposition. There were also several extracts that were more or less inhibitory towards a particular CYP such as W1 (2C8), AD03 (2C19), and W8 (2E1). This indicates that there is a specific constituent or several constituents that are present in a particular extract that are more inhibitory against a particular CYP isoform. The phytochemical analysis for these Cree plant extracts are currently being conducted at Dr. Arnason's lab. A manuscript with information on the phytochemicals present in four of the extracts is currently under review (Saleem *et al.*, in press). Phytochemicals which may possess strong potent inhibitory capabilities of particular CYP isoforms may be deduced by comparing the concentrations of the phytochemicals in these four extracts to the inhibitory effect the extracts had on a CYP isoform. By visual comparison, phytochemicals that may be inhibitory towards particular CYP isoforms are: procyanidin B2 (CYP2C19) and chlorogenic acid (CYP2E1). There are no documented studies on the inhibitory effects of procyanidin B2 of CYPs. Chlorogenic acid has not been shown to inhibit CYP1A2, 2C9, 2C19, 2D6, and 3A4 (Obach, 2000). In order to confirm if these phytochemicals are the inhibitors of the CYPs,

each phytochemical would need to be identified, quantified, isolated and then tested in the CYP inhibition assays.

Similar inhibition trends were observed among members of the same subfamily especially CYP2C and 3A. This was expected since members of the same subfamily are closely related and therefore would share similar enzyme conformation and substrate specificity (although not necessarily identical). Many of the extracts had opposite potencies towards the CYP2C and 3A subfamilies which were assessed by comparing the inhibitory values of the 17 extracts for CYP2C9 and 3A4. Of important note are AD02, AD03, AD07, and W2 because of their high potencies towards CYP2C9 and 3A4. Using two or more of these particular extracts concomitantly can increase the extent of CYP inhibition and the risk of adverse events. The extracts of AD01, AD06, AD11, W1, W3, W4, W5, W6, W7, and W8 had significant differences in inhibitory potencies for the two isoforms. Six of these extracts (AD01, AD06, AD11, W1, W4, and W8) were more inhibitory to CYP3A4, whereas the other four extracts (W3, W4, W6, and W7) were more inhibitory towards CYP2C9. This provides further evidence that there are differences in one or more constituents present in these extract that are more inhibitory against a particular CYP isoform.

The mean of the inhibitory values against each CYP isoform by the extracts ranged from 7.7% ± 1.6% to 68.4% ± 6.2% inhibition, and the ranking from the most inhibited to the least inhibited CYP isoform was 2C19 > 3A7 > 3A5 > 3A4 > 2C9 > 2C8 > 1A2 > 2E1 > 2D6 > 2B6. One possible explanation for why CYP2C and 3A members were the most inhibited is because they have large active sites with lower substrate specificity (Lewis *et al.*, 2006a) that can accommodate a range of molecules with

different sizes and shapes. The largest calculated active site volumes of CYP2C8, 2C9, and 3A4 were 1386, 1137 and 1483 $Å^3$ respectively (Lewis *et al.*, 2006a; Yano *et al.*, 2004). CYP2D6 has been found to have a medium size active site (Otyepka *et al.*, 2007). The active site volumes of the other isoforms have not been extensively studied, but can be categorized on the size of their typical substrates as small - 2E1; medium - 1A2, 2B6; large - 2C19, 3A5, 3A7 (Lewis, 2003). The inhibitory potencies of the extracts appear to be positively correlated to the size of the CYP active sites. Large active sites may also accommodate several compounds simultaneously which can block other substrates from entering the active site. CYP2C9 and 3A4, two of the major drug-metabolizing members have also been found to have flexible active sites which increase their ability to accommodate and correctly bind to substrates (Anzenbacher *et al.*, 2008). However, this increase in flexibility may also increase the number of inhibitors that can access the active site and prevent the substrate metabolism. CYP1A2 has been shown to possess an inflexible active site (Anzenbacher *et al.*, 1998).

The mean of the CYP inhibitory values obtained for each extract ranged from 5.1% ± 3.1% to 60.4% ± 8.2% inhibition. The top five inhibitory extracts were AD01 > W2 > AD07 > AD11 > W4, and the least five inhibitory extracts were W9 < W7 < AD09 < W8 < AD08. As mentioned earlier, the potencies may depend on their phytochemical content as mentioned previously.

4.6 Mechanism-Based Inhibition of CYP3A4

4.6.1 Mechanism-Based Inhibitors

In MBI, a substrate is metabolized by CYP to form an active intermediate metabolite which immediately forms covalent bonds within the CYP active site, either to

the heme group or the protein itself (Zhou *et al.*, 2004). Chronic and daily use of mechanism-based inhibitors would be expected to have serious clinical consequences, and does not provide the necessary time to sufficiently synthesize new CYPs to restore normal CYP levels (Flanagan, 2005; Greenblatt *et al.*, 2003). MBI of CYP3A4 by the 17 Cree plant extracts was studied using NADPH- and time-dependence assays. Overall from the two studies, none of the Cree plant extracts showed any significant indication of MBI towards CYP3A4 under the assay conditions. In both assays, many of the extracts had similar inhibitory trends observed with the negative control ketoconazole. Several of the extracts showed moderate differences in their ability to affect CYP3A4 activity between the two conditions (at least a 15% difference) such as AD02, AD09, W1, and W6, but were not significant.

A possible reason to explain why none of the 17 Cree plant extracts were detected as mechanism-based inhibitors was because the10 μg/mL concentration of extract tested was too low for individual constituents to be inhibitory. Further MBI tests were performed with AD02, AD09, and W3 using a higher concentration of 50 μg/mL. Minor MBI trends were observed with AD02 only, using both concentrations in both the NADPH- and time-dependent assays. It is possible that there is a constituent (or several) in AD02 that is a mechanism-based inhibitor; however its inhibitory effects may be masked by the inhibitory effects of other constituents in the extract. In order to confirm this, each phytochemical of AD02 would need to be identified, quantified, isolated and then tested for MBI of CYP3A4.

Overall, in the context of these studies none of the extracts except for possibly AD02 are mechanism-based inhibitors of CYP3A4. Even though strong inhibitory

potency against CYP3A4 was observed in the earlier assay, confirming that none of the extracts (expect for AD02) are mechanism-based inhibitors is a positive finding as this type of inhibition is irreversible. However, it is still undetermined whether the extracts are mechanism-based inhibitors of the other CYP isoforms. In addition, these results may be inconclusive as MBI is substrate dependent (Ghanbari *et al.*, 2006) and different results may be observed using another CYP3A4 substrate such testosterone or midazolam.

4.7 CYP3A4 Substrates

The time-dependent assay for MBI has been used to identify inhibitors (that are not mechanism-based inhibitors) that are also substrates of the same CYP isoform such as phenacetin (1A2) and terfenadine (3A4) (Yamamoto *et al.*, 2002). If an inhibitor is a substrate for the same CYP, after a long pre-incubation time the inhibitor would be metabolized into an inactive metabolite which is no longer inhibitory towards the enzyme. The activity of CYPs pre-incubated with the inhibitor for a longer pre-incubation time would be greater than CYPs that have been pre-incubated with the inhibitor for a shorter pre-incubation time because less inactive metabolites would be present in the former. This trend was observed with the extract AD01, indicating that inhibitory constituents of AD01 may be substrates for CYP3A4. As NADPH is required for the metabolism reaction, this prediction was further validated using the NADPH-dependence assay, which showed less inhibition when NADPH was present during pre-incubation. The time-dependent assay may be used for future studies to identify the CYPs that are involved in the metabolism of the inhibitory Cree plant compounds.

