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

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ABSTRACT

Seventeen Cree anti-diabetic medicinal plant extracts were investigated for their capability to cause adverse effects upon their use as alternative or complementary medicine. Two different aspects of safety were studied which were the ability for the extracts to effect the contraction rate of neonatal rat cardiomyocytes as a measure of heart rate in humans, and their ability to interfere with the metabolism of drugs by inhibiting the drug-metabolizing enzymes CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7 *in vitro*. Findings from these studies suggest that several of the Cree plants can have a harmful effect with diabetics through different mechanisms. The extracts W2, W4, W5, and W9 did not have an effect on the contraction rate of cardiomyocytes, however W9 was found to be toxic to the cells at physiological plasma concentrations. In addition, several of the extracts including AD01, AD07, W2, and W4 were moderate or high potent inhibitors of the most important CYP isoforms involved in metabolizing common drugs taken by diabetics, at a physiological plasma concentration of 10 μg/mL. Furthermore, the extract AD02 was identified as a possible mechanism-based inhibitor of CYP3A4. These inhibitory extracts have a great potential to cause adverse drug effects. Extracts that were low potent inhibitors of the CYP isoforms were also identified which were AD09, W7 and W9. Overall, the results suggest that several of the extracts can cause adverse events when used by the diabetics, and that the severity of the events do depend on whether the plants would be used as alternative or complementary medicine, and which pharmaceuticals would be taken concomitantly.

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LIST OF ABBREVIATIONS

AD	Anti-diabetic
ANOVA	Analysis of variance
AP	Action potential
BO	Bitter orange
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
ECG	Electrocardiogram
FF	Furafylline
FP	Field Potential
GS	Goldenseal
HERG	Human ether-à-go-go
IC_{50}	Median inhibitory concentration
KC	Ketoconazole
LDH	Lactate dehydronase
MBI	Mechanism-based inhibition
MEA	Microelectrode array
MeOH	Methanol
MFC	7-methoxy-4-(trifluoromethyl)-coumarin
NADPH	β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate
PBS	Phosphate buffer solution
PI	Positive inhibitor
QD	Quinidine
SP	Sulphaphenazole
T2D	Type II diabetes mellitus
TC	Tranylcypromine
TdP	Torsades des pointes
W	Whapmagoostui
F12	Ham's F12 medium

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 of Eeyou Istchee commonly use traditional NHPs to treat Type 2 Diabetes. Even though NHPs are commonly identified as being safe to take because they are naturally rather than synthetically produced, NHPs can cause adverse effects in the human through various mechanisms upon their use, 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 ability for the Cree NHPs to cause these adverse effects in experiments which would reflect their use 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 or respond to insulin, resulting in hyperglyceamia. 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 (WHO). 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). Type 2 Diabetes (T2D) is the most common type of diabetes, comprising of approximately 90% of all cases (CDA website). T2D is usually developed in adulthood and occurs when the pancreas does not adequately produce insulin, or the body does not respond to insulin. In Type 1 Diabetes, the pancreas cannot produce insulin at all, but 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 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 Cree of Eeyou Istchee (CEI) inhabit the northern territory of Quebec with a population of 13500 persons (Dannenbaum *et al*., 2005). They are a group from numerous Canadian First Nations that are affected by the increasing incidence of T2D in aboriginals. The prevalence of T2D is this population is approximately four times greater than the prevalence of T2D in Quebec and Canada (20.6% vs. 4.9%) and is increasing by 0.5% per year (Kuzmina & Dannenbaum, 2004; PHAC website). A contributing factor to the rise in diabetes in the CEI is their low compliance to modern pharmaceuticals, as this route of treatment 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 2005, a collaborative research group, CIHR team in Aboriginal Anti-diabetic Medicines, was created in hopes of identifying traditional Cree medicinal plants that can be used to treat T2D symptoms in the CEI. This group consists of scientists, elders and healers of the CEI, 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 anti-oxidant 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, it showed 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 (HC website). Although NHPs are widely available and frequently used, there is a lack of research on the safety of these products and their potential to interact with conventional pharmaceuticals. Even though NHPs are comprised of natural constituents (also referred to such as phytochemicals) such as flavonoids, alkaloids, diterpines, quinones, carotenoids, and phenolics, they have the

same potential to cause adverse effects upon their use in the same manner that synthetic pharmaceuticals do. In fact, NHPs and pharmaceuticals 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.

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 promotes weight loss and increasing energy (Bent *et al*., 2003). However, these alkaloids were found to be 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 commonly used such as licorice root, horseradish, etc, have been documented to cause harmful effects to the body (Chung, 2004).

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 inhibiting the normal metabolism of the pharmaceuticals and raising their blood plasma concentrations to harmful levels. One of the most notorious NHPs that has been associated with causing adverse or fatal effects by increasing the blood plasma concentrations of concomitant pharmaceuticals such as terfernadine, astemizole, felodipine, nifedipine and verapamil, is grapefruit juice (Flanagan, 2005; Wysowski *et al*., 2001). Grapefruit juice contains 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 other NHPs that have been found to inhibit drug-metabolising 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*., 2002).

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 (HC website). However, many NHPs are not manufactured such as imported TCMs or traditional medicinal plants used by aboriginal communities (Moss *et al*., 2007). These products still carry the risk to cause adverse effects upon their use and should be analyzed for their potential to cause these effects.

1.4 The Cardiac Action Potential

The heart is composed of cardiac muscle (myocardia) 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 initiated by the depolarization of the 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 of the cell. Cardiomyocytes are electrically coupled together 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 are intricately involved in 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^+), followed by calcium (Ca^{2+}) channels allowing Na^+ and Ca^{2+} ions to enter the cell to further depolarize the cell. The Na^+ channels quickly close, however the Ca^{2+} channels are slow to close, allowing 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 of the cell and restoration of the resting membrane potential to -85 mV. A simple illustration of an AP is depicted in **Figure 1**. The resting membrane potential is maintained through various ionic pumps and exchangers such as the sodium-potassium pump. 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 microelectrodes, which measures the extracellular changes in electrical activity.

1.5 Cardiac Chronotropic Effects of Xenobiotics

A xenobiotic which has an effect on the heart rate is described to have a cardiac chronotropic effect. They are also referred to as chronotropes. Many pharmaceuticals are used specifically to regulate the heart rate. ß-blockers like 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 receptors such as carbachol, can also have a negative chronotropic effect. Xenobiotics which are able to activate these

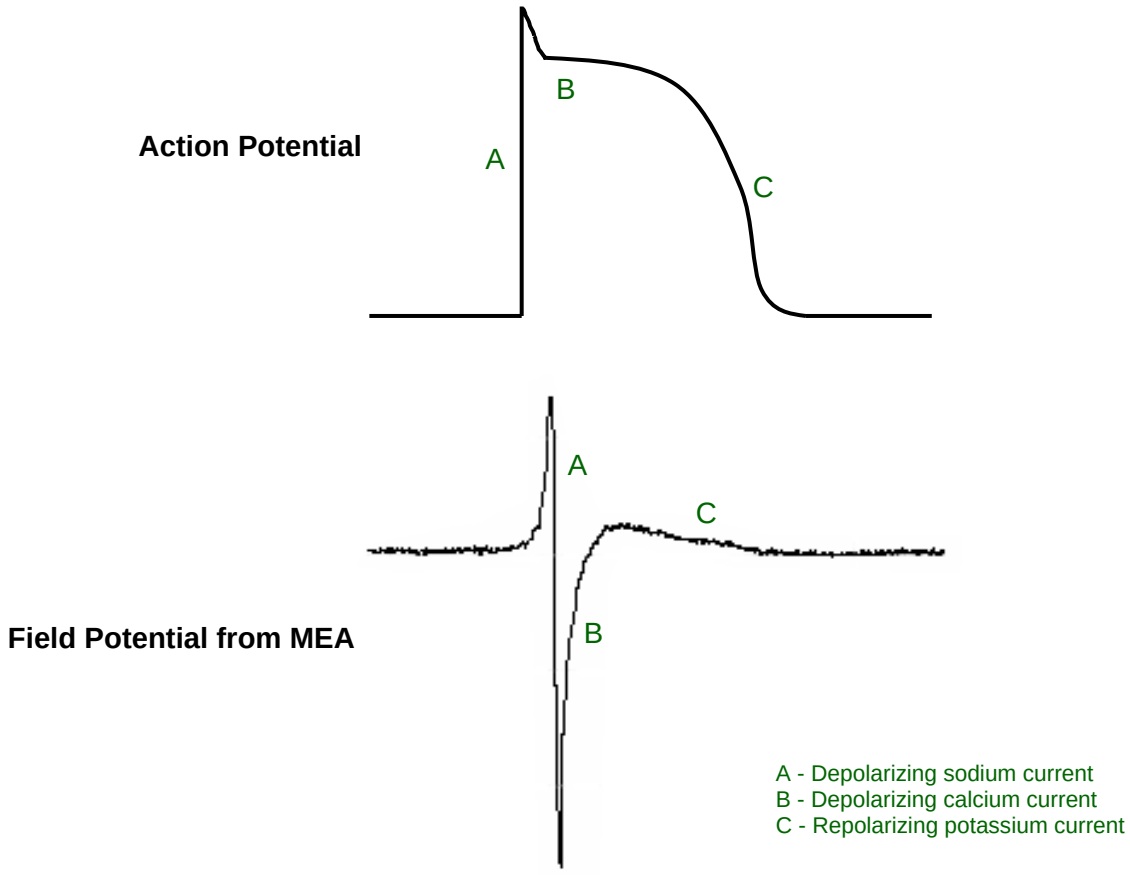


Figure 1: The relationship between an action potential and a field potential measured from a MEA. The changes in the waveforms for both the action and field potential reflect the activity of the different ion currents (A – sodium; B – calcium; C – potassium). The field potential duration is correlated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

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). There are several NHPs that have been documented to have chronotropic effects. For example, ephedra 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 called synephrine was has been associated with positive chronotropic properties (Bui *et al*., 2006; Firenzuoli *et al*., 2005; HC website). 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 (Wilkinson *et al*, 1990).

There are many documented cases where a pharmaceutical is used to treat a non-cardiac health problem, but as a side effect have an influence on the activity of the heart. The prolongation of the QT interval on an electrocardiogram is one of the most common side effects. It has 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 drug Gleevec and the anesthetic bupivacaine (Kerkela *et al*., 2006; Mather & Chang; 2001). Recently in 2007, it has been shown that the anti-hyperglycaemic drug rosiglitazone which is used to treat T2D, was found to be associated with a significant increase in the risk of myocardial infarction and death from cardiovascular disease (Nissan & Wolski, 2007).

Testing for cardiac chronotropic effects is routine during drug development because one of the three core studies of safety pharmacology studies is with the cardiovascular system (ICHS7A). However, NHPs have the same potential as drugs to cause adverse chronotropic effects.

1.6 Microelectrode Arrays

Microelectrode arrays (MEAs) are used to detect the extracellular electrical activity of electrogenic cells such as cardiomyocytes. Isolated cells or pieces of tissue are plated directed on the microelectrodes which can measure the collective extracellular field potential (FP) activity of adhered cells. The measured FP is representative of the underlying AP. The information of characteristics of an AP can also be obtained. 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*, 2004). 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 the cell membrane; the cells are kept sterile which allows for long-term experiments; 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 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 to reflect *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 mimicking the synchronous contractions in the myocardium

(Meyer *et al*., 2004). 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 allows them to be very useful for the acute and chronic screening of cardiac electrophysiological effects of xenobiotics. For example, the MEA has been used to detect the effects of ion channels blockers such as E4031, quinidine, verapamil, and nimodipine (Halbach *et al*., 2003; Meyer *et al*., 2004).

1.7 Cytochrome P450s

Cytochrome P450s (CYPs) are a family of heme-containing enzymes that are involved in the metabolism of xenobiotics. The xenobiotics are metabolized to render a metabolite that is more polar than its parent compound and is more easily excreted from the body. The 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. However, CYPs also have functions with endogenous substances such as the synthesis or metabolism of 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 (Lewis, 2004). In fact, information on

the specific CYPs involved in the metabolism of new drugs is required by drug regulatory bodies such as the Food and Drug Administration (FDA) (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 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 profoundly 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 (Tan & 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 greatly reduce the concentration of the drug entering the circulatory system as much as 95% (Flanagan, 2005). Hence, CYPs play a contributing factor in the bioavailability of drugs. 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 similarity. CYPs sharing at least a 40% or a 55% sequence identity are grouped under the same family or subfamily respectively, followed by a numerical number indicating the particular CYP in the group. For example, CYP3A4 belongs to the CYP3 family, CYP3A subfamily, and is the 4^{th} member of that subfamily. In the human genome, 57 genes coding for CYPs have been currently found, with 15 of them coding for CYPs involved

in xenobiotic metabolism: 1A1, 1A2, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 3A4, 3A5, and 3A7 (Guengerich, 2008). Metabolism of xenobiotics only occurs from members of CYP1, CYP2, and CYP3, because the substrate specificity of these isoforms is low compared to the other isoforms with endogenous roles (Lewis *et al*., 2006). Nonetheless, each of these isoforms are 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 or the benzodiazepines (Triplitt, 2006; Thummel & Wilkinson, 1998). However, there are numerous drugs that are metabolized by multiple CYPs such as amitriptyline which is metabolized by CYP2C9, 2D6 and 3A4 (Ghahramani, 1997). In the case of amitriptyline, it is metabolized through a N-methylation reaction by the three isoforms giving rise to the same metabolite, but with some drugs, different metabolites are formed with each CYP isoform (Lewis, 2003). CYP3A4 is the main isoform involved in xenobiotic metabolism, metabolizing approximately 50 % of all marketed pharmaceuticals (Zhou *et al*., 2005; Burk & Schwab, 2008). The isoforms 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7 were chosen in this study and are described below.

CYP1A2 is a member of the CYP1A family, and is greatly 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 is involved in metabolizing caffeine by N-demethylation,

as was well as the drugs theophylline, naproxen and acetominophen (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). CYP1A2 substrates typically are planar, contain aromatic rings or heterocyclic nitrogen atom rings, and are neutral or weakly basic (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). It is most known for its metabolism of the anti-depressant bupropion by oxidation (Lewis 2003). It metabolizes several other pharmaceuticals such as cyclophosphamide and propofal by hydroxylation, and ketamine by demethylation (Turpeinan *et al*., 2006). Many of its substrates are non-planar, either neutral or slightly basic, and lipophilic (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 thiazolidiones (Triplitt, 2006; CDA, 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 in the kidney (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; *S*-warfarin; and drugs from the glucose-lowering sulfonylurea family including glyburide and glimepiride (Triplitt, 2006; CDA, 2007). It is also involved in the metabolism of angiotension-II type 1 receptor blockers losartan and irbesartan, which are used to treat T2D (Triplitt, 2006). CYP2C9 plays a large role in the metabolism of drugs because of its high content in the liver (Totah & Rettie, 2005) and it accounts for approximately 60% of the CYP2C subfamily (Lin & Lu, 1998). CYP2C9 also metabolizes endogenous substances such as arachidonic acid (Zeldin *et al*., 1996; Marill *et al*., 2000). CYP2C9 has substrate specificity towards mildly acidic and lipophilic compounds with an aromatic ring (Totah & Rettie, 2005; Lewis, 2003).

CYP2C19 is most known for its metabolism of proton pump inhibitors such as omeprazole and lansoprazole via hydroxylation (Klotz *et al*., 2004). It is also involved in the metabolism of antiepileptics and tricyclic antidepressants (Goldstein, 2001; Lewis, 2003). It accounts for approximately 1% of the CYP2C subfamily (Lin & Lu, 1998) and metabolizes approximately 8% of the drugs on the market (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).

CYP2D6 is an important drug-metabolizing CYP as it metabolizes approximately 25% of marketed drugs. It is greatly 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 because it has a high affinity for alkaloids (Ingleman-Sundberg, 2005). Most CYP2D6 substrates are lipophilic bases which contain a protonable nitrogen atom (Ingleman-Sundberg, 2005).

CYP2E1 is the only member of the CYP2E subfamily in the human. It is involved in the metabolism of numerous volatile anaesthetics such as sevoflurane, halothane, diethyl ether and chloroform, and also ethanol (Chang & Kam, 1999). The activity of CYP2E1 has a potential to be harmful to the body because the metabolism of several of its substrates results in toxic metabolites (Cedarbaum, 2006). For example, the metabolism of ethanol results in 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 subfamilies 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 the this organ (Burk & Schwab, 2008).

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 calcium-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 (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 ubiquitously expressed in the human kidney, unlike CYP3A4 which is not expressed in the kidney in all humans (Haehner *et al*., 1996).

CYP3A7 is only expressed in the liver during the fetal stages of life and is absent in the adult (Lin & Lu, 1998). Its substrates are very common to CYP3A4, however its activity is much lower (Williams *et al*., 2002).

1.8 Cytochrome P450 Inhibition

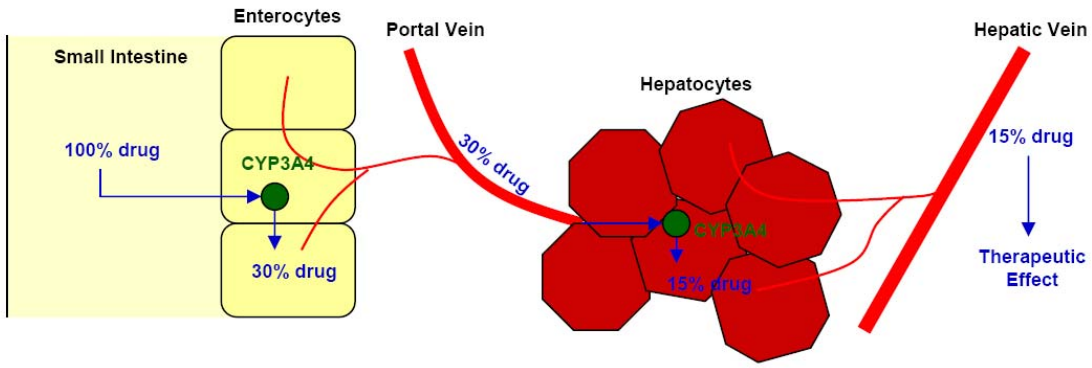
The activity of CYPs can be inhibited by xenobiotics. Since CYPs are the main enzymes involved in drug metabolism, CYP inhibition can have a major impact on the bioavailability of a drug by changing the drug's concentration in the blood **(Figure 2)**. Adverse effects can occur when the drug's plasma concentration is not within its therapeutic concentration range. For drugs with narrow therapeutic windows (a small difference between therapeutic and toxic/ineffective concentrations) such as warfarin or

cyclophosphamide (Korhonen *et al*., 2007), interfering with the metabolism of the drugs can be very harmful or even fatal. In addition, drugs which are used chronically or for life-sustenance such as antiarrhythmic ß-blockers, would also have serious effects with the interference of their normal metabolism by CYPs.

CYP inhibition can result in either an overdose or an underdose of a pharmaceutical. Pharmaceuticals which are pharmacologically active upon its administration (active drugs), require CYPs for its metabolism into inactive metabolites. Inhibition of the CYPs involved in the drug's metabolism would increase the concentration of the drug in the blood and the duration of its pharmacologic activity, resulting in a drug overdose. Pharmaceuticals can also be administered as pharmacologically inactive drugs (prodrugs) 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 blood and hence, an underdose of the drug would occur. Examples of prodrugs include Plavix and enalapril (Clarke & Waskell, 2003; Rautio *et al*., 2008).