4.8 Drug Interaction Study with Enalapril

A drug interaction study was performed with the anti-hypertensive drug enalapril. This particular drug was chosen because it is a substrate of CYP3A4 and may compete with DBF or inhibitors present in the extract for active site binding (Triplitt, 2006; Siest, 2007); or the metabolite of enalapril (enalaprate) may react with phytochemicals of the extract and affect CYP activity. In addition, as many CEI diabetics suffer from hypertension (Kuzmina & Dannenbaum, 2004), enalapril would be a likely prescribed, conventional drug in this patient population. In this study, the inhibitory effects of an extract were compared to the inhibitory effect of the same extract with enalapril to determine if enalapril affected the inhibitory effects of the extract. As mentioned previously, only the extracts of AD01, AD08, and AD09 were studied in this assay. These experiments did not use any positive or negative controls. In all three assays, the metabolism of DBF was unaffected by enalapril. The metabolism of DBF was expected to decrease because both DBF and enalapril compete for the CYP3A4 active site. It has been hypothesized that multiple substrates can bind to the CYP3A4 active site and be metabolized simultaneously (Wang *et al.*, 2000; Kenworthy *et al.*, 2001), and that CYP3A4 has multiple active sites (Kenworthy *et al.*, 1999), which may account for the lack of inhibition of DBF metabolism. It also may be possible that CYP3A4 had a greater affinity for DBF than enalapril, resulting in a lack of enalapril metabolism. In this study, enalapril had no affect on the inhibitory potency of the extracts as similar results were observed with the extract and the combination of the extract with enalapril. The inhibitory effect of AD08 with or without enalapril was not significantly different than the MeOH control in the 2^{nd} and 3^{rd} assay. There was less CYP3A4 inhibition with AD08

compared to the other extracts even though 50% of the activity of CYP3A4 was anticipated for all extracts, indicating that the IC_{50} values obtained were less accurate than expected.

There were some limitations with this study. Interaction studies are typically performed *in vivo* or clinically rather than *in vitro*, because *in vitro* conditions are very artificial and lacking in variables that can contribute to the outcome of the results. For example, only CYP3A4 was present in the assay used. Other enzymes such as other CYPs or other drug-metabolizing enzymes such as glutathione S-transferase, N-acetyltransferase and FMO, may have an impact on the interactions between the extract constituents and enalapirl, and cannot be determined in the assay used. In addition, confirmation of enalapril metabolism in the assay was not determined and should be conducted by HPLC or gas chromatography in future studies.

The results suggest enalapril does not affect the inhibitory potency of the extracts of AD01, AD08, and AD09 against CYP3A4 activity, and enalapril-NHP interactions are not expected to occur with these xenobiotics, disproving the hypothesis (4, page 25) that the Cree plants will interact with enalapril to change the plant's CYP3A4 inhibitory potential.

4.9 Comparison of Inhibitory Potencies

Typically in the literature, inhibitory potencies of pure compounds are compared using IC_{50} values, which allows for cross-comparison and analysis with other published studies. However, determining IC_{50} values of 17 complex extract solutions containing unknown constituents for 10 CYP isoforms, would not provide a more stringent comparison than the method selected where only one concentration of 10 μg/mL was

tested. The original screening assays for the CYP isoforms tested one extract concentration of 10 μg/mL which allowed comparisons to be conducted based on potency to categorize extracts as weak, moderate, or strongly potent inhibitors. From the IC_{50} values that were obtained in preparation of the drug interaction study with enalapril, a ranking of extract potencies was obtained and compared to the ranking of their potencies obtained from the original CYP3A4 inhibition study. Similar inhibitory rankings between the two studies were obtained. The extracts with low potency had identical rankings for both assays: AD09 < W3 < AD08. However, the strong potent inhibitors, AD01, AD11, W1, and W5 did not have identical rankings. Their CYP3A4 inhibitory values in the original assay ranged from 78.4% ± 1.9% to 98.2% ± 0.3%. Even though their inhibitions were not 100%, the difference in ranking may be due to CYP saturation in the original screening assay, preventing the separation of the inhibitory potential of the extracts. In other words, all active sites of the CYPs were occupied with inhibitory compounds of the extracts, and the remaining amount of inhibitory potential could not be determined. Their deviation from 100% inhibition may be due to intrinsic fluorescence or quenching, and bioavailability variability of the assay. The original screening assay was able to categorize weak, moderate and strong inhibitors, but it was not able to show the exact inhibitory potencies of the extracts; whereas in the assay which identified the IC_{50} values, the exact inhibitory potencies were obtained. The original screening assay does provide a general separation of the weak, moderate, and strongly potent inhibitors which was sufficient for determining the potential of those extracts to affect CYP-mediated metabolism for the studies of this thesis.

4.10 Comparison of Cardiac and Cytochrome P450 Studies

Extracts of the Cree plants were tested for their potential to cause adverse effects in CEI diabetics by examining their *in vitro* capability to affect the contraction rate of neonatal rat cardiomyocytes, and to inhibit the activity of 10 CYP isoforms involved in the metabolism of drugs. All of the Cree plants where examined in the CYP inhibition studies; however, only W2, W4, W5, and W9 were examined for their chronotropic and cardiotoxicity potential.

The extract from W9 was found to be toxic to cardiomyocytes at a low concentration (10 μg/mL) that may be achieved physiologically in human plasma. At the same concentration, it was observed to have low potential to inhibit CYP-mediated metabolism in all isoforms examined. W9 would not be expected to interact with anti-diabetic medications but may cause cardiotoxicity if used in high doses or chronically. The extracts of W2 and W4 displayed an opposite pattern in not being toxic to cardiomyocytes at high concentrations, yet at a low concentration of 10 μg/mL, they inhibited important CYPs (CYP2C8, 2C9, 3A4, 3A5) with moderate or high potency and may affect drug safety and efficacy. W2 was the second most potent inhibitor of all the CYP isoforms after AD01. However, W2 use as an alternative medicine may have a beneficial effect by reducing the production of ROS through the inhibition of CYP2E1. The extract of W5 was toxic to cardiomyocytes at concentrations greater than 50 μg/mL, and had strong inhibiton of CYP3A4. At pharmacologically relevant doses, W5 would not be expected to affect the heart rate or cause cardiotoxicity, but may inhibit the metabolism of CYP3A4 substrates and interfere with their safety and efficacy.

4.11 Conclusion

These studies suggest that at concentrations that may be obtained in the plasma, several of the Cree medicinal plants may have a harmful effect on diabetics by affecting the viability of cardiomyocytes, or by affecting the metabolism of drugs and other therapeutic products (such as Cree NHPs) commonly used by diabetics through the inhibition of the CYPs examined. This validates the hypothesis (3, page 25) that compounds in the Cree anti-diabetic NHPs will inhibit CYP activity. The plant species (that were selectively examined) are not expected to have an effect on the heart rate, disproving the hypothesis (1, page 25) that compounds in the Cree anti-diabetic NHPs will have a cardiac chronotropic effect. The same plant species are not expected to interact with metformin and affect cardiomyocyte contraction rate, disproving hypothesis 2 (page 25). In addition, the CYP3A4 inhibitory potencies of Cree medicinal plants (that were selectively examined) should not be affected in the presence of enalapril, disproving the hypothesis (4, page 25) that the plants will interact with enalapril to change their CYP3A4 inhibitory potencies.