Typically, enzyme inhibition can be classified as reversible or irreversible. In reversible inhibition, the inhibitor reduces the activity of the enzyme by binding to the active site preventing the substrate from entering it (competitive inhibition), or by binding to the enzyme to alter its catalytic activity without affecting substrate binding (non-competitive inhibition). The 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).

A)



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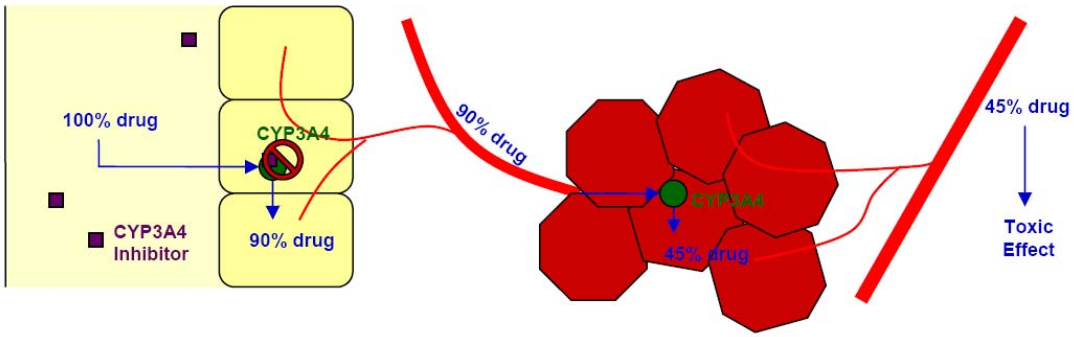


Figure 2: An illustration showing the typical metabolism of an orally administered active drug by CYP3A4 of intestinal enterocytes and liver hepatocytes in the absence and presence of CYP3A4 inhibitors. Typically, the drug is transported from the small intestines to the enterocytes where it can undergo 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 within the body for its pharmacological effect. 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 by the inhibition of CYP3A4 resulting in a high and toxic dose of the drug.

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 metabolite which then irreversibly inactivates the same CYP by 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). Examples of mechanism-based inhibitors include erythromycin, clarithromycin, tamoxifen, fluoxetine, ritonavir, diltiazem, several furanocoumarins in grapefruit juice, hydrastine in goldenseal, and glabridin in licorice root (Guo *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 modification 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 especially CYP3A4. 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, radioactivity, and antibodies. 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*., Yan & Caldwell, 2001; Yamamoto *et al.,* 2002). For these assays, the activity of the CYPs is determined by the fluorescence released by a substrate metabolized by the CYPs. In the presence of an inhibitor, the CYPs would be less active and less fluorescence would be emitted. The main disadvantage of this method is that the sample to be tested for CYP inhibition may release intrinsic fluorescence and mask the actual inhibition providing false results. On the contrary, the sample may quench the fluorescence and provide false inhibitory results. For the majority of drugs, this is not an issue as they typically dissolve colourless. 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*., were able to show that several pure constituents found in popular herbal products, isorhammetin, quercetin, vitamin, and yangonin, interfered with these assays by intrinsic fluorescence or quenching (Zou *et al*., 2002).

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 these studies, 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 b_5, which are present in concentrations that do not reflect normal physiological 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, 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 plants has not been studied and is important to identify to avoid possible adverse effects upon their use.

Two different types of safety studies were chosen to be conducted for this thesis. The first study examines the effect the Cree plants would have on the heart rate. It is important to examine their chronotropic effects because an elevated resting heart rate has been associated with in an increase in risk of mortality in Type 2 diabetics (Stettler *et al*., 2007; Linnemann & Janka, 2007). In addition, the condition of the heart in diabetics is typically unhealthy from heart disease, diabetic cardiomyopathy, or other illnesses that

affect cardiac function (CDA website, Ren *et al*., 1999). The hearts of diabetics can be more sensitive to the chronotropic effects of the plants due to their unhealthy state. The effect that the plants have on the heart is important to study because heart disease is one of the leading causes of death in type 2 diabetics, contributing to approximately 80% of all deaths (Laakso, 1999; CDA website).

The second study examines the ability for the Cree plants 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.

1.10.2 Hypotheses

1) The Cree anti-diabetic plants will have an effect on the heart rate.
2) The Cree anti-diabetic plants will inhibit the activity of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7.
3) The Cree anti-diabetic plants will interact with drugs that are used to treat diabetes and together will cause an adverse effect

1.10.3. Objectives

1) To determine the toxic concentrations of extracts of the Cree anti-diabetic plants 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 with the extracts.

3) To determine the chronotropic effect of a combination of an extract with the anti-hyperglycaemic drug 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 the 10 CYP isoforms *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 extracts are influenced by the anti-hypertensive drug enalapril.

2 MATERIALS AND METHODS

2.1 Plant Extract Preparation

2.1.1 Collection and Storage of the Cree Plants

Samples of plants 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 8 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 9 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 from the Cree plants were already prepared by members of John Arnason's lab at the University of Ottawa as mentioned in Spoor *et al.* (Spoor *et al*., 2006). These extracts were in solidified form and required dissolution in organic solutions. For the cardiotoxicity assays, the extracts were dissolved in DMSO (Sigma-Aldrich) at a concentration of 1 mg/mL, aliquoted into 0.5 mL microfuge tubes, and

Plant Species	Plant Part	Accession Number
Rhododendron groenlandicum	Leaves	AD01
Abies baslamea	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

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.

stored in -20°C. These extracts were used within three months of its preparation. On experimental days, an aliquot was removed from the freezer, thawed, and added to fresh media or Tyrode's buffer at the required concentration. 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 its preparation, whereas the working solutions were discarded after its use at the end of the day.

2.1.3 Extract Preparation of Bitter Orange and Goldenseal

Bitter orange (*Citrus aurantium*) and goldenseal (*Hydrastis Canadensis*) extracts were prepared in a different manner than the Cree plant extracts. Instead, these extracts were prepared fresh for each experiment in the lab. Bitter orange (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 representation of the entire bottle of BO, 6 capsules were opened and the ground contents were combined. Goldenseal (GS) was obtained in ground form from Kentz Farm in Waterloo, ON courtesy of Renee Leduc from Dr. Arnason's lab. Extracts were prepared by measuring the required amount of ground material in a 1 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 8 for 1 minute. The extracts were then centrifuged for 15 minutes at 12000 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 (2-3 days old) were obtained from cryopreserved vials (Lonza). 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 microelectrode arrays (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. The covered MEAs were placed in sterile glass culture dishes. Cells were cultured in a humidified incubator with an atmosphere of 5% CO_2 and 95% air in 37°C. After 4 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 was used (glucose concentration of 17.5 mM) but was supplemented with extra glucose to obtain a glucose concentration of 25.5 mM. For experiments using low glucose media, the media had to be made in a different manner than the normal and high glucose media because the normal

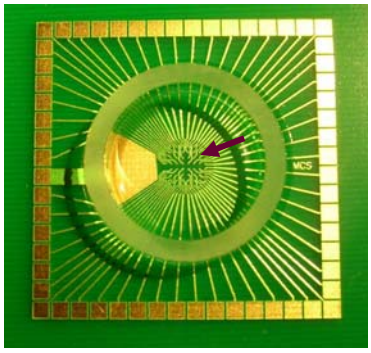


Figure 3: **An image of the microelectrode array (EcoMEA) used for the cardiomyocyte experiments.** The arrow points to the location of the 60 microelectrodes which is surrounded by an enclosed well.

media already had 17.5 mM of glucose. The low glucose media was prepared by combining DMEM (without glucose) and F12 in a 1:1 ratio, followed by the addition of the other constituents.

2.2.2 Gathering of Extracellular Field Potential Activity

The MEA system is a device that allows the *in vitro* recording of the extracellular field potential (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 culture dish and connected to a MEA60 System (Multi Channel Systems) set at 37°C and also in the biological hood. Recordings from the MEA were then performed with the well being covered with the white cap. Signals were recorded at 25 Hz, 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, only selected microelectrodes 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).**

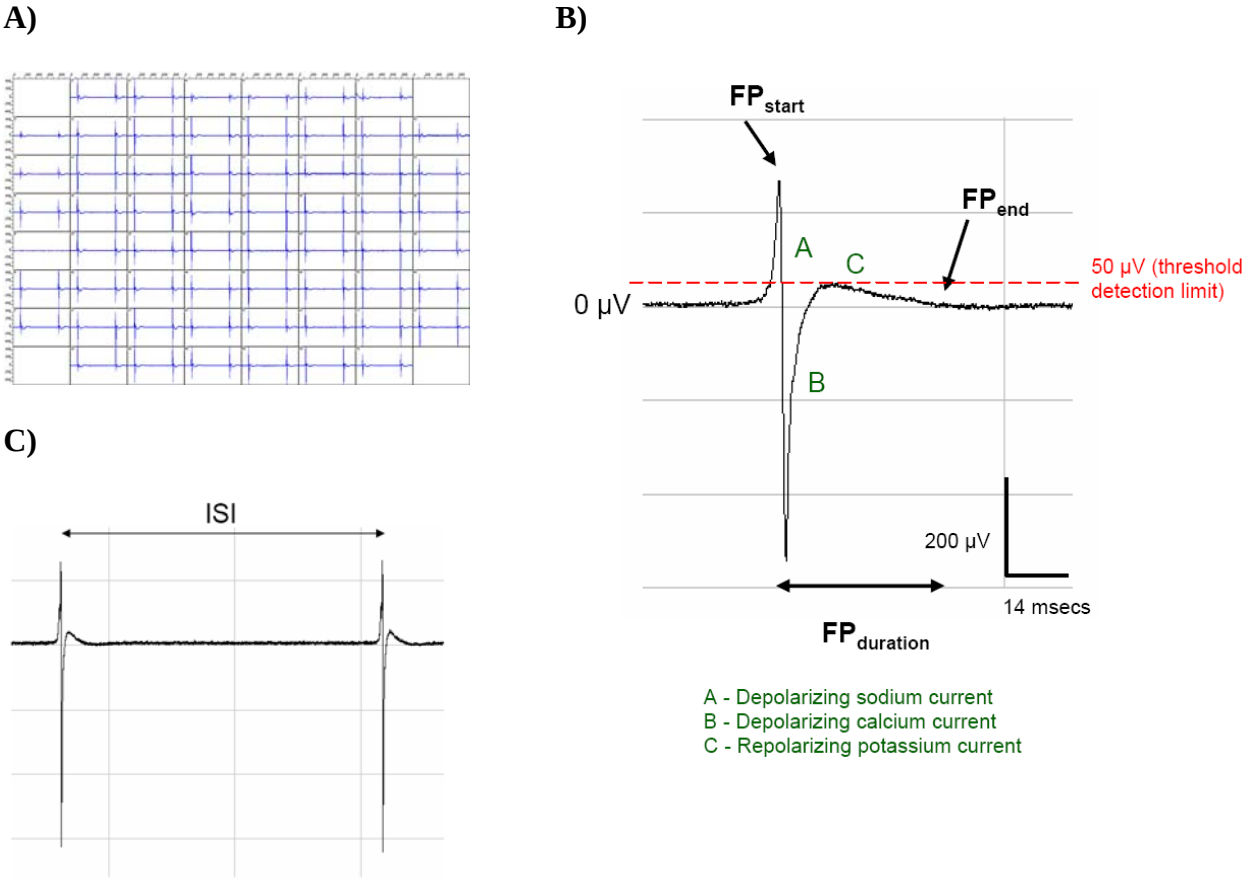


Figure 4: Illustrations of the extracellular field potential activity obtained using the MEA system. A screen view of the FP activity measured from the 60 microelectrodes during one second of a recording is shown in **(A)**. A magnified image and a description of the FP activity changes that occur during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

2.2.4 Determining the Acute Chronotropic Effects

A MEA with a culture of cardiomyocytes at 5 to 7 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, 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 culture 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 on once and then discarded. If the cardiomyocytes were used again, they were used 24 hours later and then discarded. Typically on an experimental day, numerous (up to eight) cultures of cardiomyocytes in

MEAs were analyzed one after the other. The drugs isoproterenol (Sigma-Aldrich) carbachol (Sigma-Aldrich), and metformin were dissolved in water.

2.2.5 Determining the 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 brought back to the incubator for a chronic (18 hour) 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 Determination of Cytotoxic Extract Concentrations

Cardiomyocytes were plated at 5 x 10^5 cells/mL per well in 96-well plates. At 5 days *in vitro* (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 18000 rpm. The supernatants 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 and allowed to incubate at room temperature in the absence of light. After 30 minutes, the stop solution was added 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 1X Stockholm 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 PBST overnight at 4°C in the absence of light. The cardiomyocytes were then rinsed as previous with Stockholm 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.

2.2.9 Statistical Analysis

In most cases, statistical analysis for the cardiac chronotropic effect studies 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 chrontropy 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 hypotheses 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 hypotheses tested for the production of diabetic-like cardiomyocytes study was: no difference in the FP duration between the different media. The null hypotheses 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.* and Leduc *et al*. (Budzinski *et al.,* 2005; Leduc *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), which 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), which 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 bottoms and white walls (Corning). All 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, and the substrate in 0.5 M potassium phosphate buffer solution (pH 7.4). Solution B contained the enzyme in the 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 shaken for 3 seconds and the initial fluorescence ($T_{initial}$) measured using a Cytofluor4000 Plate Reader (Applied Biosystems) at various excitation and emission wavelengths depending on the substrate used. The solutions were 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**.

CYP isoform	CYP Concentration (pmol/mL)	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	Sulfaphenazole	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] 0.5 mM Tris buffer (pH 7.5) was used instead of potassium phosphate buffer
[b] 0.54 mM NADPH was used in Solution A

Table 2: Description of the experimental condition for the CYP inhibition studies

2.3.2 Mechanism-based Inhibition of CYP3A4

A similar method to the CYP3A4 inhibition assay mentioned above 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 0.1 pmol/μL CYP3A4 in buffer; and an incubation solution containing 1.08 mM NADPH and 1 μM DBF in 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 bottoms and white walls and allowed to warm to 37°C for 5 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 5 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 3 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 aliquoted into empty wells of the assay plate and allowed to pre-incubate for a longer period of time at 37°C. At 5 and 10 minutes, 20 μL from each pre-incubation solution was added to another 3 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 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 1, 5 and 10 min pre-incubation times, to determine if there were differences in activity between the different pre-incubation conditions. For the Cree plant extract samples exhibiting time- and NADPH-dependence, other concentrations were tested. Goldenseal extract (2.3 to 9.7 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.

2.3.3 Determination of the 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 dilutions had the same content of 100% MeOH (1 uL) 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 an extract can affect the activity of CYP3A4 compared to the extract alone. 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 expect 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 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 or the drug interaction study, 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 MBI of CYP3A4 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 1 minute of pre-incubation time and 5 or 10 minutes of pre-incubation times. For the drug interaction study with enalapril,

the null hypotheses tested was: no difference between the inhibition by the extract and the inhibition by the extract with enalapril.

3 RESULTS

3.1 Identifying Cardiac Chronotropic Effects of Cree Plant Extracts

3.1.1. Screening for Cardiac Chronotropic Effects of Chronotropes

To confirm that neonatal rat cardiomyocytes plated on MEA chips can be used for the detection of chronotropic effects of the Cree plant extracts, experiments were conducted with known chronotropes to observe if the expected changes in cardiac contraction rate 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 natural product which activates 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 produced 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 did not have a significant acute effect on the contraction rate of the cardiomyocytes, however there was a 14% decrease relative its contraction rate in Tyrode's buffer **(Figure 5)**. Acute dose-dependent results were observed for isoproterenol, carbachol and 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 36.58% ± 14.15% of the mean contraction rate of cardiomyocytes treated with the water vehicle

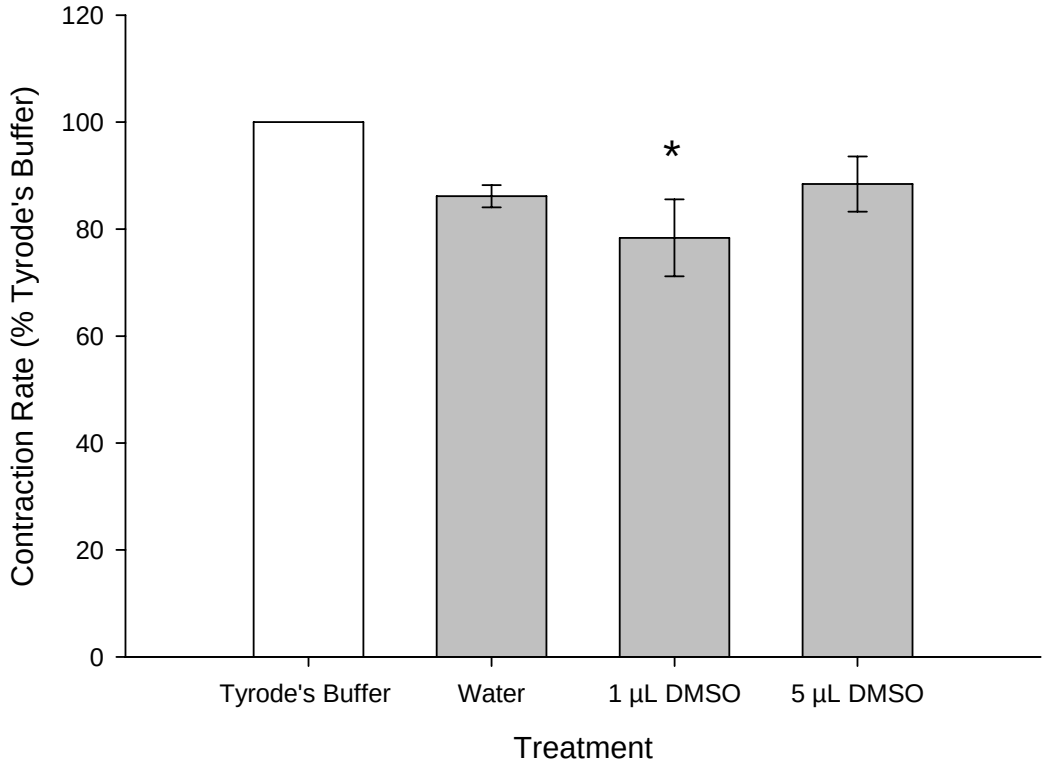


Figure 5: The acute chronotropic effects of the vehicle controls with neonatal rat cardiomyocytes. The cardiomyocytes were treated with the vehicle controls (1 µL water or DMSO, and 5 µL DMSO) for one minute and the resultant contraction rates were measured using the MEA system and counted using the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

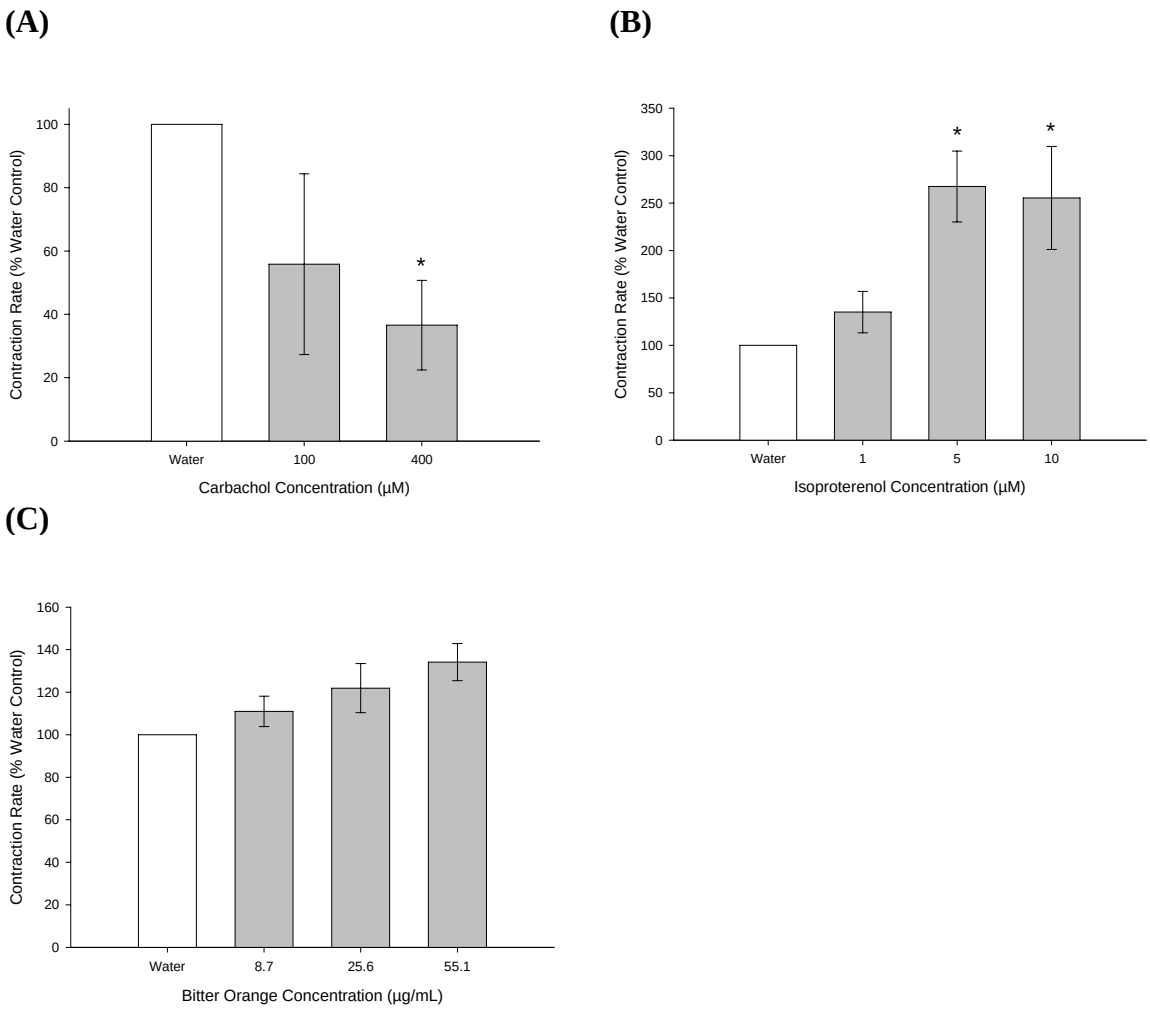


Figure 6: The acute chronotropic effects of carbachol, isoproterenol and bitter orange extract with neonatal rat cardiomyocytes. The cardiomyocytes were treated with different concentrations of the cardioactive substances **(A)** carbachol, **(B)** isoproterenol, and **(C)** bitter orange extract for 1 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 (n = 4-6). *$p \leq 0.05$ with respect to the mean contraction rate in the water vehicle control, using one-way ANOVA followed by the Tukey test.

control. Significant effects were observed with 5 and 10 μM isoproterenol ($p \leq 0.05$) **(Figure 6B)**. The mean contraction rates of cardiomyocytes treated with 5 and 10 μM isoproterenol were 267.48% ± 37.39% and 255.36% ± 54.39% respectively of the mean contraction rate of cardiomyocytes treated with the water vehicle control. Increases in the contraction rate were observed with increasing BO concentration, however these increases were not significantly different ($p > 0.05$) 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 on MEAs.

3.1.2 Determination of the Highest Non-toxic Concentration

DMSO was used to solubilize the Cree plant extracts for the cardiac experiments. To confirm that the DMSO itself was not toxic to the cardiomyocytes, the cardiomyocytes 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. Concentrations up to 6 μL/mL DMSO did not significant effect cell viability ($p > 0.05$) and therefore, the concentrations of DMSO to be used for the experiments were not toxic **(Figure 7)**.

Due to time limitations, only four extracts were chosen to be studied: W2, W4, W5, and W9. Only their highest non-toxic concentrations were planned to be studied for chronotropic effects. The highest non-toxic concentrations of these extracts were identified by measuring the amount of LDH released in the media from the

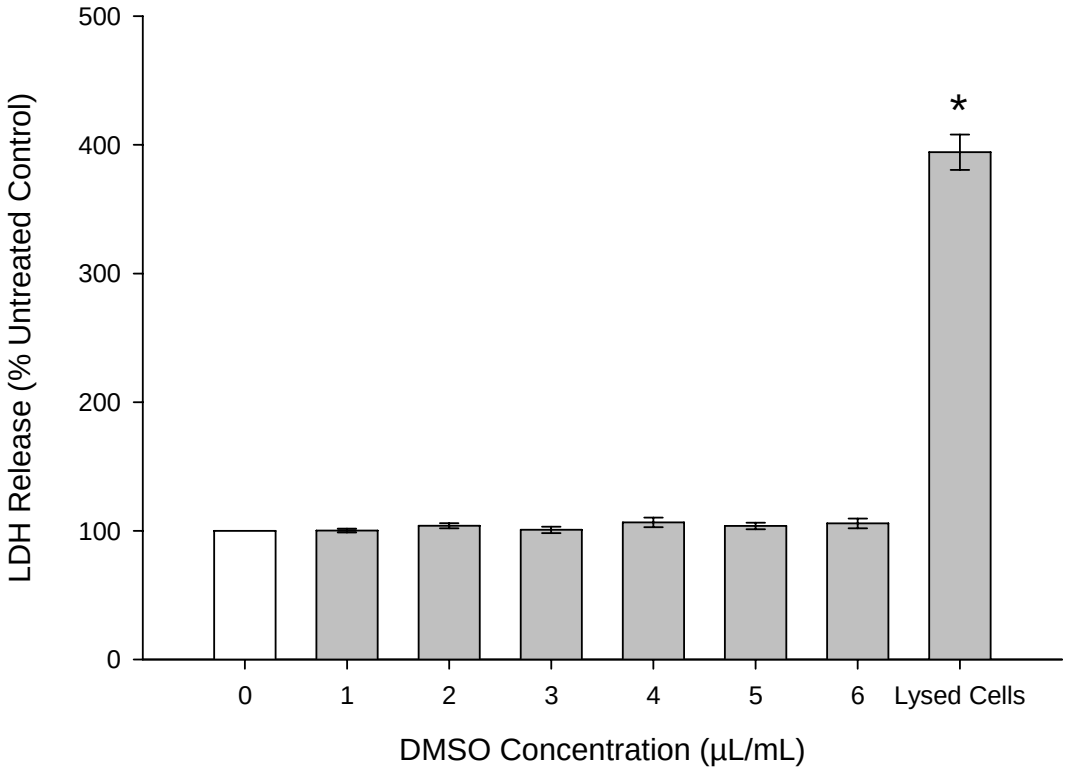


Figure 7: The cytotoxic effects of various concentrations of DMSO with neonatal rat cardiomyocytes. Cardiomyocytes were treated with various concentrations of DMSO (1 to 6 µL/mL) for 18 hours, and their cytotoxic effects were determined by the measurement of the resultant LDH released. Lysed cells were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

cardiomyocytes treated with various concentrations of the extracts. Different toxic concentrations were observed for the 4 extracts **(Figure 8)**. The lowest significant toxic concentrations for W2, W5 and W9 respectively were 200, 50, and 10 µg/mL respectively ($p \leq 0.05$). For W4, toxic concentrations were not determined up to concentrations of 600 µg/mL. It is physiologically impossible for extract levels to reach that concentration in the blood plasma and therefore, further studies to determine the lowest toxic concentration was not conducted. Dose-dependent release of LDH was observed with increasing extract concentrations for the extracts W2, W5, and W9. Overall, the highest non-toxic concentrations to be tested on the cardiomyocytes were 100, 500, 25, and 5 µg/mL for the extracts W2, W4, W5, and W9 respectively.

3.1.3 Screening for Cardiac Chronotropic Effects of the Cree Plant Extracts

Similar to the water vehicle control, 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. A significant decrease ($p \leq 0.05$) in the contraction rate was observed with 1 µL/mL of DMSO (average contraction rate of 78.35 ± 7.20 contractions/minute), but not 5 µL/mL (average contraction rate of 88.43 ± 5.15 contractions/minute) **(Figure 5)**. Since the chronotropic results from the Cree plant extracts will be relative to its vehicle control, this significant decrease should not affect the results. The concentrations of the extracts that were tested were 100, 500, 25, and 5 µg/mL for the extracts W2, W4, W5, and W9 respectively, which corresponded to their highest non-toxic concentrations. At these concentrations tested, there were no significant changes ($p > 0.05$) in acute chronotropy observed **(Figure 9)**. W2 had a minor positive chronotropic effect (108.25% ± 8.50% of vehicle control), while W4, W5, and W9 had

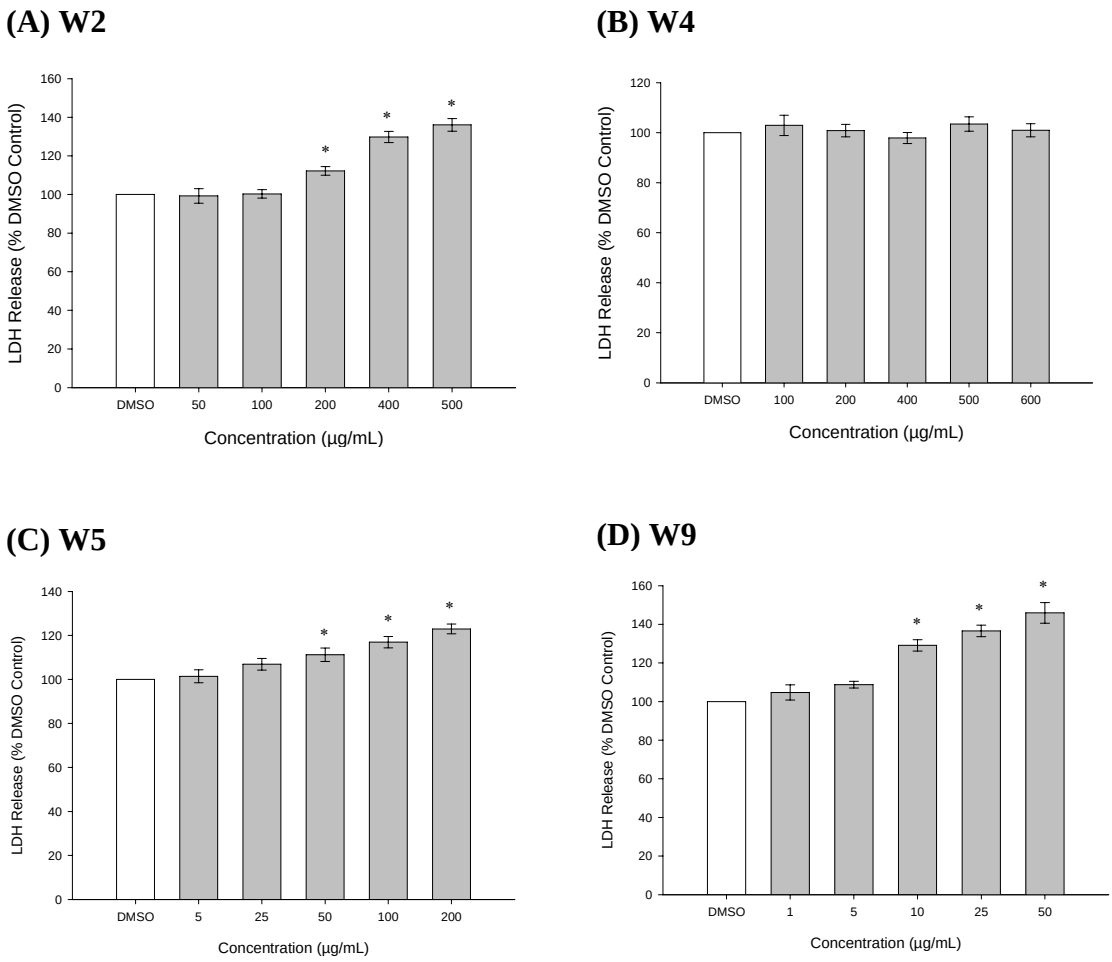


Figure 8: The cytotoxic effects of various concentrations of the extracts W2, W4, W5, and W9 with neonatal rat cardiomyocytes. Cardiomyocytes were treated with various concentrations of the extracts **(A)** W2, **(B)** W4, **(C)** W5, and **(D)** W9 for 18 hours, and their cytotoxic effects were determined 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 (n = 7-9). *$p \leq 0.05$ with respect to the DMSO vehicle control, using one-way ANOVA followed by the Tukey test.

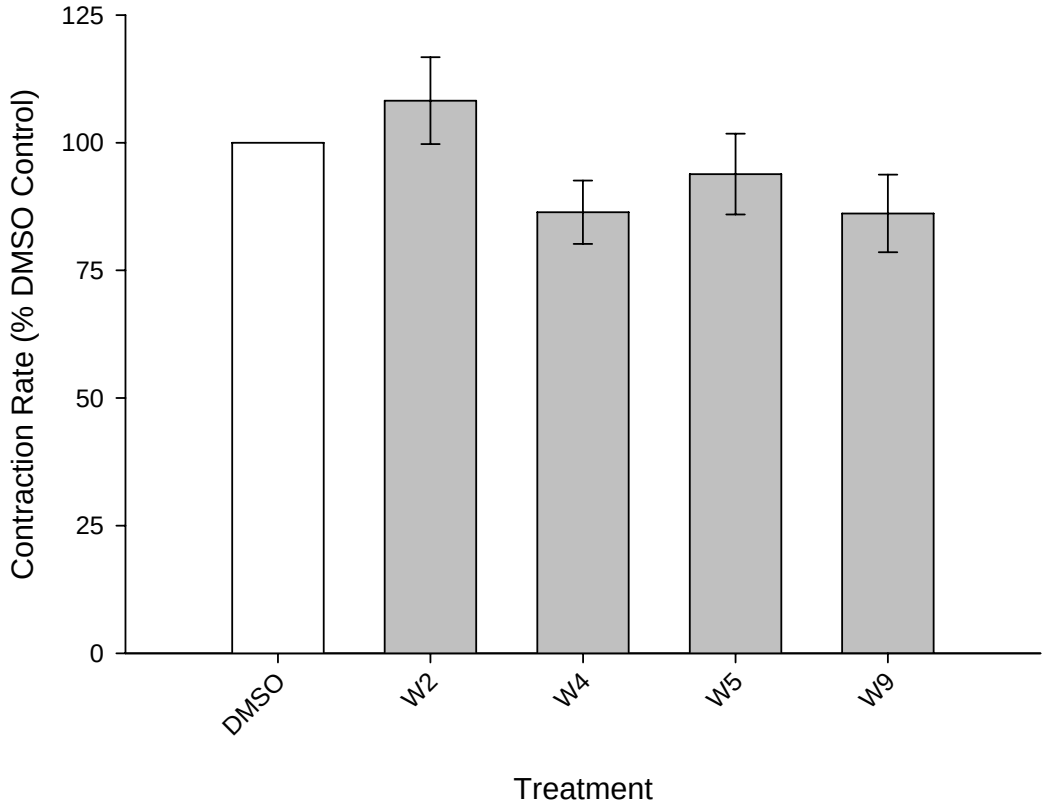


Figure 9: The acute chronotropic effects of the extracts W2, W4, W5 and W9 with neonatal rat cardiomyocytes. The cardiomyocytes were treated with the highest non-toxic concentrations of the extracts W2, W4, W5 and W9 for one minute, and the resultant contraction rates were measured using the MEA system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

minor negative chronotropic effects (86.37% ± 6.21%, 93.85% ± 7.91%, and 86.15% ± 7.59% respectively of vehicle control).

The extract W9 was chosen for additional analysis because it had the greatest effect on the contraction rate and it had a non-toxic dose (5 μg/mL) that can be reasonably be within blood plasma levels. The extract W4 had a similar 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 than10 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. Both of these concentrations did not significantly affect 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 95.01% ± 4.75% and 97.20% ± 8.35% of the vehicle control respectively.

The chronic chronotropic effects of the extracts were also studied. For these experiments, the extracts at their highest non-toxic concentrations were added to the media and the cardiomyocytes were treated for 18 hours. 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 (mean contraction rate of 62.07% ± 5.38% contractions/minute), but not 1 μL/mL (mean contraction rate of 84.56% ± 6.71% contractions/minute) relative to their contraction rate after their initial exposure to the vehicle controls **(Figure 11)**. Since the chronotropic results from the extracts will be relative to the vehicle control, this significant decrease should not affect the results. At the highest non-toxic concentrations of the extracts tested, there were no

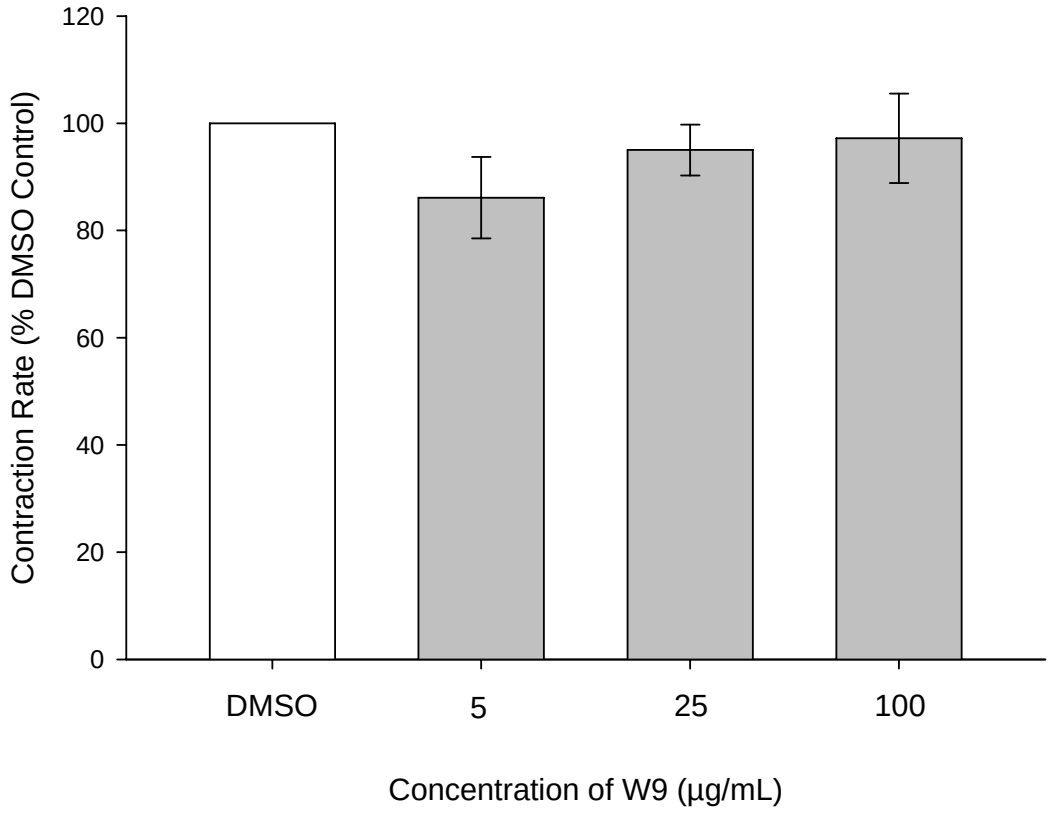


Figure 10: The acute chronotropic effects of various concentrations of the extract W9 with neonatal rat cardiomyocytes. The cardiomyocytes were treated with 5, 25 or 100 µg/mL of W9 for 1 minute, and the resultant contraction rates were measured using the MEA system and counted using the software AxoScope. The contraction rates were expressed relative to the contraction rate of the DMSO vehicle control, as the mean contraction rate ± SEM (n = 4-7). *$p \leq 0.05$ with respect to the contraction rate in the DMSO vehicle control, using one-way ANOVA followed by the Tukey test.