Several of the plant species investigated displayed noteworthy results: W9 was cardiotoxic, but had weak inhibitory potency against all CYP isoforms; W2 and W4 were found to be non-toxic to cardiomyocytes, but had moderate or strong inhibitory potency against important CYP isoforms; AD01 had the greatest potential to cause adverse drug effects as it was a moderate or strong inhibitor for the greatest number of CYP isoforms; AD07 had strong inhibitory potency towards important CYP isoforms; AD02 inhibited CYP3A4 with moderate potency, but may be a CYP3A4 mechanism-based inhibitor; AD09, W7 and W9 were shown to have the least potential to inhibit drug metabolism.

Overall, the results suggest that several of the Cree anti-diabetic medicinal plants have the potential to cause adverse events when used by diabetic patients. However, determining which of the Cree plants would have the greatest risk of toxicity is difficult to ascertain due to the different effects the plants had in the assorted studies. In addition, their risk of toxicity also depends on whether the plants are used as an alternative or complementary medicine, which pharmaceuticals are concomitantly administered, and if multiple plants are used concomitantly. It is important to note that the results were obtained by testing crude ethanolic extracts of the anti-diabetic medicinal plants, rather than samples that were prepared in a traditional manner. Ethanolic extracts were used because they contain the phenolics which are typically the active constituents of NHPs. In addition, ethanolic extracts do not easily undergo decomposition unlike water extracts. The activity of traditionally prepared samples which are prepared using water may differ than the activity observed in these experiments due to differences in their phytochemical composition (Foster *et al.*, 2005). Further studies with other solvent extracts, and traditionally prepared plant samples are warranted, to determine if there are risks of clinical adverse effects.

4.12 Future Directions

Only four of the extracts were studied for their effects with the neonatal rat cardiomyocytes. Future studies should study the remaining 13 extracts and should also include the screening for their ability to prolong the QT interval using adult guinea pig cardiomyocytes. Screening for the prolongation of the QT interval is a mandatory study for all new pharmaceuticals due to the fact that many non-cardiac drugs can cause this prolongation which can lead to events including sudden cardiac death (ICH, 2005; Yap &

Camm, 2003). In addition, further studies using diabetic-like or diabetic cardiomyocytes should be performed as previously mentioned in this chapter, in order to determine whether these dysfunctional cells are more susceptible to the effects of the extracts.

It would also be important to determine which phytochemical(s) in the extracts is causing the potent inhibition against the different CYP isoforms. As these phytochemicals may be present in other NHPs, these findings would help predict the inhibitory potency of other NHPs against the CYP isoforms. Determining if any of the extracts or their constituents are mechanism-based inhibitors of CYP2C8, 2C9 and 3A5 should also be performed, as well as repeating the MBI assays for CYP3A4 using another probe substrate (Ghanbari *et al.*, 2006). In addition, confirming that enalapril is metabolized by CYP3A4 in the drug interaction study should be performed using HPLC or gas chromatography. With the consent of the CEI to test traditionally prepared plant material, many of the experiments should also be repeated with these samples as they have greater clinical relevance.

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APPENDIX

A1 Determination of the Extract Concentrations of Bitter Orange and Goldenseal

A1.1 Materials and Methods

The extracts of BO and GS were prepared in a different manner than the extracts of the Cree plants as mentioned in the materials and methods section in Chapter 2. Using this method, the final concentration of extract produced is unknown because the amount of phytochemicals extracted from the bulk material was uncertain. To determine the final extract concentrations, the phytochemicals in the extract solutions were quantified by evaporating the extract solvent. Empty 1.5 mL microfuge tubes were weighed and 250 µL of extract was added to each tube. The solvent was then evaporated to dryness using a vacuum centrifuge (Savant SpeedVac SVC100) leaving behind precipitated extract in the microfuge tubes. The microfuge tubes containing the precipitated extract were then weighed again to determine the extract concentration produced.

A1.2 Results

The extract concentration produced from 15, 50, and 100 mg of BO extracted in 1 mL of water, was 8.7 ± 1.0, 25.5 ± 0.5, and 55.1 ± 1.1 mg/mL respectively **(Figure A1A)**. The percent yield ranged from 51 to 58 %.

The extract concentration produced from 5, 10, and 25 mg of GS extracted in 51 mL of 55% MeOH, was 2.3 ± 0.4, 3.7 ± 0.3, and 9.7 ± 0.8 mg/mL respectively **(Figure A1B)**. The percent yield was lower than the percent yield observed with BO with a range of 37 to 46 %.

Table A1: Determining the concentrations of the bitter orange and goldenseal extracts. The final extract concentrations of **(A)** BO and **(B)** GS produced from their initial concentrations were determined by evaporating the extracts to dryness and measuring the weight of the precipitated extracts (n = 3)

(A) Bitter Orange

Initial Concentration (mg/mL)	Final Concentration (mg/mL ± SEM)	% Yield
15	8.7 ± 1.0	58
50	25.5 ± 0.5	51
100	55.1 ± 1.1	55

(B) Goldenseal

Initial Concentration (mg/mL)	Final Concentration (mg/mL ± SEM)	% Yield
5	2.3 ± 0.4	46
10	3.7 ± 0.3	37
25	9.7 ± 0.8	39

A2 Optimal Period of Neonatal Rat Cardiomyocyte Development

The period of neonatal rat cardiomyocyte development that was most suitable for the cardiac chronotropic experiments was investigated because cardiomyocytes can undergo changes in their cellular and electrophysiological characteristics as they are kept in culture due to their maturation into adult cardiomyocytes or their dedifferentiation into a more embryonic or fetal stage of growth (Ueno *et al.*, 1988; Guo *et al.*, 1996). The different stages of development are associated with changes in electrophysiological properties such as their AP duration, ion channel expression, and contractility, as well as the expression of genes (Guo *et al.*, 1996; Wahler *et al.*, 1994; Yokota *et al.*, 1995). Therefore, it is possible that cardiomyocytes at different stages of development may respond to chronotropes differently. In order to reduce this possibility, a specific period of cardiomyocyte development was chosen to be used for the chronotropic screening experiments to eliminate any age-dependent effects.

A2.1 Materials and Methods

Gathering Parameter Data

After a MEA recording, data on two parameters (contraction rate and ISI deviation) was obtained using the software Spanner XBD (Result) using a threshold detection limit of 50 μV. In other words, the measured extracellular FP activity would require an amplitude height of at least 50 μV in order to be detected as a contraction **(Refer to Figure 4)**.

Measuring Glucose Concentrations

The glucose concentration of used media was measured using an Ascensia Elite XL Glucose Meter (Bayer HealthCare) and Ascensia elite test strips.

A.2.2 Results

The contractions rates and ISI deviations from 1 to 9 DIV were studied from four separate cultures of cardiomyocytes grown under identical conditions **(Figure A1)**. Detectable FP activity was observed after 3 or 4 DIV. Differences in contraction rate between the cultures were observed daily. In particular, major inter-culture differences in contraction rates were observed at 5 and 8 DIV. In addition, intra-culture differences in the daily contraction rate were observed for all cultures, especially Cultures 1 and 2. The ISI deviation provides an indication of how consistent the cardiomyocytes are contracting, with a low value indicating a constant contraction rate. Daily changes in inter-culture and intra-culture ISI deviation values were observed. However, Culture 4 had very low and consistent daily ISI deviations values. A negative correlation between contraction rate and ISI deviation was identified ($r^2 = 0.630$, $p < 0.001$) indicating that an inverse relationship existed between the contraction rate and the ISI deviation **(Figure A2)**. In other words, a high contraction rate was associated with a low ISI deviation.