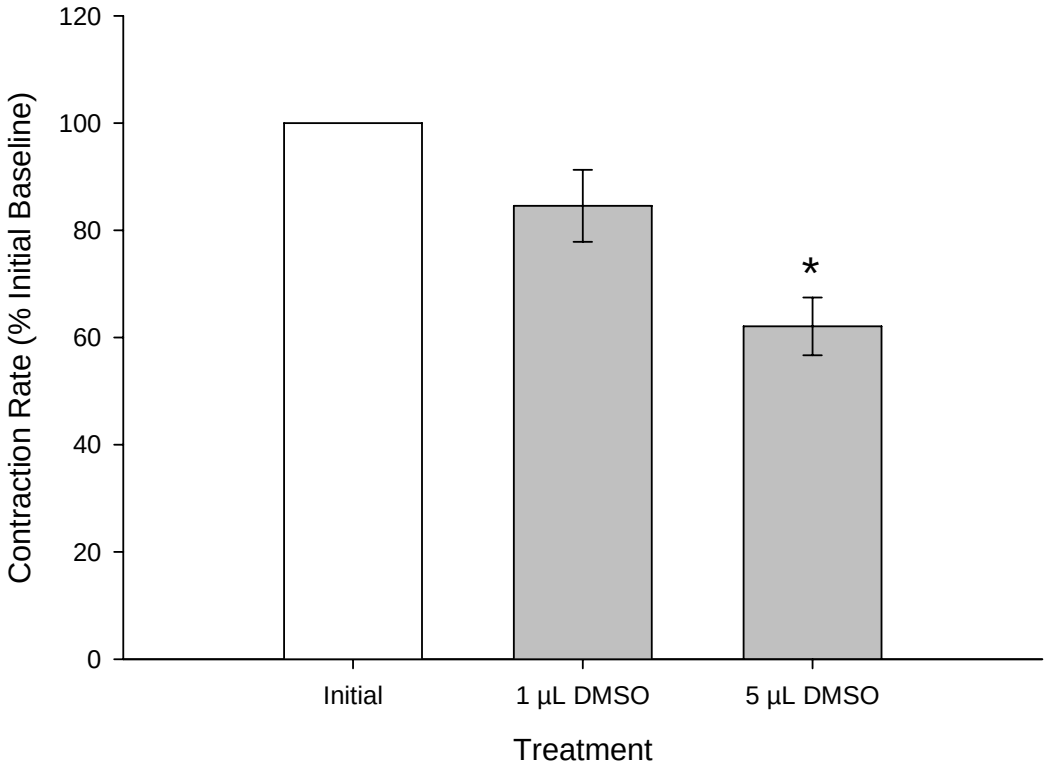


Figure 11: The chronic chronotropic effects of the vehicle controls with neonatal rat cardiomyocytes. The cardiomyocytes were treated with the vehicle controls (1 µL DMSO, and 5 µL DMSO) for 18 hours and the resultant contraction rates were measured using the MEA system and counted using the software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

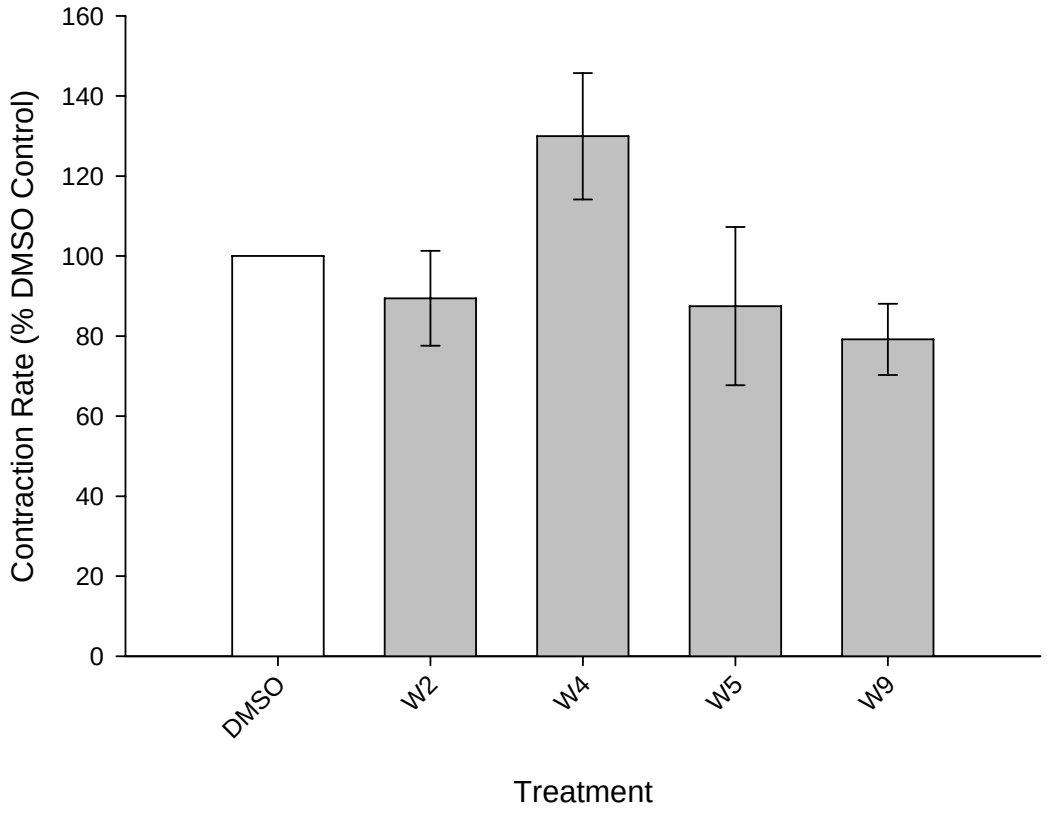


Figure 12: The chronic chronotropic effects of the extracts W2, W4, W5 and W9 with neonatal rat cardiomyocytes. The cardiomyocytes were treated with the highest non-toxic concentrations of the extracts W2, W4, W5 and W9 for 18 hours, and the resultant contraction rates were measured using the MEA system and counted using the software AxoScope. The contraction rates were expressed relative to the contraction rate of the DMSO vehicle control, as the mean contraction rate ± SEM (n = 3-4). *$p \leq 0.05$ with respect to the contraction rate in the DMSO vehicle control, 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 (129.93% ± 15.79% of vehicle control), while W2, W5, and W9 had minor negative chronotropic effects (89.44% ± 11.82%, 87.48% ± 19.77%, and 79.19% ± 8.88% respectively of vehicle control).

Overall, from the results of **Figure 9 to 12**, the extracts W2, W4, W5, and W9 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-hyperglycaemic drug metformin, and the extract W9 (used as complementary medicine) had an effect on the contraction rate. Metformin was first tested to determine if it had an effect on the contraction rate. Relative to its water vehicle control (the solvent), metformin did not significantly have a chronotropic effect (94.48% ± 8.15% of vehicle control) ($p > 0.05$) **(Figure 13)**. To determine if the combination of metformin and the extract W9 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 (5 or 100 μg/mL) were then added to the Tyrode's buffer. After 1 minute of exposure to both metformin and W9, the contraction rate of the cardiomyocytes was measured. There was a dose-dependent negative chronotropic effect when the cardiomyocytes were exposed to both metformin and W9, but not when W9 was present without metformin **(Figure 14)**. In addition, the combination of 50 μM metformin and 100 μg/mL W9 significantly decreased the contraction rate relative to the

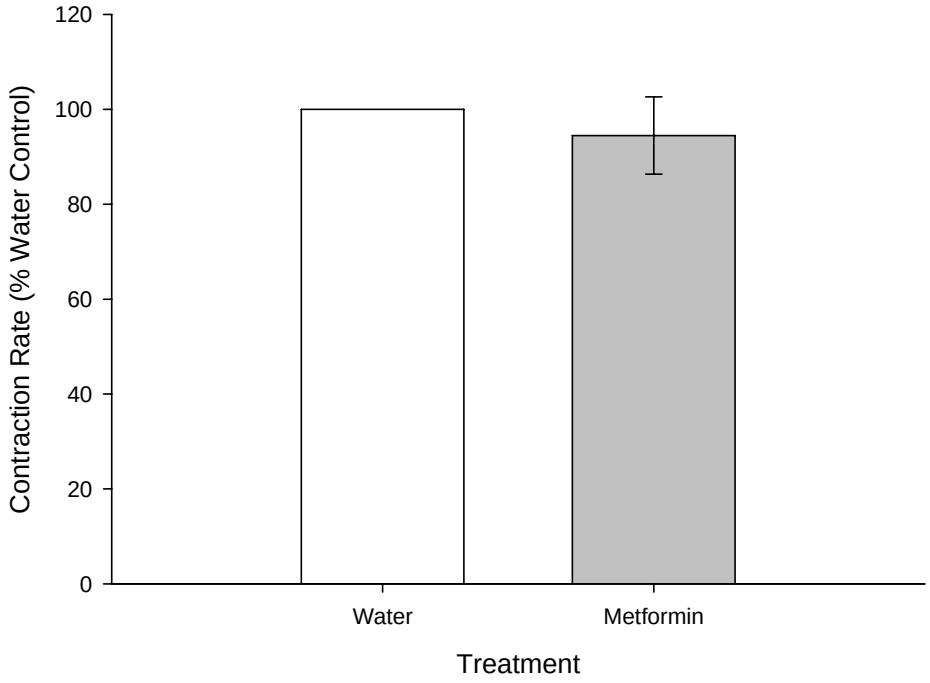


Figure 13: The acute chronotropic effect of 50 µM metformin with neonatal rat cardiomyocytes. The cardiomyocytes were treated with 50 µM of metformin for one minute, and the resultant contraction rates were measured using the MEA system and counted using the software AxoScope. The contraction rates were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

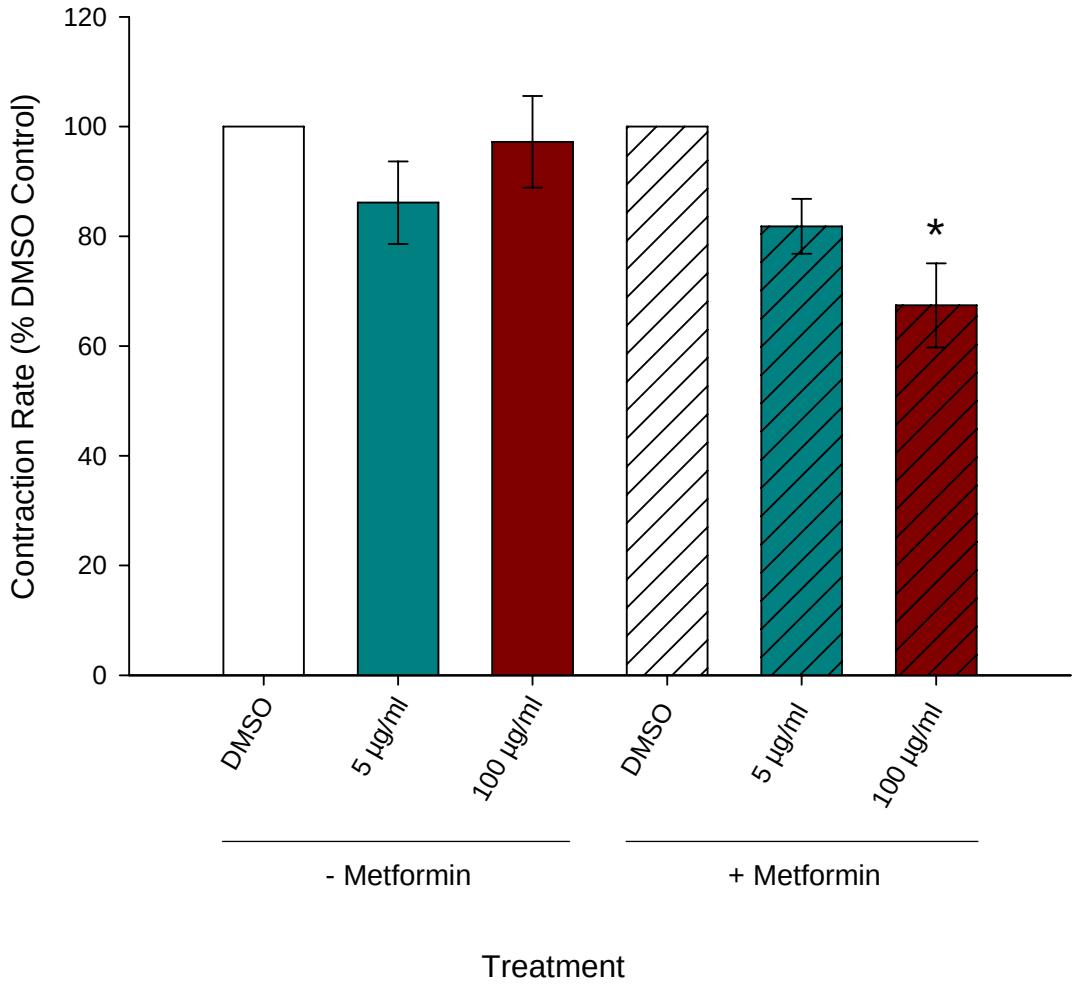


Figure 14: The acute chronotropic effect of the combination of metformin and the extract W9 with neonatal rat cardiomyocytes. The cardiomyocytes were treated with 50 µM of metformin and 5 or 100 µg/ml of the extract W9 for one minute, and the resultant contraction rates were measured using the MEA system and counted using the software AxoScope. The contraction rates were expressed relative to the contraction rate of the DMSO vehicle control as the mean contraction rate ± SEM (n = 4-7). *$p \leq 0.05$ 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 ($p \leq 0.05$).

combination of 50 μM metformin and the DMSO vehicle control (contraction rate was 67.41% ± 7.66% of the vehicle control) ($p \leq 0.05$). However, there was not a significant difference between 100 μg/mL W9, and 50 μM metformin with 100 μg/mL W9 (67.41% ± 7.66% vs. 97.22% ± 8.35% respectively) ($p > 0.05$).

The mechanism behind the decrease in contraction rate 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 which 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 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 observed. After the cardiomyocytes were treated with metformin and W9, the drug and extract were washed out once with Tyrode's buffer, and then the media was re-added to the cardiomyocytes. If there was a toxic effect and not a cellular signaling effect from the interactions between metformin and W9, there should be a recovery observed 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 for the cardiomyocytes treated with 5 μg/mL W9 (98.28% ± 9.46% of vehicle control), and a minor recovery with 100 μg/mL W9 (76.08% ± 10.76% of vehicle control) **(Figure 15)**. A significant difference in recovery was not observed between the DMSO vehicle control and 100 μg/mL W9

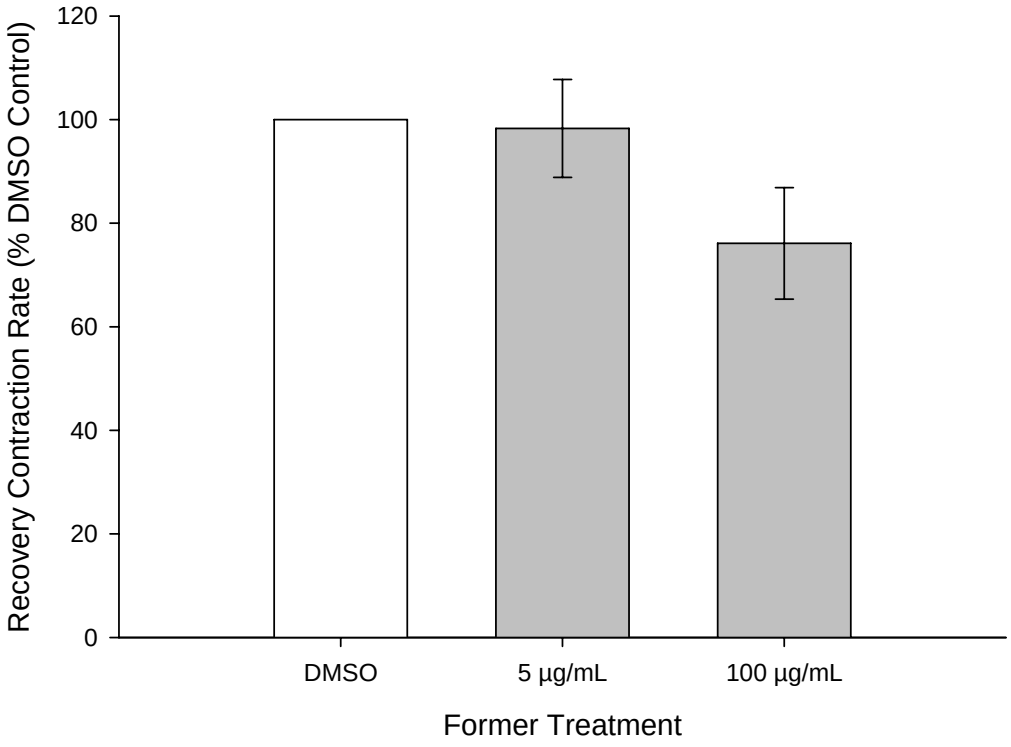


Figure 15: The acute recovery contraction rate of neonatal rat cardiomyocytes in media following the washout of metformin and the extract W9. Metformin and the extract W9 (5 or 100 µg/mL) were washed out with Tyrode's buffer after a 1 minute treatment with the cardiomyocytes. The buffer was then replaced with media and the resultant recovery contraction rates were measured using the MEA system and counted using the software AxoScope. The recovery contraction rates were expressed relative to the recovery contraction rate of the DMSO vehicle control as the mean contraction rate ± SEM (n = 5-6). *$p \leq 0.05$ with respect to the recovery DMSO vehicle control, using one-way ANOVA followed by the Tukey test.

indicating that toxicity most likely occurred.