To estimate the metabolic activity of the cardiomyocytes from 5 to 11 DIV, the glucose concentrations of the used media from four cultures were measured following media changes using a glucose meter. The glucose concentrations of the used media after 5 DIV (< 7.60 mM) were significantly lower ($p \leq 0.05$) than the glucose concentration of the used media measured at 3 DIV (12.05 mM) **(Figure A3)**. Glucose concentrations remained relatively constant and significant differences were not observed

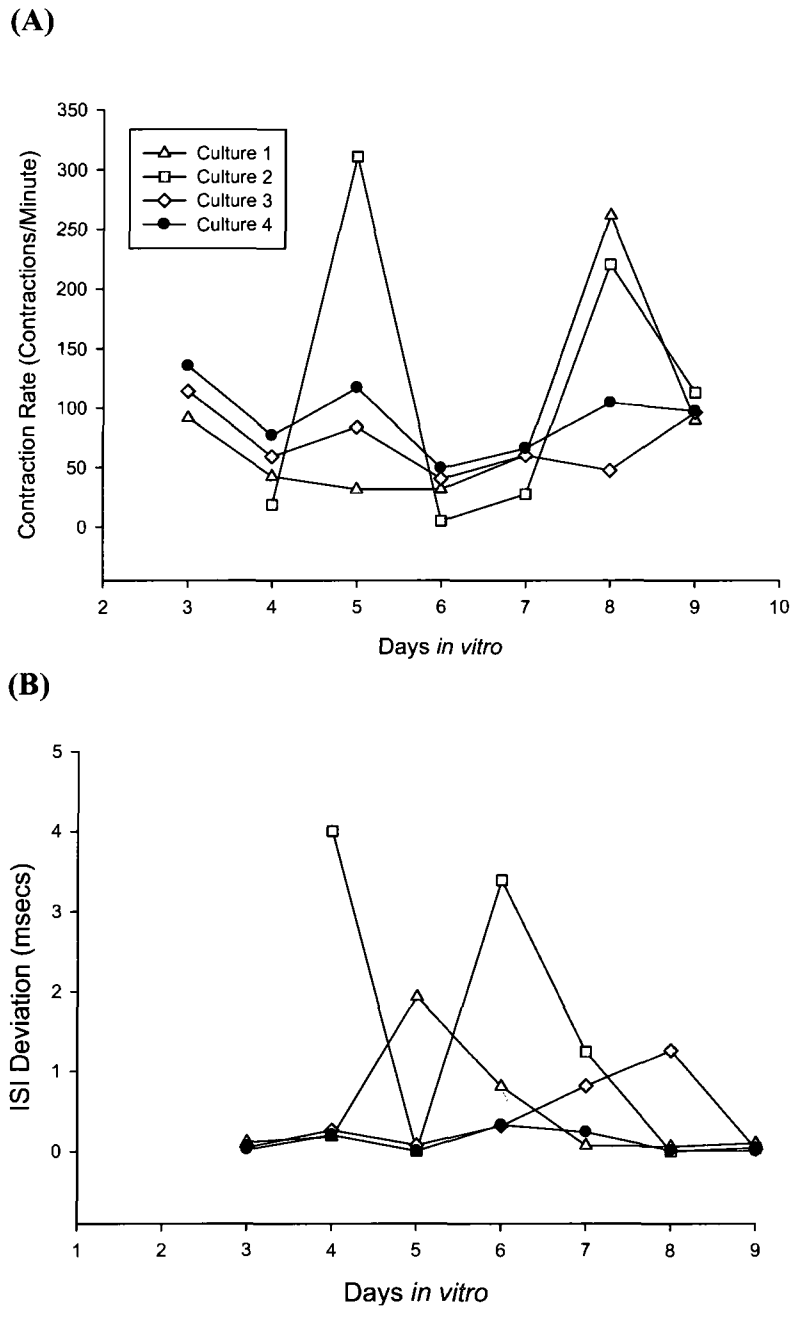

Figure A1: The contraction rate and inter-spike interval deviations of four cultures of neonatal rat cardiomyocytes. The electrical activity characteristics of four separate cultures of neonatal rat cardiomyocytes from 1 to 9 DIV based on the parameters: **(A)** contraction rate and **(B)** ISI deviation were obtained using the MEA system and the program Spanner XBD.

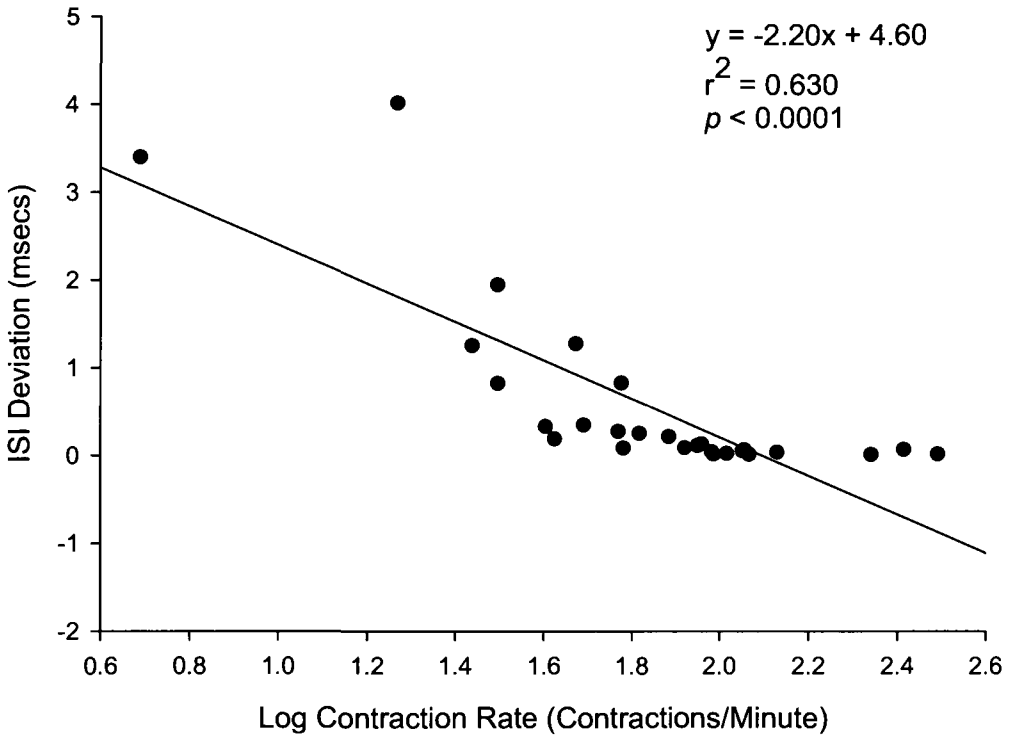

Figure A2: A Pearson correlation of neonatal rat cardiomyocyte contraction rate to inter-spike interval deviation. The log contraction rate was plotted as a function of the ISI deviation. A line of best fit was obtained using linear regression. The equation ($y = -2.20x + 4.60$), r^2 value (0.630) indicating how well the line fits the plotted values, and the p value (<0.001) indicating that the slope of the linear is significantly different than a slope value of 0, were obtained using the software SigmaPlot.

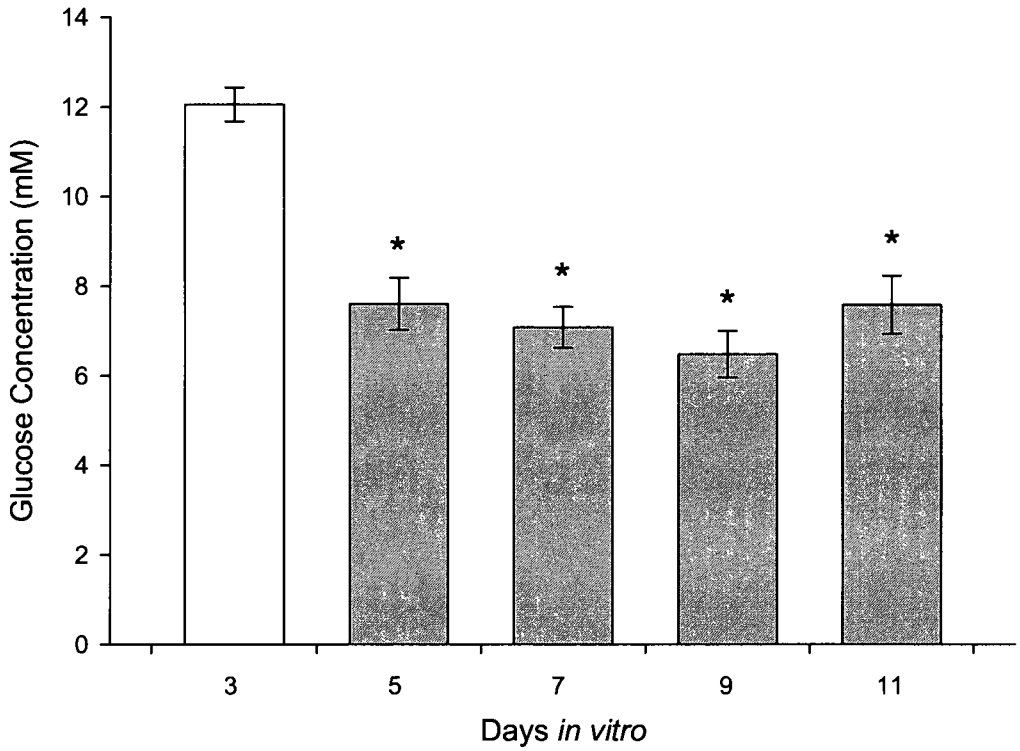

Figure A3: The glucose concentrations of used media collected during media changes from 3 to 11 DIV. After media changes, the used media was saved and their glucose concentrations were measured using as Ascensia Elite XL Glucose Meter. The results were expressed as the mean glucose concentration (mM) ± SEM. Pooled data (n = 4) is presented. *$p \leq 0.05$ with respect to the mean glucose concentration at 3 DIV, using one-way ANOVA followed by the Tukey test.

at 5, 7, 9 and 11 DIV indicating relatively constant metabolic activity from 5 to 11 DIV.

From the above studies, it was decided that the optimal period of neonatal rat cardiomyocyte development for the chronotropic experiments was from 5 to 7 DIV. During the time period of 5 to 7 DIV, the FP activity is detectable and stable, the cardiomyocytes should not have undergone an extensive dedifferentiation **(Figure 18)**, and their metabolic activity is constant. In addition, consistently contracting cultures (i.e. low ISI deviation values) were desired and therefore, cultures with contraction rates greater than 60 contractions/minute were chosen to be used for future experiments. Since contractions rates were observed to reach as high as 311 contractions/minute, chosen cultures also had to have less than 130 contractions/minute. Cultures with very low contraction rates may not provide a reasonable response to negative chronotropes, and vice versa.

A3 Limitations of the Experimental Method

Several experiments were conducted to determine if the experimental procedure that was to be used for the chronotropic screening experiments had any unfavorable effects on the experimental results.

A.3.1 Results

Tyrode's buffer was used as the electrophysiological buffer for the experiments. During the experimental process, Tyrode's buffer (with or without a xenobiotic) is added to the cardiomyocytes, and then removed from the cardiomyocytes as many as three times. To determine if the subsequent additions and removals of Tyrode's buffer have an effect on the contraction rate, an experiment was conducted which mimicked the

experimental procedure but without xenobiotics present in the Tyrode's buffer **(Figure A4)**. The first step of the experiment requires the replacement of media with Tyrode's buffer. This replacement results in an increase in contraction rate, but it is not significant **(Tyrode's Buffer in Figure A4)**. The second step of the experiment requires the removal of 300 μL of the Tyrode's buffer from the MEA to which a xenobiotic is added to, and then the re-addition of the Tyrode's buffer to the cardiomyocytes **(Addition 1 in Figure A4)**. This re-addition decreases the contraction rate relative to the initial contraction rate in Tyrode's buffer but is not significant. Several experiments require the immediate addition of a second xenobiotic and therefore, the second step was repeated again **(Addition 2 in Figure A4)**. This re-addition significantly decreased the contraction rate relative to the initial contraction rate in Tyrode's buffer ($p \leq 0.05$). These results show that additional additions of xenobiotics should be limited to one addition, and that chronotropic effects of xenobiotics should be compared to the chronotropic effects of the vehicle controls.

Another experiment was conducted to determine if the Tyrode's buffer had an effect on the contraction rate of a culture of cardiomyocytes after a period of time. For this experiment, cultures of cardiomyocytes were left in Tyrode's buffer outside of the incubator for 15 minutes and their contraction rate were measured every 5 minutes **(Figure A5)**. At 5 minutes, there was an increase in contraction rate compared to the rate after the initial exposure of Tyrode's buffer (at 0 minutes). At 10 and 15 minutes, there was a significant decrease in contraction rate compared to the rate after the initial exposure of Tyrode's buffer ($p \leq 0.05$). Therefore, the experimental time to complete the