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

3.3 The Production of Diabetic-like Cardiomyocytes

3.3.1 Confirming Their Presence

The extracts W2, W4, W5, and W9 were tested on healthy cardiomyocytes, but these extracts will be used by diabetics and therefore, these extracts should be tested on diabetic cardiomyocytes. The 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 affected by the extracts. Jun Ren *et al*., 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 used for the experiments but supplementing the media with extra glucose to obtain a glucose concentration of 25.5 mM. The 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*., 1998; D'Amico *et al*., 2001). These FP durations were compared to the FP durations of cardiomyocytes cultured in the normal media which has 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 are expected to have the shortest FP durations. The low glucose media was prepared in a different manner from the normal and high glucose media because of the required low glucose media, but otherwise were identical in

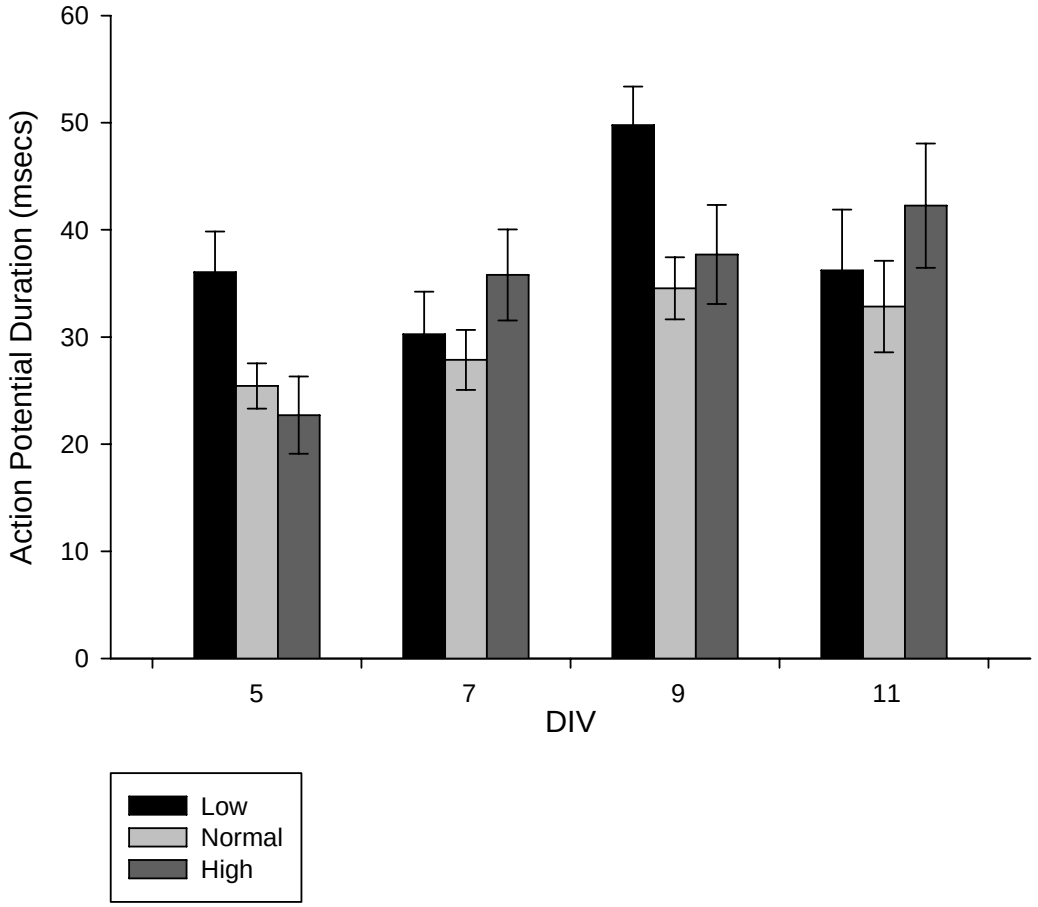


Figure 16: The measured field potential durations of neonatal rat cardiomyocytes cultured in media with various concentrations of glucose from 5 to 11 DIV. Cultures of cardiomyocytes were grown in low (5 mM), normal (17.5 mM), or high (25.5 mM) glucose media, and field potential activity recordings were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

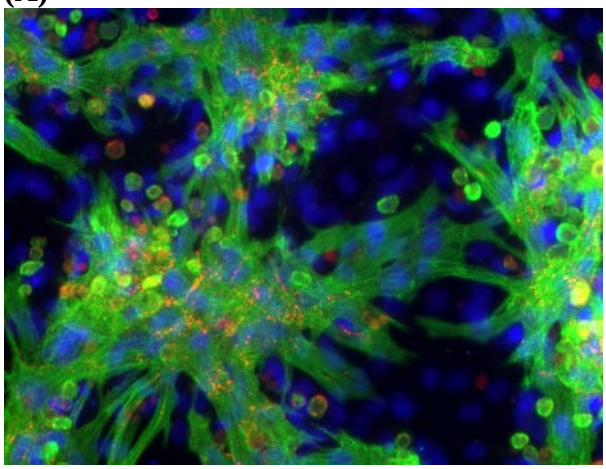
constituents. The FP durations were measured at 5, 7, 9, and 11 DIV **(Figure 16)**. Overall, there were no significant differences in FP durations between the cardiomyocytes cultured in different media for each day analyzed. In addition, the cardiomyocytes cultured in the low glucose media had the longest FP durations compared to the other cardiomyocytes cultured in the two types of media. In general, the cardiomyocytes cultured in the high glucose media did have 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 increase in FP duration the longer the cardiomyocytes were in culture for the ones 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 The Viability of Cardiomyocytes

It was also observed that the viability for cardiomyocytes cultured in the and high glucose media was lower than those cultured in normal glucose by visual analysis **(Figure 17)**. This can be seen by the greater amounts of detached, rounded up cardiomyocytes observed. These cells were not fibroblasts or endothelial cells because these rounded up cells were positive for α-actinin staining (green) which is only expressed in cardiomyocytes. The cultures did contain non-cardiac cells such as fibroblasts or endothelial cells indicated by the numerous stained nuclei in cells that did not express α-actinin. A similar decrease in viability was observed with the cardiomyocytes cultured in the low glucose media, but no data was collected to verify this observation.

(A)



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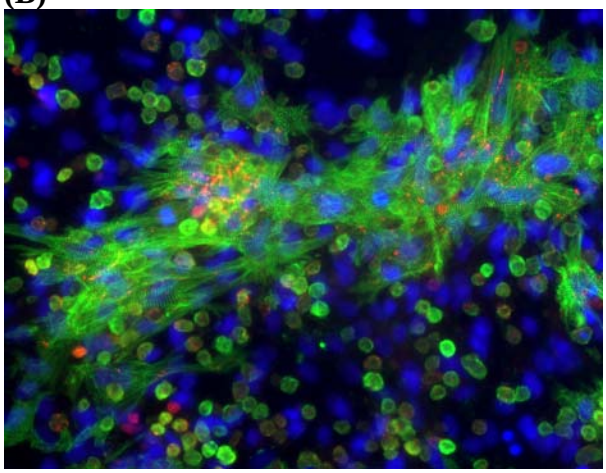


Figure 17: Images of rat neonatal rat cardiomyocytes at 7 DIV cultured in normal and high glucose media. Cardiomyocytes were cultured in **(A)** normal glucose media (17.5 mM), and **(B)** high glucose media (25.5 mM) for 7 DIV, fixed with Lana's fixative, and stained for α-actinin (green), connexin43 (red), 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 chip where the FP durations were measured from eight separate microelectrodes on various days. Eight microelectrodes were analyzed because the changes in extracellular electrical activity from different regions of cardiomyocytes of the one culture can be 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)**. It was observed that there was an increase in FP duration the longer the cardiomyocytes were kept in culture, which was also 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 same at 5 DIV (6.1msecs).

3.5 The Inhibition of the Various Cytochrome P450 Isoforms

3.5.1 Identifying the Inhibitory Potency of the Cree Plant Extracts

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

CYP1A2

Overall, the majority of the extracts had low inhibitory potencies against CYP1A2 activity **(Figure 19)**. Only four extracts (AD01, AD06, AD11, and W5) had moderate

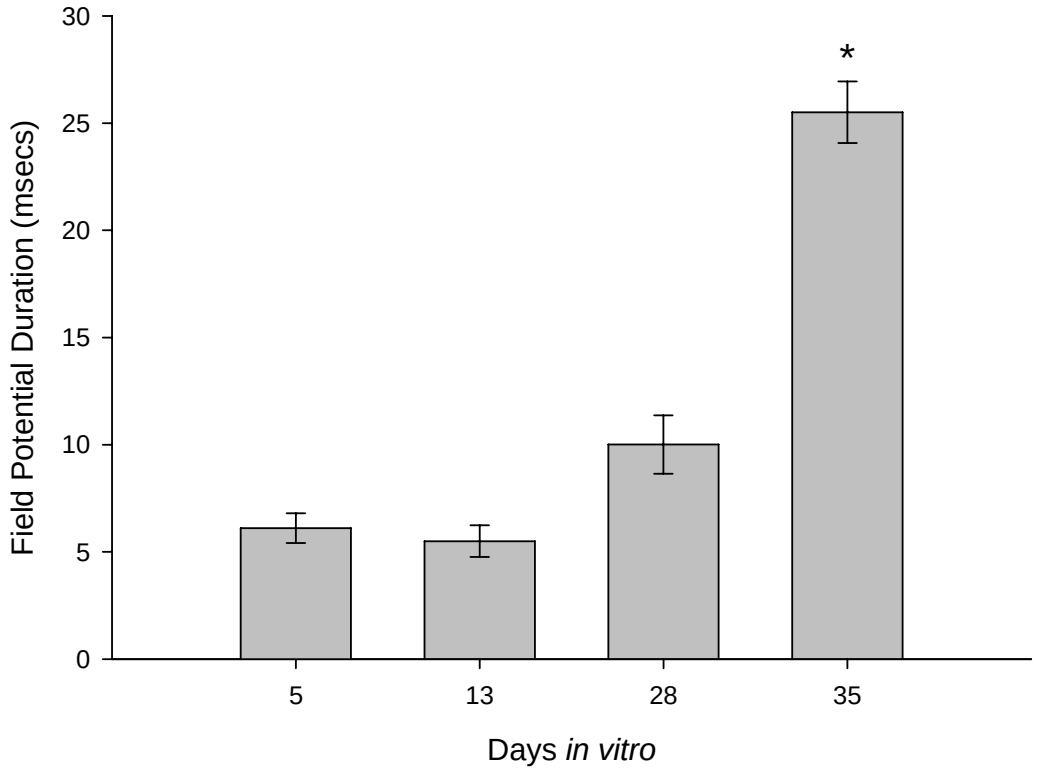


Figure 18: The field potential durations of a long-term culture of neonatal rat cardiomyocytes up to 35 DIV. A culture was kept in culture for 35 DIV, and field potential activity recordings were performed at 5, 13, 28, and 35 DIV using the MEA system. The field potential durations were measured using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

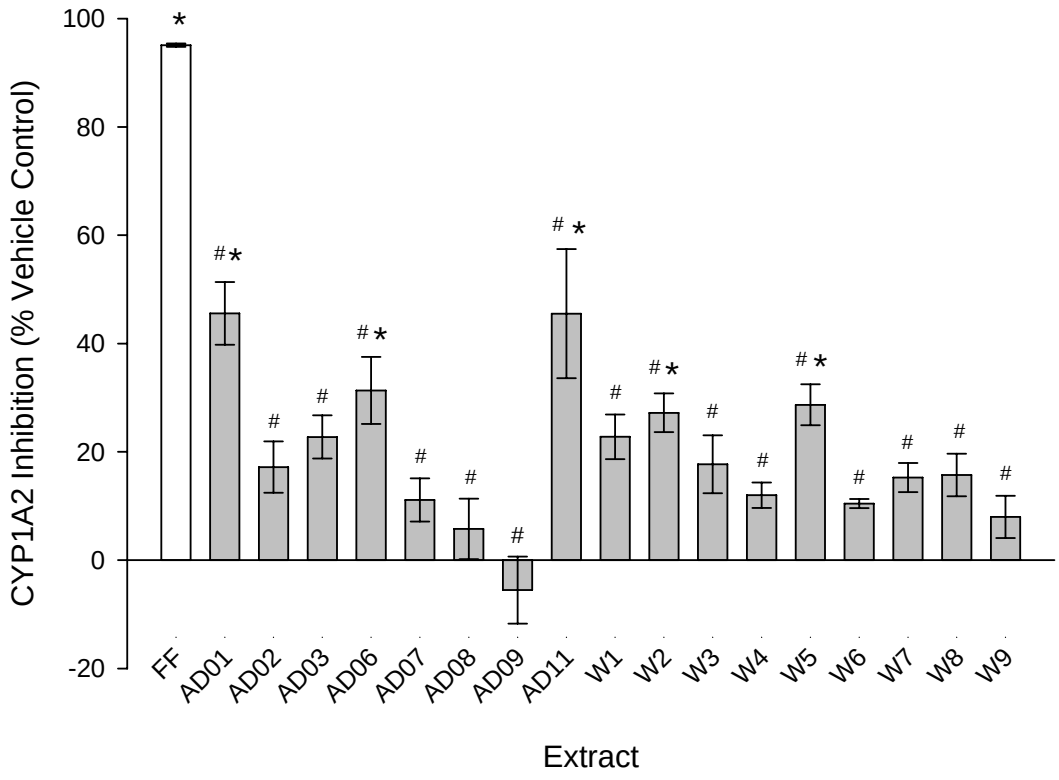


Figure 19: The inhibition of CYP1A2 by the 17 Cree plant extracts. The Cree plant extracts were tested for their potency to inhibit CYP1A2 by measuring the metabolism of the CYP1A2 substrate CEC in a microtitre fluorometric assay. A single concentration of 10 µg/mL was tested for each extract. Furafylline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

inhibitory potencies. The mean inhibition of CYP1A2 for the 17 extracts was 19.50% ± 3.24% **(Table 3)**. Furafylline at a concentration of 50 μM was used as a positive control and inhibited CYP1A2 at 95.06% ± 0.31%. None of the extracts had comparable inhibitory potencies to furafylline. Statistical analyses using ANOVA were performed by comparing the inhibitory values of the extracts to the inhibitory value of the positive control (furafylline), and to the inhibitory value of the negative control (100% MeOH) which was set at 0% inhibition. All of the extracts were significantly different than furafylline ($p \leq 0.05$). The extracts AD01, AD06, AD11, W2, and W5 were significantly different than 100% MeOH ($p \leq 0.05$).

CYP2B6

CYP2B6 had the least overall inhibition by the 17 extracts for all of the isoforms studied **(Figure 20A)**. All of the extracts were low potent inhibitors of CYP2B6. The mean inhibition of CYP2B6 for the 17 extracts was 7.66% ± 1.62% **(Table 3)**. Tranylcypromine at a concentration of 1 mM was used as a positive control and inhibited CYP2B6 at 69.00% ± 0.65%. None of the extracts had comparable inhibitory potencies to tranylcypromine. Statistical analyses using ANOVA were performed by comparing the inhibitory values of the extracts to the inhibitory value of the positive control (tranylcypromine) and to the inhibitory value of the negative control (100% MeOH) which was set at 0% inhibition. All of the extracts were significantly different than tranylcypromine ($p \leq 0.05$). the extracts AD01, AD02, AD11, W1, and W4 were significantly different than 100% MeOH ($p \leq 0.05$).

CYP	Mean Inhibition (% ± SEM)	Top High Inhibitors (% Inhibition ± SEM)	Top Low Inhibitors (% Inhibition ± SEM)
1A2	19.50 ± 3.24	AD01 (45.58 ± 5.80) AD11 (45.52 ± 9.55) AD06 (31.32 ± 6.18)	AD09 (-5.53 ± 6.19) AD08 (5.76 ± 5.58) W9 (7.98 ± 3.90)
2B6	7.66 ± 1.62	W4 (20.41 ± 2.25) AD01 (18.76 ± 2.62) W1 (14.32 ± 0.56)	W8 (-1.77 ± 2.05) W9 (-1.06 ± 1.98) W7 (-0.08 ± 0.29)
2C8	49.03 ± 5.81	AD07 (83.54 ± 3.26) W6 (77.09 ± 3.97) W1 (67.43 ± 11.26)	W9 (-16.30 ± 5.28) W8 (23.73 ± 10.14) AD09 (25.66 ± 6.95)
2C9	53.32 ± 6.26	W6 (97.09 ± 0.86) AD07 (86.92 ± 1.68) W4 (83.13 ± 2.24)	W9 (14.17 ± 1.53) W5 (21.92 ± 5.15) W8 (25.26 ± 4.07)
2C19	68.40 ± 6.21	W6 (97.36 ± 0.54) W4 (94.36 ± 1.23) AD03 (94.06 ± 4.05)	W9 (14.03 ± 5.57) W8 (23.72 ± 0.95) W7 (39.31 ± 1.40)
2D6	12.34 ± 3.17	AD01 (46.54 ± 8.28) AD09 (23.21 ± 8.74) W2 (22.21 ± 0.73)	W1 (-11.89 ± 12.07) W9 (-2.44 ± 11.08) W7 (-0.53 ± 9.99)
2E1	18.09 ± 2.59	W2 (35.32 ± 2.09) W8 (34.60 ± 4.31) AD01 (31.10 ± 4.72)	AD02 (-2.06 ± 2.39) AD08 (1.04 ± 2.09) W7 (9.66 ± 1.97)
3A4	58.25 ± 7.99	AD11 (98.17 ± 0.28) AD06 (96.67 ± 0.45) W5 (92.28 ± 1.23)	W7 (-5.20 ± 5.91) W9 (0.56 ± 1.49) AD09 (17.78 ± 0.98)
3A5	63.12 ± 6.11	AD07 (90.57 ± 0.60) AD01 (88.01 ± 2.52) W2 (84.81 ± 3.00)	W9 (11.03 ± 2.65) W7 (12.49 ± 1.23) AD09 (34.61 ± 2.33)
3A7	66.38 ± 5.54	W4 (96.87 ± 7.09) AD02 (88.73 ± 0.97) AD07 (86.87 ± 1.90)	W9 (12.02 ± 3.09) W7 (34.08 ± 3.46) AD09 (35.36 ± 1.74)

Table 3. The mean inhibition values of the 17 extracts for each CYP isoform, with the highest and lowest three inhibitors for each CYP isoform (n = 3).

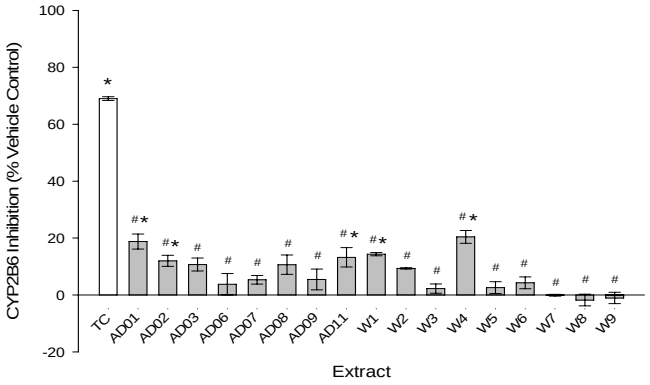
CYP2D6

CYP2D6 had the second least overall inhibition by the 17 extracts for all of the isoforms studied **(Figure 20B)**. All of the extracts were low potent inhibitors except for AD01 which inhibited CYP2D6 with moderate potency. The mean inhibition of CYPD6 for the 17 extracts was 12.34% ± 3.17% **(Table 3)**. Quindine at a concentration of 2 μM was used as a positive control and inhibited CYP2D6 at 93.74% ± 2.89%. None of the extracts had comparable inhibitory potencies to quinidine. Statistical analyses using ANOVA were performed by comparing the inhibitory values of the extracts to the inhibitory value of the positive control (quindine) and to the inhibitory value of the negative control (100% MeOH) which was set at 0% inhibition. All of the extracts were significantly different than quinidine ($p \leq 0.05$). The extract AD01 was the only extract significantly different than 100% MeOH ($p \leq 0.05$).