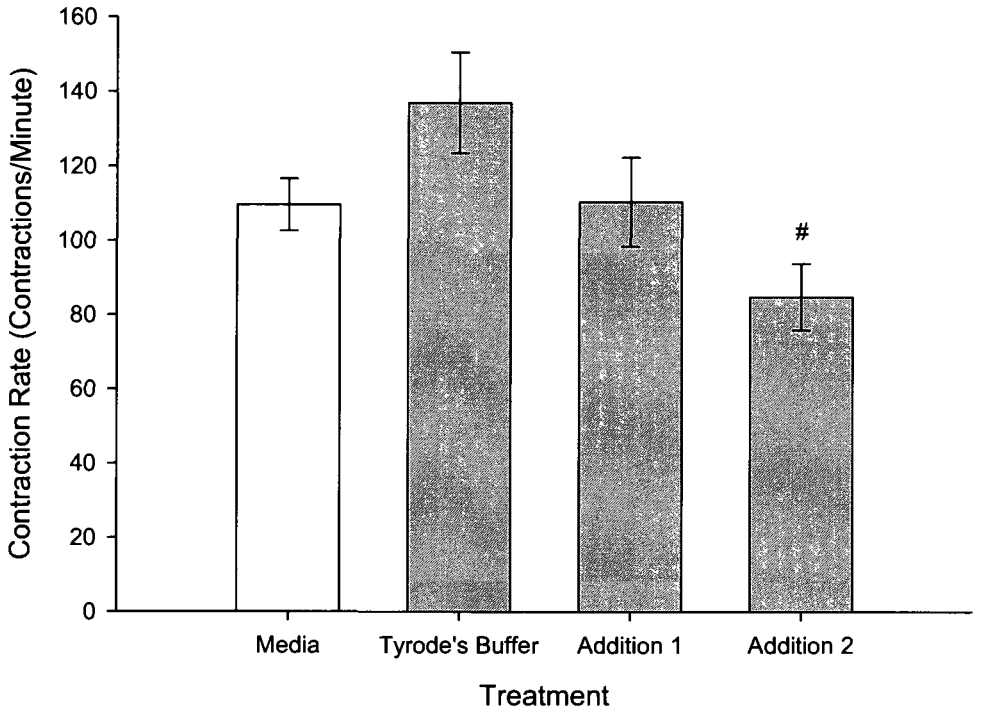

Figure A4: The chronotropic effect of the experimental technique with neonatal rat cardiomyocytes. The experimental technique for drug or extract testing was mimicked using Tyrode's buffer without drugs or extracts to determine if the technique itself affected the contraction rate of the cardiomyocytes. The Tyrode's buffer represents the initial replacement of media to Tyrode's buffer. Addition 1 and 2 represent the first and second addition of drugs or extracts respectively. The results are expressed as the mean contraction rate ± SEM. Pooled data ($n = 5$) is presented. $^{*}p \leq 0.05$ with respect to the contraction rate in media, using one-way ANOVA followed by the Tukey test. $^{\#}p \leq 0.05$ with respect to the contraction rate in the initial Tyrode's buffer's, using one-way ANOVA followed by the Tukey test.

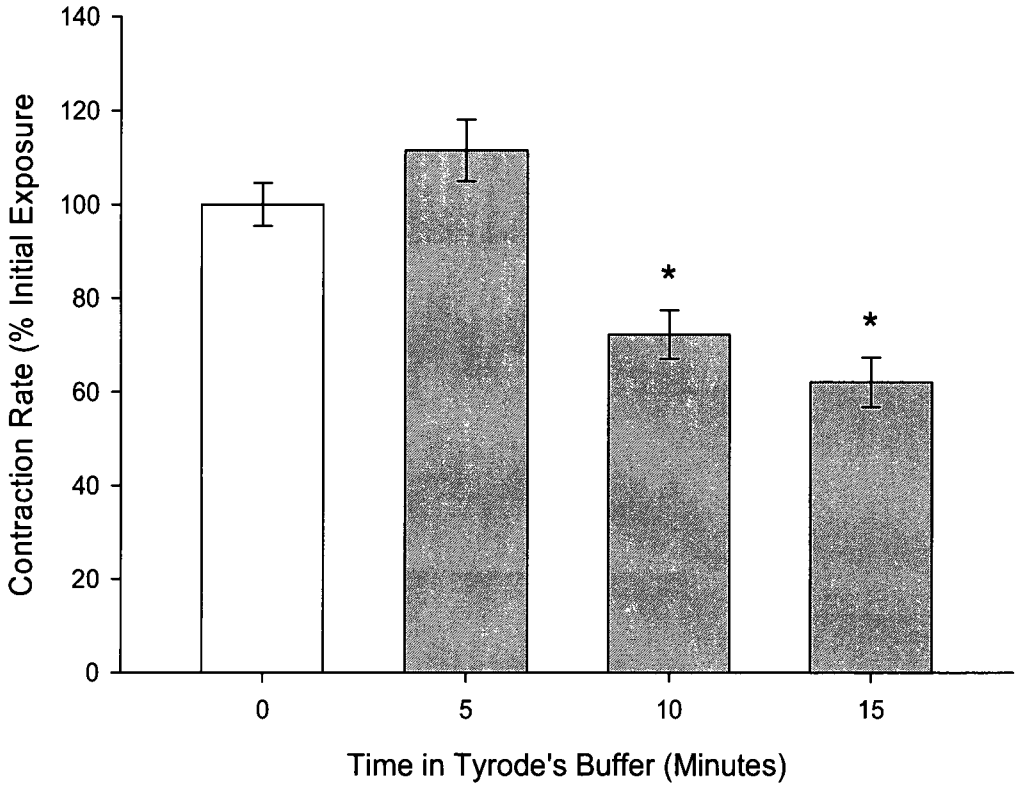

Figure A5: The changes in contraction rate that occur upon 15 minutes of exposure to Tyrode's buffer. The contraction rate of neonatal rat cardiomyocytes after 0 to 15 minutes of exposure to Tyrode's buffer was measured using the MEA system and counted using the software AxoScope. The contraction rates at 5, 10 and 15 minutes were expressed relative to the contraction rate at 0 minutes, as the mean contraction rate ± SEM. Pooled data ($n = 6$) is presented. *$p \leq 0.05$ with respect to the contraction rate at 0 minutes, using one-way ANOVA followed by the Tukey test.

experiment does affect the experimental results and all the experiments should be completed in a set amount of time.

A4 Identifying the Linear Region of the Activity Curves of the Metabolism of Cytochrome P450 Substrates by Various Cytochrome P450 Isoforms

A4.1 Materials and Methods

The activity of the CYPs should be analyzed when the metabolism of CYP substrates follows a linear fashion to ensure that none of the assay reagents are limiting the metabolism reaction. The activity curves were produced by following the typical CYP inhibition assay for each isoform testing 1 μL 100% MeOH per well (in a total volume of 200 μL). The reactions were allowed to run for a set amount of time depending on the CYP isoform tested, and fluorescent measurements were made every 5 or 10 minutes. The reaction time for CYP3A4 and 3A5 was 20 minutes; for CYP1A2, 2B6, 2D6, and 3A7 it was 40 minutes; and for CYP2C8, 2C9, 2C19, and 2E1 it was 60 minutes. For CYP3A4 the reaction time was extended to 30 minutes which was the incubation time for the MBI and drug interaction studies. The reaction time was plotted against the fluorescent units measured to obtain the activity curves.

A4.2 Results

The assays used to study the activity of the CYP isoforms were previously optimized in the lab. The activity curves are provided here to confirm that the results obtained for the CYP inhibition studies were obtained during the linear region of the activity curves.

Variations in the activity curves were observed among the different CYP isoforms **(Figure A6 and A7)**. CYP1A2, 2C8, 2D6, and 3A5 showed immediate linearity, whereas CYP2B6, 2C9, 2C19, 2E1, 3A4, and 3A7 had a slight delay before linearity of the reaction occurred. The activity of the CYP isoforms based on the amount of fluorescence released also differed. Fluorescent counts ranged from as low as 8645 units for CYP2C19 to as high as 60001 units for CYP2B6. Higher fluorescent counts are favoured because they can provide a better separation of the inhibition values obtained for the 17 Cree plant extracts. However, high fluorescent counts were not attained for all isoforms because of their low activity or its specificity to the substrate used. At the end of the reaction times for the different CYP isoforms, the activities were within the linear portion of the activity curves. Therefore, the results obtained for the CYP inhibition studies were obtained from the linear region of the activity curves.