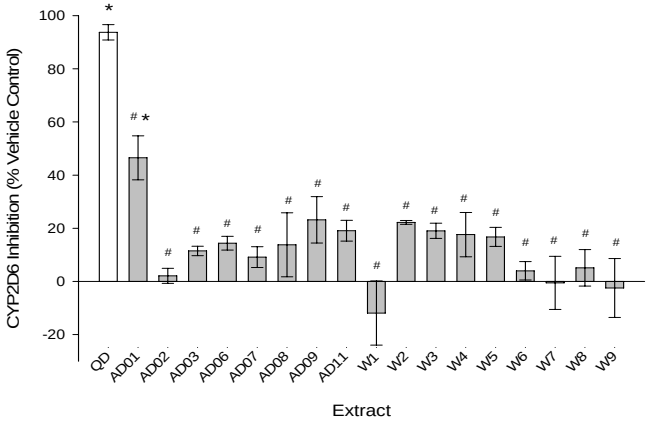
CYP2E1

Overall, the majority of the extracts had low inhibitory potencies against CYP2E1 activity **(Figure 20C)**. Only four extracts (AD01, AD03, W2, and W8) had moderate inhibitory potencies. The mean inhibition of CYP2E1 for the 17 extracts was 18.09% ± 2.59% **(Table 3)**. Diethyldithiocarbamate at a concentration of 100 μM was used as a positive control and inhibited CYP2E1 at 82.02% ± 0.88%. None of the extracts had comparable inhibitory potencies to diethyldithiocarbamate. Statistical analyses using ANOVA were performed by comparing the inhibitory values of the extracts to the inhibitory value of the positive control (diethyldithiocarbamate) and to the inhibitory value of the negative control (100% MeOH) which was set at 0% inhibition. All of the extracts were significantly different than diethyldithiocarbamate ($p \leq 0.05$). The extracts

(A) CYP2B6



(B) CYP2D6



(C) CYP2E1

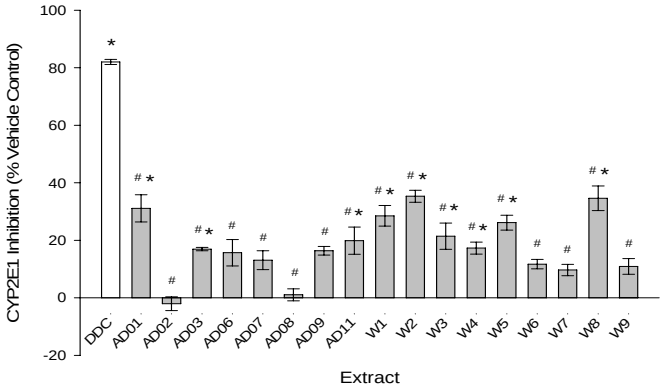


Figure 20: The inhibition of CYP2B6, 2D6, and 2E1 by the 17 Cree plant extracts. The Cree plant extracts were tested for their potency to inhibit **(A)** CYP2B6, **(B)** CYP2D6, and **(C)** CYP2E1 by measuring the metabolism of the substrates MFC (2B6 and 2E1) or AMMC (2D6) in a microtitre fluorometric assay. A single concentration of 10 µg/mL was tested for each extract. The positive controls tranylcypromine (1 mM) (TC), quinidine (2 µM) (QD), and diethyldithiocarbamate (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 ($n = 3$). *$p \leq 0.05$ 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.

AD01, AD03, AD11, W1, W2, W3, W4, W5, and W8 were significantly different than 100% MeOH ($p \leq 0.05$).

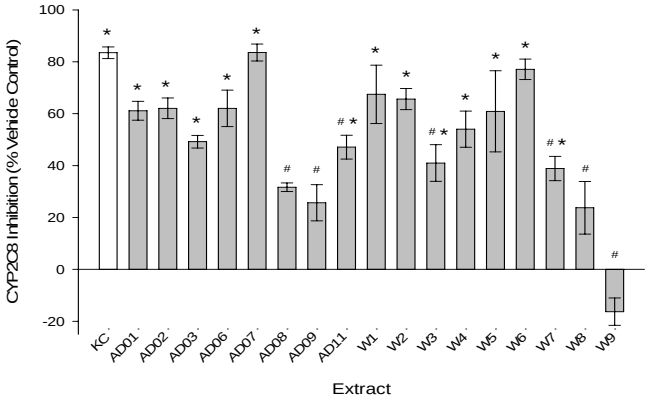
CYP2C8

There was a broad range of inhibition against CYP2C8 by the 17 extracts ranging from -16.30% ± 5.28% inhibition to 83.54% ± 3.26 % inhibition **(Figure 21A)**. The majority of the extracts (12/17: AD01, AD02, AD03, AD06, AD08, AD11, W1, W2, W3, W4, W5, and W7) were moderately potent inhibitors. Two extracts (AD07 and W6) were high potent inhibitors, whereas three extracts (AD09, W8 and W9) were low potent inhibitors. The mean inhibition of CYP2C8 for the 17 extracts was 49.03% ± 5.81% **(Table 3)**. Statistical analyses using ANOVA were performed by comparing the inhibitory values of the extracts to the inhibitory value of the positive control (ketoconazole) and to the inhibitory value of the negative control (100% MeOH) which was set at 0% inhibition. The extracts AD08, AD09, AD11, W3, W4, W7, W8 and W9 were significantly differentthan ketoconazole ($p \leq 0.05$). The extracts AD01, AD02, AD03, AD06, AD07, AD11,W1, W2, W3, W4, W5, W6, and W7 were significantly different than 100% MeOH ($p \leq 0.05$).

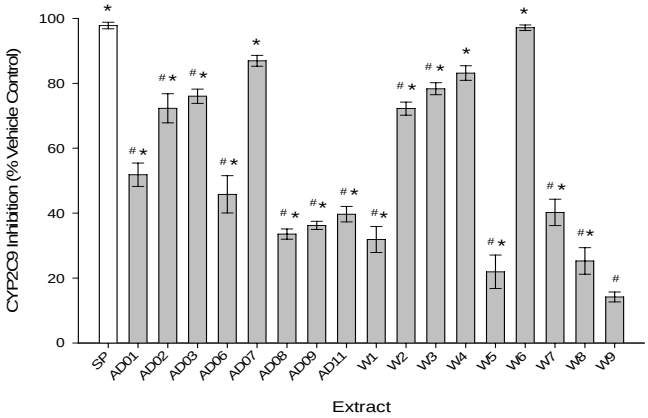
CYP2C9

There was also a broad range of inhibition against CYP2C9 from the 17 extracts ranging from 14.17% ± 1.5% to 97.09% ± 0.86% inhibition **(Figure 21B)**. More than half of the extracts (9/17: AD01, AD02, AD06, AD08, AD09, AD11, W1, W2, and W7) were moderately potent inhibitors. Five extracts (AD03, AD07, W3, W4, and W6) were high potent inhibitors, whereas three extracts (W5, W8 and W9) were low potent inhibitors. The mean inhibition of CYP2C9 for the 17 extracts was 53.32% ± 6.26% **(Table 3)**.

(A) CYP2C8



(B) CYP2C9



(C) CYP2C19

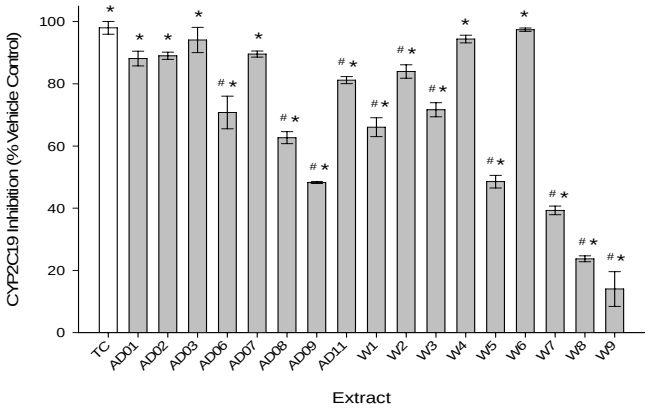


Figure 21: The inhibition of CYP2C8, 2C9, and 2C19 by the 17 Cree plant extracts. The Cree plant extracts were tested for their potency to inhibit **(A)** CYP2C8, **(B)** CYP2C9, and **(C)** CYP2C19 by measuring the metabolism of the substrates DBF, MFC and CEC respectively, in a microtitre fluorometric assay. A single concentration of 10 µg/mL was tested for each extract. The positive controls ketoconazole (10 µM) (KC), sulphaphenazole (100 µM) (SP), and tranylcypromine (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 ($n = 3$). *$p \leq 0.05$ 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.

Sulphaphenazole at a concentration of 100 μM was used as a positive control and inhibited CYP2C9 at 97.79% ± 1.01%. The extract W4 had comparable inhibitory potencies to sulphaphenazole (within 10% of the inhibitory value of sulphaphenazole).Statistical analyses using ANOVA were performed by comparing the inhibitory values of the extracts to the inhibitory value of the positive control (sulphaphenazole) and to the inhibitory value of the negative control (100% MeOH) which was set at 0% inhibition. The extracts AD01, AD02, AD03, AD06, AD08, AD09, AD11, W1, W2, W3, W5, W7, W8 and W9 were significantly different than sulphaphenazole ($p \leq 0.05$). All the extracts except for W9 were significantly different than 100% MeOH ($p \leq 0.05$).

CYP2C19

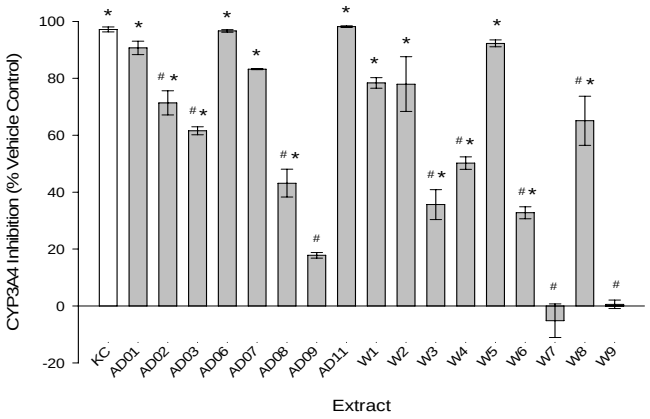
CYP2C19 was inhibited by the 17 extracts from a range of 14.03% ± 5.57% to 97.36% ± 0.54% inhibition **(Figure 21C)**. Almost half of the extracts (8/17: AD01, AD02, AD03, AD07, AD11, W2, W4, and W6) were high potent inhibitors. Seven extracts (AD06, AD08, AD09, W1, W3, W5, and W7) were moderate potent inhibitors, whereas two extracts (W8 and W9) were low potent inhibitors. The mean inhibition of CYP2C19 for the 17 extracts was 68.40% ± 6.21% **(Table 3)** which was the highest average inhibition among the CYP isoforms. Tranylcypromine at a concentration of 100 μM was used as a positive control and inhibited CYP2C19 at 97.98% ± 2.06%. Several of the extracts had comparable inhibitory potencies to tranylcypromine (within 10% of the inhibitory value of tranylcypromine): AD01, AD02, AD03, AD07, W4 and W6. Statistical analyses using ANOVA were performed by comparing the inhibitory values of the extracts to the inhibitory value of the positive control (tranylcypromine) and to the

inhibitory value of the negative control (100% MeOH) which was set at 0% inhibition. The extracts AD06, AD08, AD09, AD11, W1, W2, W3, W5, W7, W8 and W9 were significantly different than tranylcypromine ($p \leq 0.05$). All the extracts were significantly different than 100% MeOH ($p \leq 0.05$).

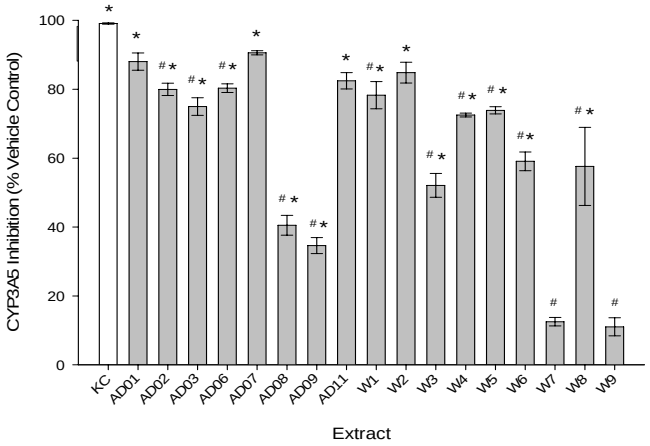
CYP3A4

There was also a broad range of inhibition against CYP3A4 from the 17 extracts ranging from -5.20% ± 5.91% to 98.17% ± 0.28% inhibition **(Figure 22A)**. Seven of the extracts were high potent inhibitors: AD01, AD06, AD07, AD11, W1, W2 and W5; seven of the extracts were moderate potent inhibitors: AD02, AD03, AD08, W3, W4, W6 and W8; and three of the extracts were low potent inhibitors: AD09, W7, and W9. The mean inhibition of CYP3A4 for the 17 extracts was 58.25% ± 7.99% **(Table 3).** Ketoconazole at a concentration of 1.9 μM was used as a positive control and inhibited CYP3A4 at 97.18% ± 0.86%. Several of the extracts had comparable inhibitory potencies to ketoconazole (within 10% of the inhibitory value of ketoconazole): AD01, AD06, AD11, and W5. Statistical analyses using ANOVA were performed by comparing the inhibitory values of the extracts to the inhibitory value of the positive control (ketoconazole) and to the inhibitory value of the negative control (100% MeOH) which was set at 0% inhibition. The extracts AD02, AD03, AD08, AD09, W3, W4, W6, W7, W8 and W9 were significantly different than ketoconazole ($p \leq 0.05$). All the extracts except for AD09, W7 and W9 were significantly different than 100% MeOH ($p \leq 0.05$).

(A) CYP3A4



(B) CYP3A5



(C) CYP3A7

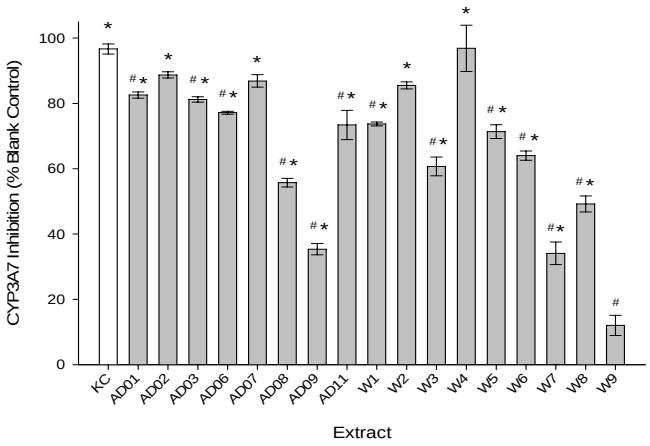


Figure 22: The inhibition of CYP3A4, 3A5, and 3A7 by the 17 Cree plant extracts. The Cree plant extracts were tested for their potency to inhibit **(A)** CYP3A4, **(B)** CYP3A5, and **(C)** CYP3A7 by measuring the metabolism of the substrate DBF in a microtitre fluorometric assay. A single concentration of 10 µg/mL was tested for each extract. The positive control ketoconazole (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 ($n = 3$). *$p \leq 0.05$ with respect to the MeOH vehicle control, using one-way ANOVA followed by the Tukey test. $^{\#}p \leq 0.05$ with respect to ketoconazole, using one-way ANOVA followed by the Tukey test.

CYP3A5

CYP3A5 was inhibited by the 17 extracts from a range of 11.03% ± 2.65% to 90.57% ± 0.60% inhibition **(Figure 22B)**. Almost half of the extracts (8/17: AD01, AD02, AD03, AD07, AD11, W2, W4, and W6) were high potent inhibitors. Seven extracts (AD08, AD09, W3, W4, W5, W6 and W8) were moderate potent inhibitors, whereas two extracts (W7 and W9) were low potent inhibitors. The mean inhibition of CYP3A5 for the 17 extracts was 63.12% ± 6.11% **(Table 3).** Ketoconazole at a concentration of 1.9 μM was used as a positive control and inhibited CYP3A5 at 99.06% ± 0.20%. The extract W7 had comparable inhibitory potencies to ketoconazole (within 10% of the inhibitory value of ketoconazole). Statistical analyses using ANOVA were performed by comparing the inhibitory values of the extracts to the inhibitory value of the positive control (ketoconazole) and to the inhibitory value of the negative control (100% MeOH) which was set at 0 % inhibition. The extracts AD02, AD03, AD06, AD08, AD09, W1, W3, W4, W5, W6, W7, W8 and W9 were significantly different than ketoconazole ($p \leq 0.05$). All the extracts except for W7 and W9 were significantly different than 100% MeOH ($p \leq 0.05$).

CYP3A7

CYP3A7 was inhibited by the 17 extracts from a range of 12.02% ± 3.09% to 96.87% ± 7.09% inhibition **(Figure 22C)**. Seven of the extracts were high potent inhibitors: AD01, AD02, AD03, AD06, AD07, W2 and W4; nine of the extracts were moderate potent inhibitors: AD08, AD09, AD11, W1, W3, W5, W6, W7 and W8; and one of the extracts was a low potent inhibitors: W9. The mean inhibition of CYP3A7 for the 17 extracts was 66.38% ± 5.54% **(Table 3)** which was the second highest average

inhibition among the CYP isoforms. Ketoconazole at a concentration of 1.9 μM was used as a positive control and inhibited CYP3A7 at 96.69% ± 1.55%. Two of the extracts had comparable inhibitory potencies to ketoconazole (within 10% of the inhibitory value of ketoconazole): AD02 and W4. Statistical analyses using ANOVA were performed by comparing the inhibitory values of the extracts to the inhibitory value of the positive control (ketoconazole) and to the inhibitory value of the negative control (100% MeOH) which was set at 0 % inhibition. The extracts AD01, AD03, AD06, AD08, AD09, AD11 W1, W3, W5, W6, W7, W8 and W9 were significantly different than ketoconazole ($p \leq 0.05$). All the extracts except for W9 were significantly different than 100% MeOH ($p \leq 0.05$).

3.5.2 The Inhibition Trends of the 10 Cytochrome P450 Isoforms

There was a broad range of overall mean inhibition of the different CYP isoforms by the 17 Cree plant extracts from 7.66% ± 1.62% to 68.40% ± 6.21% 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 top three high inhibitors of the different CYP isoforms such as AD01, W4, and W6. However, there were also several extracts that were one of the top three inhibitors for only one isoform: W1 – CYP2C8; AD03 – CYP2C19; W8 – CYP2E1; W5 – CYP3A4; and AD02 – CYP3A7. Similarly, there were several extracts that were common top three low inhibitors of the different CYP isoforms such as AD09, W7, W8, and W9, and extracts which were more potent to a particular CYP isoform: W5 – CYP2C9; W1 – CYP2D6; AD02 – CYP2E1.

Similar inhibition trends were observed among members of the same subfamily. For example, W6 and AD07 were common high potent inhibitors of CYP2C8, 2C9 and 2C19 which all belong to the CYP2C subfamily. W8 and W9 were common low potent inhibitors of CYP2C8, 2C9, and 2C19. Similar observations were observed with the CYP3A subfamily. Across the subfamilies CYP2C and 3A, there were differences in the inhibitory potency of the extracts. 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 inhibitions from the extracts were compared **(Figure 23)**. CYP2C9 and 3A4 were chosen because they contribute the most to the metabolism of drugs in their subfamilies, and they also had similar overall mean inhibitions from the extracts (53.32% ± 6.26% and 58.25% ± 7.99% mean inhibition respectively). 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 ($p \leq 0.05$). Six of the extracts were more potent towards CYP3A4: AD01, AD06, AD11, W1, W5, and W8. The other 4 extracts, W3, W4, W6, and W7, were more potent to CYP2C9.