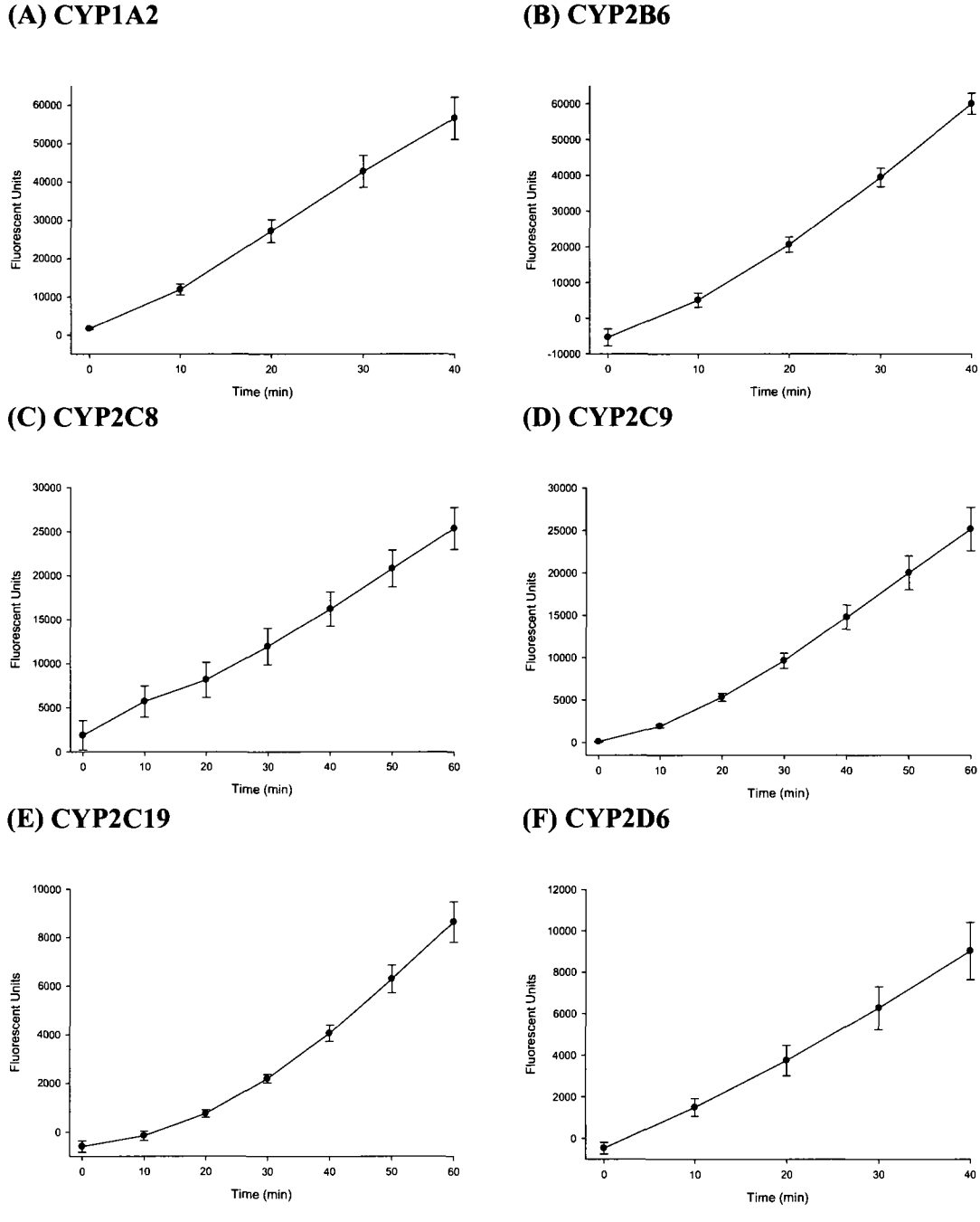

Figure A6: The activity curves of the CYP isoforms 1A2, 2B6, 2C8, 2C9, 2C19, and 2D6. Activity curves for the CYP isoforms **(A)** 1A2, **(B)** 2B6, **(C)** 2C8, **(D)** 2C9, **(E)** 2C19, and **(F)** 2D6 were plotted based on the fluorescence released from metabolized substrates of the CYPs at various time points. The results are expressed as the mean CYP isoform activity in fluorescent units ± SEM ($n = 3$).

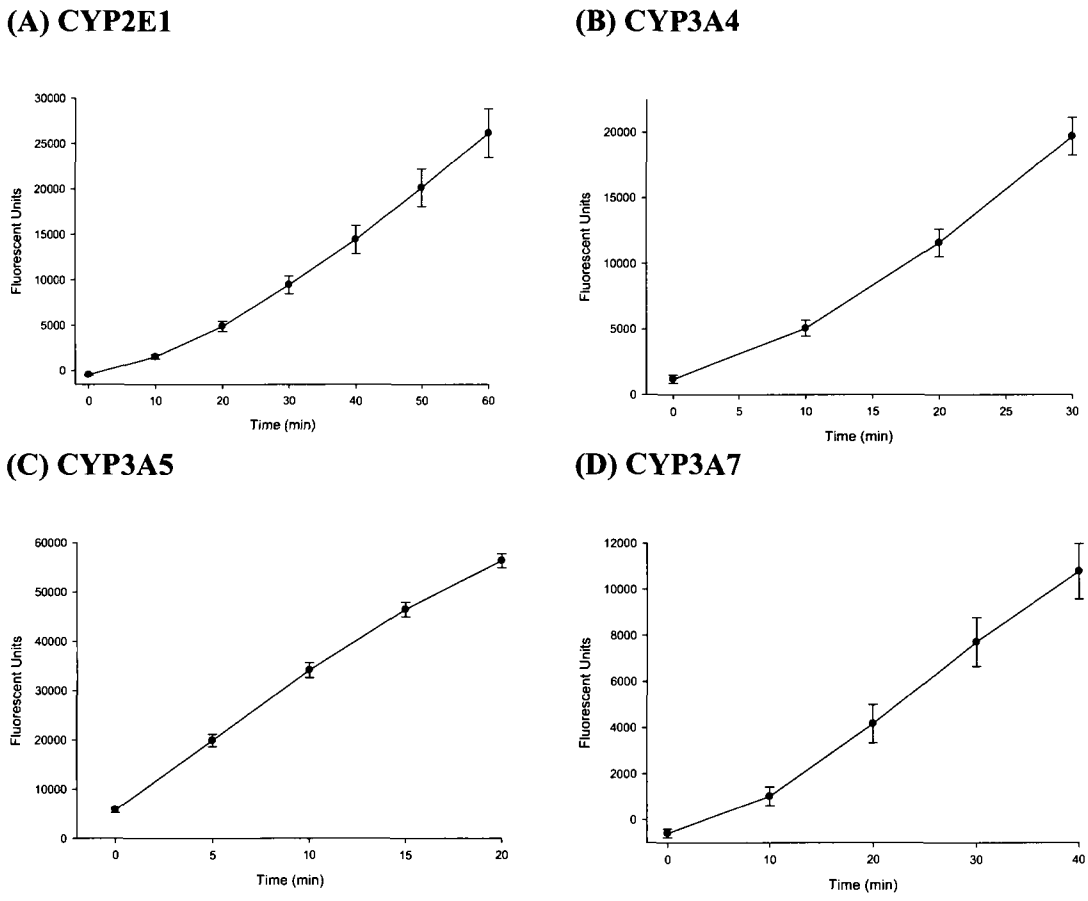

Figure A7: The activity curves of the CYP isoforms 2E1, 3A4, 3A5, and 3A7. Activity curves for the CYP isoforms **(A)** 2E1, **(B)** 3A4, **(C)** 3A5, and **(D)** 3A7 were plotted based on the fluorescence released from metabolized substrates of the CYPs at various time points. The results are expressed as the mean CYP isoform activity in fluorescent units ± SEM (n = 3).