The extracts had different mean inhibitory potencies of the 10 CYP isoforms ranging from 5.09% ± 3.09% to 60.43% ± 8.18% mean inhibition **(Table 4)**. The top five inhibitory extracts were AD01 > W2 > AD07 > AD11 > W4, and the least five inhibitory extracts were W9 < W7 < AD09 < W8 < AD08.

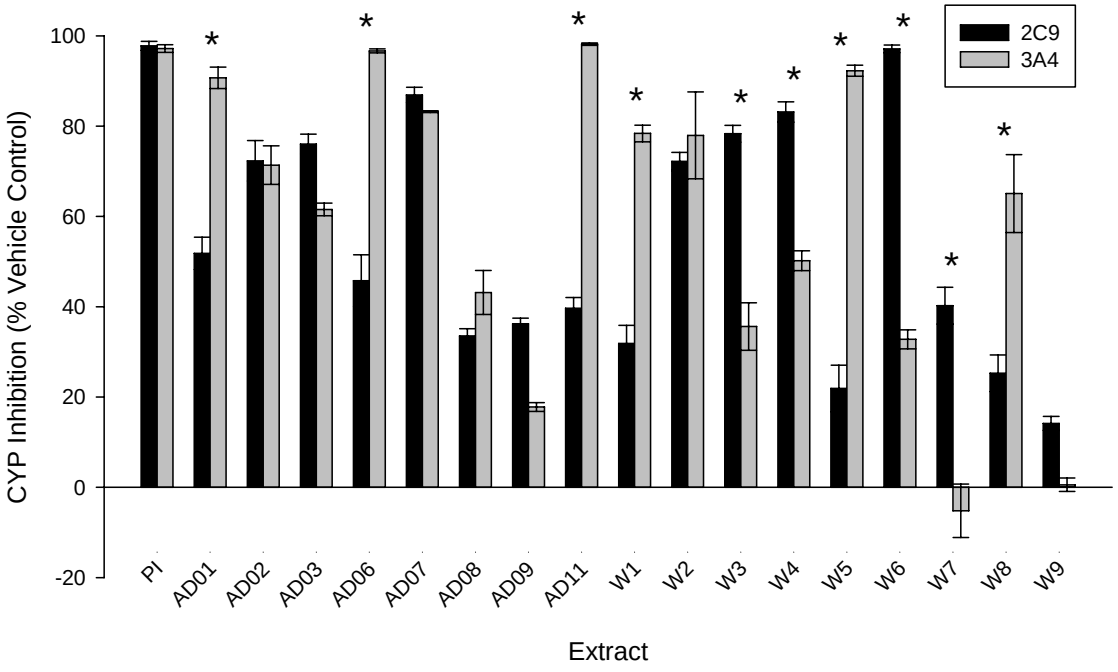


Figure 23: A comparison of the inhibition of CYP2C9 and 3A4 by the 17 Cree extracts. The inhibitory values of the 17 extracts for CYP2C9 and 3A4 were graphed together for a comparison analysis. The positive inhibitors (PI) used were sulphaphenazole (100 μM) and ketoconazole (1.9 μM) for CYP2C9 and 3A4 respectively. The results were expressed relative to a 100% MeOH vehicle control, as the mean inhibition ± SEM (n = 3). 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 (*).

Extract	Mean Inhibition (% ± SEM)	Ranking
AD01	60.43 ± 8.18	1
AD02	49.26 ± 11.80	8
AD03	49.89 ± 10.12	7
AD06	49.79 ± 10.20	6
AD07	55.93 ± 12.63	3
AD08	29.85 ± 6.74	13
AD09	23.74 ± 5.05	15
AD11	51.96 ± 9.55	4
W1	44.94 ± 10.06	10
W2	56.41 ± 9.36	2
W3	39.97 ± 8.01	12
W4	51.84 ± 10.64	5
W5	44.30 ± 9.29	11
W6	45.78 ± 11.94	9
W7	18.40 ± 5.73	16
W8	29.83 ± 6.92	14
W9	5.09 ± 3.09	17

Table 4. The mean inhibition values of the 10 CYP isoforms tested for each extract. The extracts were ranked according to their inhibitory capability ($n = 3$).

3.6 Mechanism-Based Inhibition of CYP3A4

3.6.1 Identifying 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 (2.3 μg/mL) and azamulin (0.1 μM). Ketoconazole (0.2 μM) was used as a negative control. A single concentration of 10 μg/mL of extract was tested for both the NADPH- and time-depedence assays.

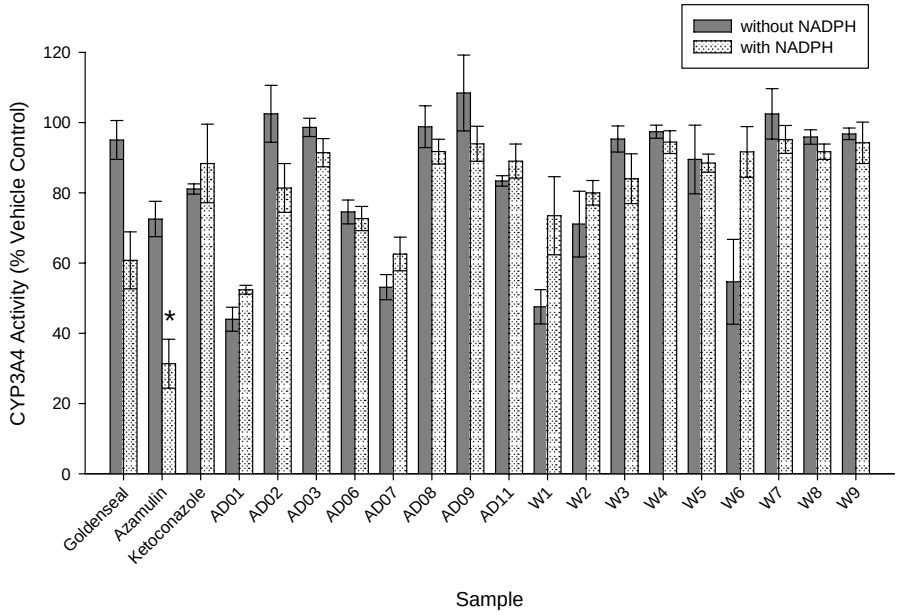
Mechanism-based inhibitors require NADPH to form covalent bonds with the enzyme which would inhibit CYP activity. In the absence of NADPH, the enzyme cannot be inhibited through MBI. CYP3A4 was pre-incubated with the extracts or the positive/negative control in the presence or absence of NADPH. Decreases in activity were observed with the samples pre-incubated with NADPH and GS (95.04% ± 5.55% for no NADPH vs. 60.78% ± 8.10% for with NADPH) or azamulin (72.54% ± 5.03% for no NADPH vs. 31.37% ± 6.96% for with NADPH) as expected **(Figure 24A)**. However, only a significant decrease was observed with azamulin ($p \leq 0.05$). There was no significant difference in activities for ketoconazole (81.10% ± 1.46% for no NADPH vs. 88.34% ± 11.16% for with NADPH) as expected. In the presence of the Cree plant extracts, there were no significant differences in CYP3A4 activity between the two pre-incubation conditions ($p > 0.05$) and hence, no mechanism-based inhibitors were detected among the extracts in the NADPH-dependence assay. Several of the extracts showed greater 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.

All of the other extracts had almost identical effects on CYP3A4 activity similar to the negative control ketoconazole.

Mechanism-based inhibitors cause greater inhibition of CYPs the longer they are in contact 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 short pre-incubation time. CYP3A4 was pre-incubated with the extracts or the positive/negative controls in the presence of NADPH for either 1 or 10 minutes. Decreases in activity were observed with the samples pre-incubated with NADPH for 10 minutes in GS (80.96% ± 4.64% with 1 minute pre-incubation vs. 60.78% ± 8.10% with 10 minutes pre-incubation) or azamulin (62.89% ± 11.27% with 1 minute pre-incubation vs. 31.37% ± 6.96% with 10 minutes pre-incubation) as expected, but they were not significant **(Figure 24B)** ($p > 0.05$). There was no significant difference in activities for ketoconazole (84.00% ± 9.32% with 1 minute pre-incubation vs. 88.34% ± 11.19% with 10 minutes pre-incubation) as expected. In the presence of the Cree plant extracts, there were no significant differences in CYP3A4 activity between the two pre-incubation times and hence, no mechanism-based inhibitors were once detected among the Cree plant extracts in the time-dependent assay. The extract AD02 showed a greater difference in their ability to effect CYP3A4 activity between the two conditions (at least a 15% difference) but was not significant ($p > 0.05$).

Overall, none of the 17 Cree plants extracts were significant mechanism-based inhibitors of CYP3A4.

(A) NADPH-dependence



(B) Time-dependence

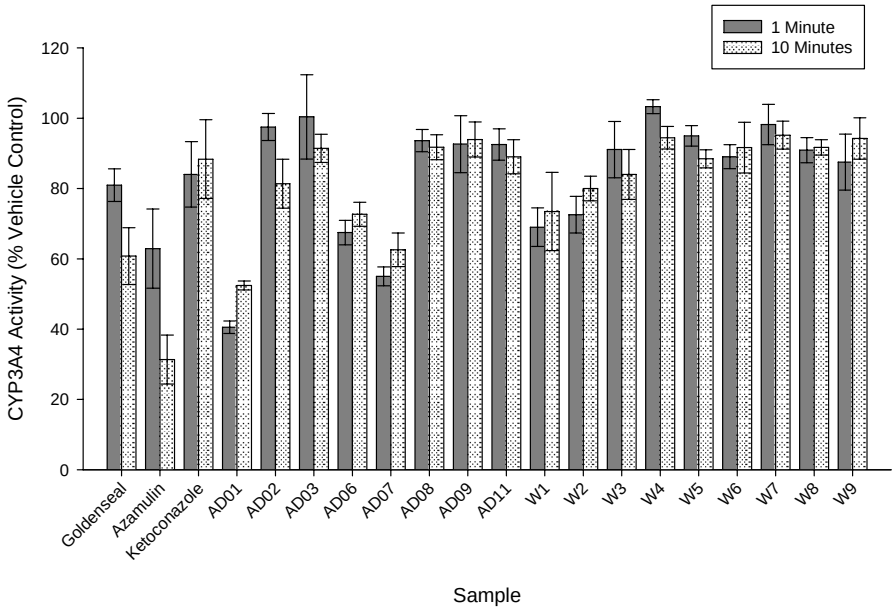


Figure 24: Identifying mechanism-based inhibitors of CYP3A4 of the 17 Cree plant extracts. The activity of CYP3A4 after a pre-incubation with each extract **(**10 µg/mL) **(A)** in the presence or absence of NADPH for 10 minutes, and **(B)** in the presence of NADPH for 1 or 10 minute(s). The results were expressed relative to a 100% MeOH vehicle control, as the mean CYP3A4 activity ± SEM (n = 3). Goldenseal (2.3 µg/mL) and azamulin (0.1 µM) were used as positive controls. Ketoconazole (0.2 µM) was used as a negative control. 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 (*).

3.6.2 Confirmation of Mechanism-Based Inhibitors

A possible reason for why none of the 17 Cree plant extracts were detected as mechanism-based inhibitors was because the concentration tested (10 µg/mL) was too low for inhibition to occur. In **Figures 24A and 25B**, many of the extracts had low inhibition of CYP3A4 since the CYP3A4 retained relatively close to 100% activity. Further MBI tests were performed with three of the extracts that showed signs of mechanism-based inhibition in **Figures 24A and/or 25B**, using a higher concentration of 100 µg/mL. The extracts chosen were AD02, AD09, and W3. These additional tests also included another pre-incubation time of 5 minutes, and two additional concentrations of the controls were tested: GS at concentrations of 3.7 and 9.7 µg/mL, azamulin at concentrations of 1 and 10 µM, and ketoconazole at concentrations of 0.9 and 1.9 µM. Significant differences in CYP3A4 activity were not observed with 100 µg/mL of AD02, AD09, and W3 in both the NADPH- and time-dependence assays **(Figures 25)**. Decreases in activity were observed with all concentrations of GS and azamulin with NADPH during pre-incubation, and with a 5 or 10 minute pre-incubation time, but the decreases in activity were only significant with 2.3 µg/mL GS (95.04% ± 3.26% for no NADPH vs. 52.50% ± 8.10% for with NADPH) and 0.1 µM azamulin (72.54% ± 5.03% for no NADPH vs. 31.37% ± 6.96% for with NADPH) with the NADPH- dependence assay ($p \leq 0.05$). There were no differences observed with ketoconazole as expected in both the NADPH- and time-dependence assays. Interestingly, at both concentrations tested for AD02, there were decreases in CYP3A4 activity with NADPH in the pre-incubation solution (10 µg/mL - 102.50% ± 8.09% for no NADPH vs. 81.37% ± 6.95% for with NADPH; 100 µg/mL – 43.98% ± 6.56% for no NADPH vs. 31.46% ±

3.31% for with NADPH). In addition, general decreases in CYP3A4 activity were observed with increasing pre-incubation times at both concentrations indicating that AD02 may contain compounds which are mechanism-based inhibitors.

3.8 Drug Interaction Study with Enalapril

3.8.1 Identifying the Median Inhibitory Concentrations

The next study was performed to determine if the extracts (used as complementary medicine) and the hypertensive drug enalapril interacted with each other to affect CYP3A4 activity. The experiments were performed by comparing the activity of CYP3A4 in the presence of the extract, to the activity of CYP3A4 in the presence of the extract and enalapril. A single concentration of 0.3 μM was tested. The extracts chosen were AD01, AD08, AD09, AD11, W1, W3, and W5, because they showed the greatest anti-diabetic potential in previous studies from collaborators (Spoor *et al*., 2006; Harbilas *et al*., in press). The median inhibitory concentration (IC_{50}) was chosen to be tested so that changes in CYP3A4 activity could be better detected when comparing the extract and the extract with enalapril. The IC_{50} values were determined by testing a range of concentrations in the CYP3A4 assay to obtain sigmoidal curves by graphing the log concentrations as a function of CYP3A4 inhibition. The data points of the linear portion of the curves were then plotted to obtain linear regression lines with 95% confidence intervals **(Figures 26)**. The IC_{50} values were 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 the extracts 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.

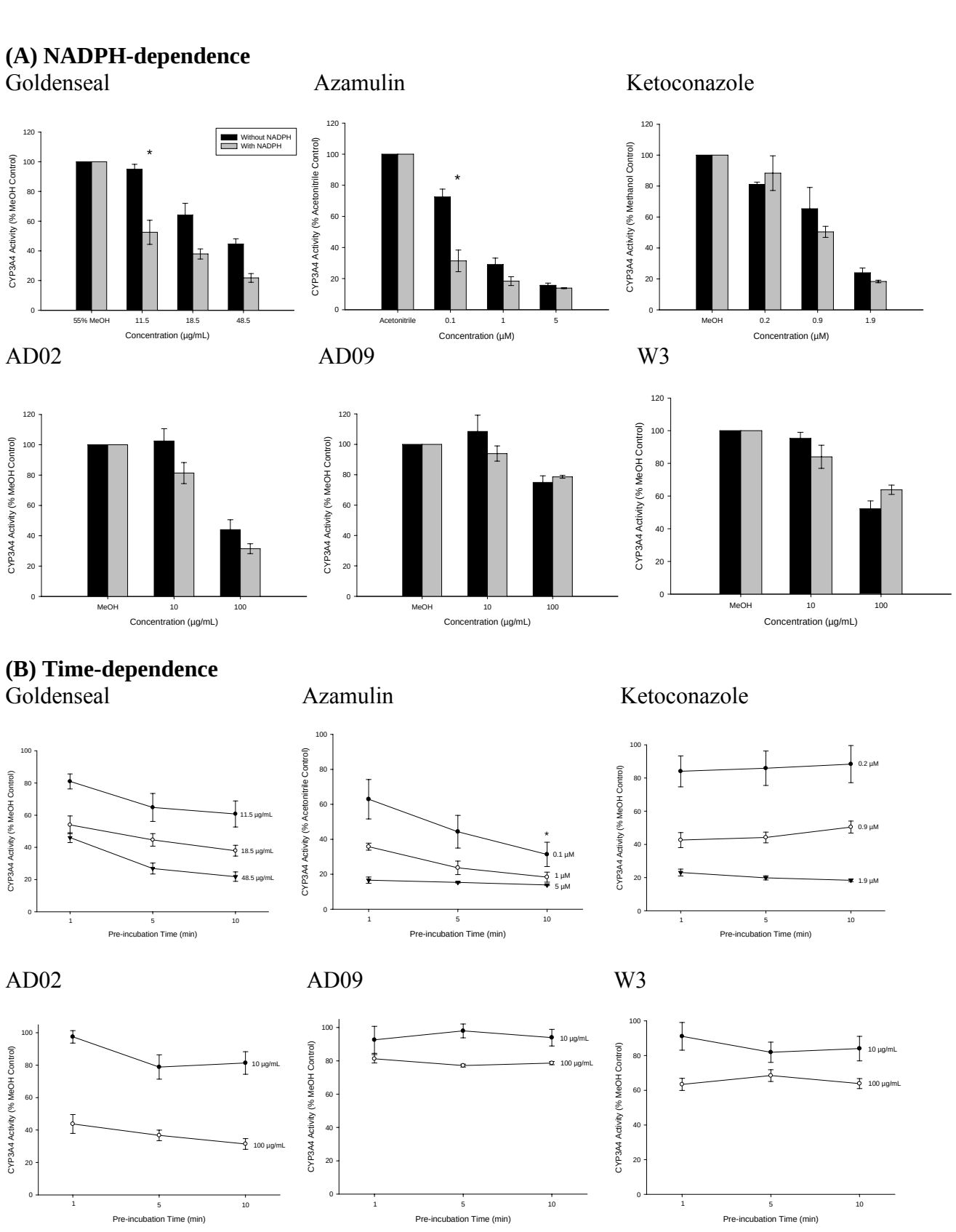


Figure 25: Identifying mechanism-based inhibition of CYP3A4 of the extracts AD02, AD09, and W3 using a higher concentration. The activity of CYP3A4 after a pre-incubation with different concentrations (10 and 100 µg/mL) of AD02, AD09 and W3 **(A)** for 10 minutes in the presence or absence of NADPH, 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 (n = 3). 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 (*).