A5 Summary of the Cytochrome P450 Inhibition Values

Results from **Figures 19-22** and **Tables 3-4** were compiled to provide one table to summarize the CYP inhibition results **(Table A2)**.

Table A2: A summary of the inhibition values of the 10 CYP isoforms by the 17 Cree plant extracts. The mean inhibition of the 10 CYP isoforms by the 17 extracts, and the mean of the inhibition of the 17 extracts for each CYP isoform are also provided (% inhibition ± SEM). The values are colour-coded based on their inhibition values and potency: weak (<30%) – green; moderate (31-74%) - yellow; strong (>75%) – red.

Extract	1A2	2B6	2C8	2C9	2C19	2D6	2E1	3A4	3A5	3A7	Mean Inhibition	Ranking
AD01	45.6 ± 5.8	18.8 ± 2.6	61.1 ± 3.6	51.8 ± 3.6	88.1 ± 2.4	46.5 ± 8.3	31.1 ± 4.7	90.7 ± 2.4	88.0 ± 2.5	82.6 ± 0.9	60.4 ± 8.2	**1**
AD02	17.2 ± 4.7	12.0 ± 1.9	62.1 ± 4.0	72.3 ± 4.5	89.0 ± 1.1	2.1 ± 2.8	-2.1 ± 2.4	71.4 ± 4.3	80.0 ± 0.9	88.7 ± 1.2	49.3 ± 11.8	8
AD03	22.8 ± 4.0	10.7 ± 2.3	49.2 ± 2.4	76.0 ± 2.2	94.1 ± 4.1	11.5 ± 1.8	16.9 ± 0.6	61.6 ± 1.4	75.0 ± 2.6	81.2 ± 0.9	49.9 ± 10.1	7
AD06	31.3 ± 6.2	3.8 ± 3.7	62.0 ± 7.0	45.8 ± 5.7	70.8 ± 5.2	14.4 ± 2.6	15.7 ± 4.6	96.7 ± 0.5	80.3 ± 1.2	77.2 ± 0.4	49.8 ± 10.2	6
AD07	11.11 ± 4.0	5.3 ± 1.5	83.5 ± 3.3	86.9 ± 1.7	89.5 ± 1.0	9.2 ± 3.9	13.1 ± 3.3	83.2 ± 0.2	90.6 ± 0.6	86.9 ± 1.9	55.9 ± 12.6	**3**
AD08	5.8 ± 5.6	10.6 ± 3.4	31.6 ± 1.7	33.6 ± 1.6	62.7 ± 1.9	13.8 ± 12.1	1.0 ± 2.1	43.1 ± 4.9	40.5 ± 2.9	55.7 ± 1.3	29.9 ± 6.7	13
AD09	-5.5 ± 6.2	5.5 ± 3.7	25.7 ± 7.0	36.2 ± 1.3	48.3 ± 0.4	23.2 ± 8.7	16.4 ± 1.5	17.8 ± 1.0	34.6 ± 2.3	35.4 ± 1.7	23.7 ± 5.0	**15**
AD11	45.5 ± 11.9	13.2 ± 3.4	47.1 ± 4.6	39.7 ± 2.4	81.2 ± 1.1	19.1 ± 3.9	19.9 ± 4.7	98.2 ± 0.3	82.4 ± 2.4	73.4 ± 4.5	52.0 ± 9.6	4
W1	22.8 ± 4.1	14.3 ± 0.6	67.4 ± 11.3	31.9 ± 4.0	66.0 ± 3.0	-11.9 ± 12.1	28.5 ± 3.6	78.4 ± 1.9	78.3 ± 4.0	73.7 ± 0.6	44.9 ± 10.1	10
W2	27.1 ± 3.6	9.4 ± 0.2	65.6 ± 4.1	72.2 ± 2.0	83.9 ± 2.2	22.2 ± 0.7	35.3 ± 2.1	77.9 ± 9.6	84.8 ± 3.0	85.5 ± 1.1	56.4 ± 9.4	**2**
W3	17.7 ± 5.3	2.3 ± 1.6	41.0 ± 7.1	78.3 ± 1.9	71.6 ± 2.3	19.1 ± 2.9	21.4 ± 4.6	35.6 ± 5.3	52.1 ± 3.5	60.7 ± 2.9	40.0 ± 8.0	12
W4	12.0 ± 2.4	20.1 ± 2.3	54.0 ± 7.0	83.1 ± 2.2	94.4 ± 1.2	17.7 ± 8.3	17.3 ± 2.1	50.2 ± 2.2	72.5 ± 0.6	96.9 ± 7.1	51.8 ± 10.6	5
W5	28.7 ± 3.8	2.6 ± 2.1	60.9 ± 15.6	21.9 ± 5.2	48.5 ± 2.0	16.8 ± 3.6	26.2 ± 2.6	92.3 ± 1.2	73.9 ± 1.8	71.4 ± 2.1	44.3 ± 9.3	11
W6	10.4 ± 0.8	4.3 ± 2.1	77.1 ± 40.0	97.1 ± 0.9	97.4 ± 0.5	4.0 ± 3.5	11.7 ± 1.7	32.8 ± 2.1	59.1 ± 2.7	64.0 ± 1.4	45.8 ± 11.9	9
W7	15.3 ± 2.7	-0.1 ± 0.3	33.8 ± 4.7	40.2 ± 4.1	39.3 ± 1.4	-0.5 ± 10.0	9.7 ± 2.0	-5.2 ± 5.9	12.5 ± 1.2	34.1 ± 3.5	18.4 ± 5.7	**16**
W8	15.7 ± 3.9	-1.8 ± 2.1	23.7 ± 10.1	25.3 ± 4.1	23.7 ± 1.0	5.1 ± 6.9	34.6 ± 4.3	65.1 ± 8.6	57.6 ± 11.3	49.2 ± 2.5	29.8 ± 6.9	14
W9	8.0 ± 3.9	-1.1 ± 2.0	-16.3 ± 5.3	14.2 ± 1.5	14.0 ± 5.6	-2.44 ± 11.1	10.9 ± 2.7	0.6 ± 1.5	11.0 ± 2.7	12.0 ± 3.1	5.1 ± 3.1	**17**
Positive Inhibitor	95.1 ± 0.3	69.0 ± 0.7	83.5 ± 2.3	97.8 ± 1.0	98.0 ± 2.1	93.7 ± 2.9	82.0 ± 0.9	97.2 ± 0.9	99.1 ± 0.2	96.7 ± 1.6	94.9 ± 3.7	
Mean Inhibition	19.5 ± 3.2	7.7 ± 1.6	49.0 ± 5.8	53.3 ± 6.3	68.4 ± 6.2	12.3 ± 3.2	18.1 ± 2.6	58.3 ± 8.0	63.1 ± 6.1	40.9 ± 7.6	41.6 ± 7.7	