3.8.2 Identifying Changes of CYP3A4 Activity with Enalapril

Only the extracts AD01, AD08, and AD09 were chosen for the drug interaction studies with enalapril 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. As mentioned previously, the activity of CYP3A4 in the presence of an extract was compared to the activity of CYP3A4 in the presence of the same extract and 0.3 µM enalapril after 30 minutes of treatment. Three different versions of the basic CYP3A4 assay were used because it was unsure what would be the best method to test for interactions *in vitro*. The first assay was identical to the original CYP3A4 assay, but the extract and enalapril were added together (whereas in the original assay only the extract was added) **(Figure 27A)**. In the second assay, the extract and enalapril were incubated together for 15 minutes at 37°C in the absence of the experimental reagents before the addition of Solution A, B, and C **(Figure 27B)**. In the third assay, the extract and enalapril were incubated together for 15 minutes at 37°C with buffer and NADPH (Solution A without the DBF) **(Figure 27C)**. The substrate was instead added to Solution B and C. 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 ($p > 0.05$), and hence did not inhibit CYP3A4 activity. In all three assays, there was no significant difference in CYP3A4 activity when comparing the extract and the extract with enalapril indicating that there were no interactions between the extract and enalapril ($p > 0.05$). 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 assay,

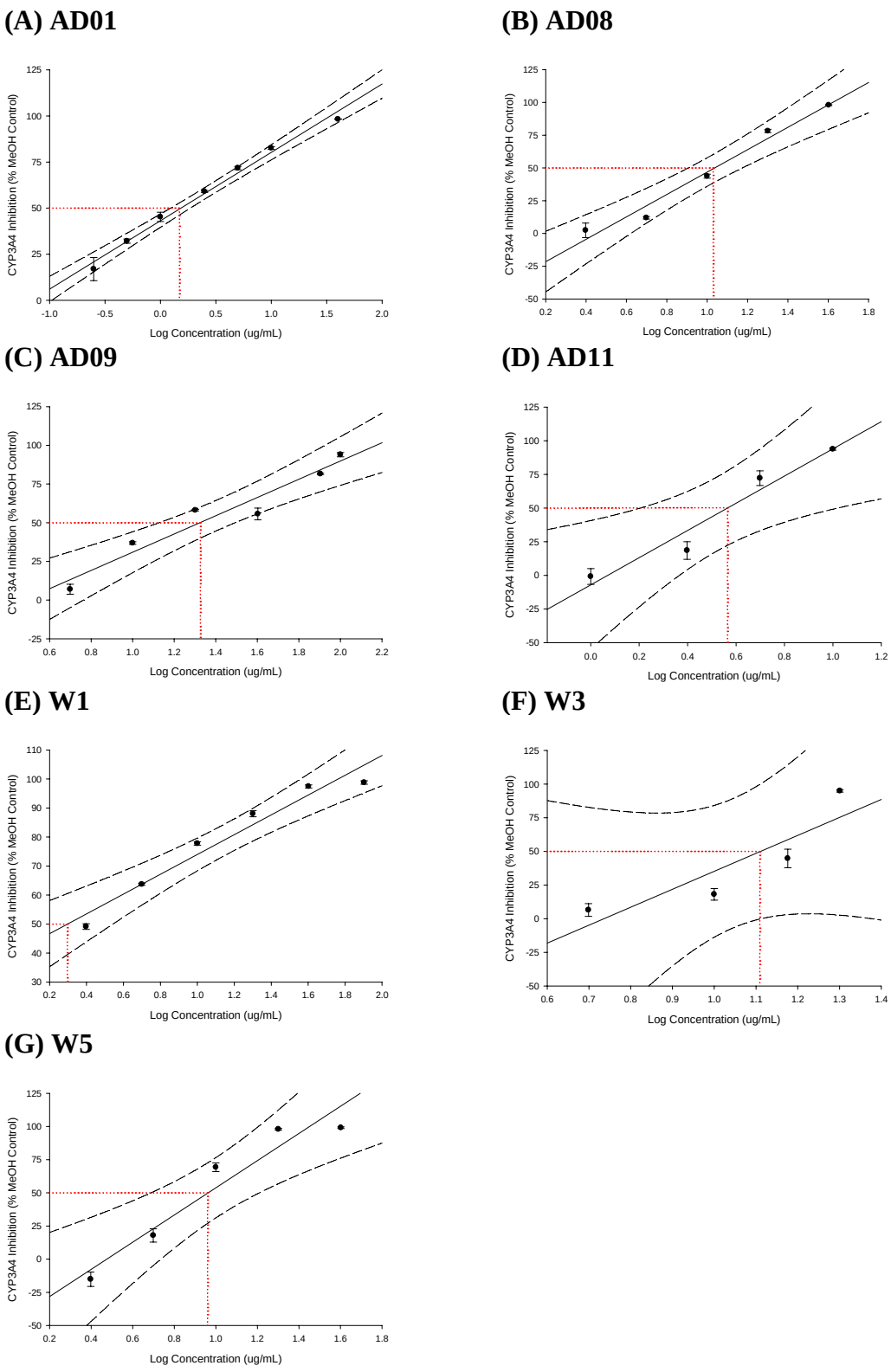


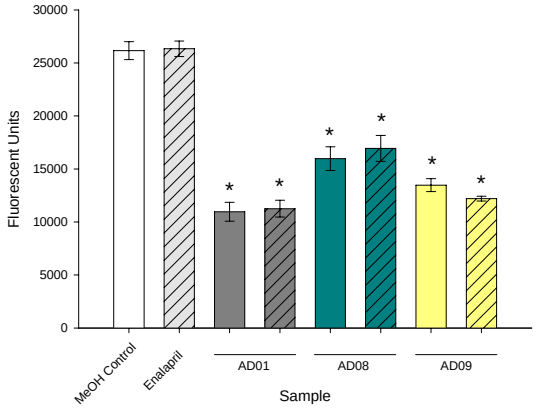
Figure 26: Linear regression lines with the 95% confidence intervals used in determining the median inhibitory concentration of seven of the Cree plant extracts. Various concentrations (0.25 to 160 µg/mL) of the extracts (A) AD01, (B) AD08, (C) AD09, (D) AD11, (E) W1, (F) W3, and (G) W5 were tested for 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 (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

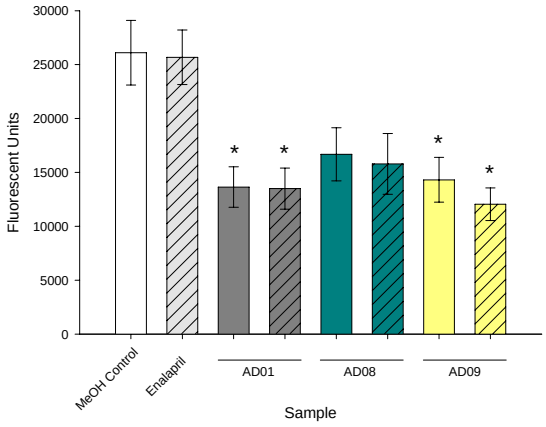
[a] Values in brackets correspond to the lower and upper 95% confidence limits respectively. Note that the confidence limit values could not be obtained with W3

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).

(A) No incubation



(B) 15 minutes of incubation



(C) 15 minutes of incubation with buffer and NADPH

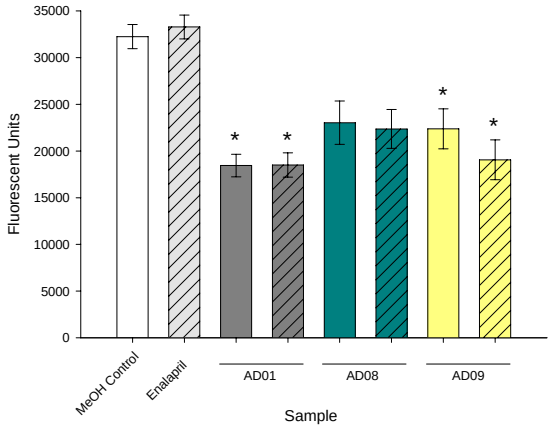


Figure 27: Identifying the effect of enalapril on the inhibitory effects of the Cree plant extracts AD01, AD08, and AD09 against CYP3A4. Enalapril (0.3 µM) was tested for its interaction with the extracts AD01, AD08, and AD09 to determine if the drug affected the inhibitory potencies of the extracts 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 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 ($n = 3$). *$p \leq 0.05$ 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)

there were significant decreases 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 ($p \leq 0.05$). In the third assay, there were significant decreases 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 ($p \leq 0.05$).

4 DISCUSSION

4.1. Identifying Cardiac Chronotropic Effects of the Cree Plant Extracts

The first study investigated the effects the Cree plants would have on the contraction rate of neonatal rat cardiomyocytes *in vitro*, which would represent the clinical heart rate in humans. The ethanolic extracts of the Cree plants were tested.

4.1.1. Screening for Cardiac Chronotropic Effects of Chronotropes

The expected acute chronotropic effects of carbachol, isoproterenol, and BO were observed after one minute of treatment. A significant negative chronotropic effect was observed with 400 μM carbachol which is much higher than the concentrations used to elicit a negative chronotropic affect in other studies using neonatal rat cardiomyocytes (1-100 μM) (Kohashi *et al*., 2003). Similarly, the significant concentration of isoproternol which had a positive chonotropic effect (greater than 5 μM) were much higher than the concentrations used to elicit a positive chronotropic effect in other studies using neonatal rat cardiomyocytes (10^{-10} to 10^{-11} M) (Simpson & Savion, 1981). 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 chronotopes (Simpson & Savion, 1981). The wash-in time of the chronotrope of one minute may not have been long enough 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., 2004; Simpson & Savion, 1981; Kohasi et al., 2003). A short wash-in time was desired for the

experiments to reduce the time 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 an NHP that possesses sympathomimetic activity. BO is closely related to ephedra and contains synephrine alkaloids which act as agonists on α- and ß-adrenergic receptors (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 that I am aware of. 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 metabolism are unknown. However, based on the dose-dependent result obtained, multiple dosing or chronic use of BO can result in a positive chronotropic effects.

4.1.2. Determination of the Highest Non-toxic Concentration

Only four extracts were chosen to be tested for chronotropic effects due to time limitations. The extracts chosen were W2 (*Kalmia angustifolia*), W4 (*Juniperus communis*), W5 (*Salix planifolia*), and W9 (*Vaccinium vitis-ideae*). These extracts were chosen because of their greater association to affect cardiac function, or to interact with anti-hyperglycaemic pharmaceuticals. In W2, a diterpenoid called kalmanol was found to have cardiotoxic properties (Burke *et al*., 1989). W4 was chosen because oil from a related species was associated with causing tachycardia (Koruk *et al*., 2005). W5 was chosen because it can be found in natural weight loss supplements containing ephedra and it was important to confirm that cardiotoxic effects of the supplements were from

ephedra rather than W5 (Coffey *et al*., 2004). Lastly, W9 was chosen because W9 and its related species have hypoglycaemic properties and may exacerbate the effects of anti-hyperglycaemic pharmaceuticals (Cignarella *et al*., 1996).

The highest non-toxic concentrations of each extract were tested for chronotropic effects. This concentration was chosen because it would represent an overdose of the extract which may occur from chronic use, an overdose, or the inhibition of CYPs involved in its metabolism. 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.

The toxicity of the DMSO vehicle control and the extracts was assessed by measuring the amount of LDH released from treated 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 non-toxic concentration was not obtained even at 600 µg/mL. Since it is physiologically impossible for extract levels to reach that concentration in the plasma, further studies to determine the toxic concentrations of W4 were not pursued. 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). The plasma levels of the Cree plants after a typical dose are currently unknown due to the present lack of clinical studies with these plants. W9 was the most potent in terms of toxicity to the cardiomyocytes and its toxic

concentrations (>10 µg/mL) can possibly be obtained 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 obtained in the plasma but would be extremely rare. However, this would greatly depend on the quantity and frequency of W5 administration, which is also 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 a known cardiotoxin is found 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 at all in the extract. For example, extracts can be produced using water, ethanol, methanol, hexane, or ethyl acetate, which can extract different phytochemicals based on their polarity. Most extracts are performed with ethanol or methanol because many of the active phytochemicals are extracted in these solvents. In addition, growing conditions such as the season of collection, the altitude, and location can affect the phytochemical prolifes of plants from the same species (Adams, 1987; Williamson, 2006).

4.1.3 Screening for Cardiac Chronotropic Effects of the Cree Plant Extracts

The extracts were solubilized in DMSO and the final DMSO concentration during the screening tests were 1 µL/mL for the majority of the extracts except for the extract W4 which had a DMSO concentration of 5 µL/mL because of the high concentration tested for this extract. A significant acute negative chronotropic effect was observed with the vehicle control 1 µL DMSO, 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 negative chronotropic effect, and it may be possible that with an increase in

the number of cultures tested (n greater than 5) a significant affect would not be observed. 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). However, after an 18 hour treatment, 5 µL/mL DMSO did significantly have a negative chronotropic effect. An 18 hour treatment is a sufficient time to allow DMSO to interact with cellular signaling pathways or cellular processes within the cardiomyocytes, which may account for the negative chronotropic effect observed. Since the chronotropic effect of an extracts were 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 did not have any acute significant chronotropic effects relative to its DMSO vehicle control. W2 had a minor positive chronotropic effect, whereas the other three extracts had minor negative chronotropic effects. Oil from related species to W4 has been associated with a positive chronotropic effects which was the opposite effect observed in this study. It is possible that different constituents are present in the oil which are absent in the extract, or that the active constituents differ among the species.

The extract W9 was chosen for further analysis for numerous reasons: it had the greatest negative chronotropic effect; and its highest non-toxic concentration can reasonably be within plasma levels. In addition, if W9 had a dose-dependent negative chronotropic effect, W9 would be an ideal plant to treat T2D if used as alternative medicine. Recent results from two separate follow-up studies in Switzerland and Germany, have shown that a high resting heart rate in Type 2 diabetics is associated with

in an increase in mortality (Stettler *et al*., 2007; Linnemann & Janka, 2007). If W9 can decrease the heart rate, this extract can be beneficial for diabetics because it also has hypoglycaemic properties. However, if used concomitantly with anti-hyperglycaemic pharmaceuticals, a drastic drop in glucose levels may ensue and can be harmful to the patient.

Two toxic concentrations of W9 were tested for acute chronotropic effects: 25 and 100 µg/mL. Both of these concentrations did not significantly affect the contraction rate and therefore it is very unlikely that W9 would have an impact on the heart rate even at high doses.

The four extracts did not have a significant chronic chronotropic effect**.** However, these results are not conclusive. The contraction rate of a single culture changed on a daily basis **(Figure A1)** which increases the difficulty of observing chronic chronotropic effects. In addition, a low number of samples were tested for this study (n of 3).

Overall, the results indicate that the extracts W2, W4, W5 and W9 do not have an affect on cardiac contraction rate at their highest non-toxic concentrations after acute or chronic use. 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 blood plasma and should not be used frequently in high doses. W2 and W4 would have any toxic affects on cardiomyocytes, but W5 may have a toxic effect in very rare cases of extreme overdose. 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 complementary medicine and used concomitantly with drugs used to treat diabetes. Interactions may occur between the drug and the plant and affect the heart rate. One of the most commonly used pharmaceuticals used by diabetics is metformin (Glucophage), an anti-hyperglycaemic medication. Both metformin and the extract W9 were tested together on cardiomyocytes to determine if the combination of the two had an affect on the contraction rate. A single concentration of 50 μM metformin was chosen for numerous reasons. Firstly, the typical blood concentration of metformin can reach as high as 40 μM (Sum *et al*., 1992). In addition, metformin does not undergo metabolism by CYPs because of its hydrophilic nature and typically is excreted unchanged from the kidney (Kirpichnikov *et al*., 2002). Also, approximately half of the CEI diabetics suffer from renal dysfunction and hence, have a reduced capability to excrete drugs such as metformin from the body (Kuzmina & Dannenbaum, 2004). This can result in higher levels of metformin in the blood up to 80 μM (Kazory *et al*., 2007). Lastly, a concentration of 50 μM was found to be non-toxic in adult rat cardiomyocytes, but higher concentrations were found to be toxic (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 affect on the contraction rate of the cardiomyocytes as expected. 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 affect. 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, and this concentration of W9 along with 50 µM metformin may have been too strenuous 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 at a later time such 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 blood plasma, but not 100 µg/mL of W9. Therefore, the combination of metformin and W9 should not have an affect on the heart rate of diabetics in the CEI. In addition, since W9 did not have a chronotropic effect individually, the results signify that any extract at an extremely high dose with 50 µM metformin can have a toxic effect on cardiomyocytes and affect their contraction rate. In addition, the 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 effects of the extracts. 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, 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 The Production of Diabetic-Like Cardiomyocytes

4.3.1. Confirming Their Presence and Viability

Diabetic-like cardiomyocytes were used as a model of diabetic cardiomyocytes. These cardiomyocytes exhibit the typical properties of diabetic cardiomyocytes including a prolonged AP duration and slowed calcium clearing (Ren *et al*., 1997; Shimoni *et al*., 1994). Overall, the method used to produce diabetic-like cardiomyocytes was not successful. There was not a great prolongation of the FP duration in the cardiomyocytes cultured in high glucose media compared to the cardiomyocytes cultured in the normal glucose media. However, there was not a great difference in glucose concentration between the normal (17.5 mM) and high (25.5 mM) glucose media which may account for the small increase in FP duration observed with the cardiomyocytes cultured in high glucose media. In addition, a patient is diagnosed with diabetes when his or her 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 many already have occurred with the normal glucose media.

In Ren *et al.'s* studies, their low glucose media had a glucose concentration of 5 mM. Interestingly, the cardiomyocytes cultured in the 5 mM glucose media had longer FP durations than the cardiomyocytes cultured in the normal glucose media. Even though the constituents between the media were identical expect for the glucose concentration, it appeared that the low glucose media was more toxic to the cardiomyocytes for unknown reasons because the viability of these cardiomyocytes was much lower. 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 diabetic-like cardiomyocytes did not appear to be produced using a similar method that Ren *et al*., used to produce adult diabetic-like cardiomyocytes, 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

It was observed that the FP durations were increasing the longer the cardiomyocytes were kept in culture which is inconstant with results previously presented in other studies. The AP durations of fetal or neonatal cardiomyocytes are extremely long compared to the AP durations of adult cardiomyocytes (Kilborn & Fedida, 1990; Guo *et al*., 1996). By 7 DIV, the AP duration of cultured neonatal rat cardiomyocytes can be extremely short like the adult AP ranging from 100 to 200 msecs (Meiry *et al*., 2001; Kilborn & Fedida, 1990). In a similar study using cultured neonatal rat cardiomyocytes and MEAs performed by Meiry *et al*., the FP durations of the cardiomyocytes progressively decreased and ranged from 200 to 300 msecs (Meiry *et al*., 2001) during 3 to 8 DIV. In patch-clamp studies using the same cells, the AP durations ranged from 300 to 600 msecs (Kamiya *et al*., 1999; Kilborn & Fedida, 1990; Guo *et al*., 1996). As it has

been shown that there a direct relationship between the FP and AP duration can be observed using MEAs, it is uncertain why the AP durations measured in the experiments were extremely short (Halbach *et al*., 2001). It is possible that the type of MEA used (EcoMEA) was not sensitive enough to detect the minute changes in FP; however, the bulk of the AP duration is from the calcium current which can be easily detected since the AP transits from a depolarized to repolarized state during this time. In these studies, the FP duration does not appear to be a direct measure of the AP duration and therefore, the validity of the confirmation of the production of diabetic-like cardiomyocytes can be faulty.

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 also did not contain any anti-proliferating agents such as bromodeoxyuridine (BrdU) or cytosine 1-ß-D arabinofuranoside (Ara C), which favours the growth of fibroblasts. There was many non-cardiac cells observed in the cultures, most likely being fibroblasts. Fibroblasts also have an influence on the contraction rate and the membrane potential of cardiomyocytes (Simpson & Savion, 1981; LaFromboise *et al*., 2007).

4.5 The Inhibition of the Various 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-hyperglycaemic drugs which include various classes: bigunides, sulfonylureas, meglitanides, thiazolidiones, and α-glucosidase inhibitors. Reducing glucose levels in the blood can help alleviate symptoms of diabetes. Hypertension or renal dysfunctions are treated by angiotension-converting enzymes inhibitors, angiotension-II type 1 receptor blockers, calcium channel blockers, and diuretics. Cardiac complications are treated by adrenergic ß-blockers, anti-arrhythmic drugs, angiotension-converting enzymes inhibitors, calcium 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 very 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 resulting in a dosage of the drug which is toxic or inefficient to elicit its pharmacological activity, both which can have adverse effects. The CYP isoforms studied were: 1A2, 2B6, 2C8, 2C9, 2C19, 2D5, 2E1, 3A4, 3A5, and 3A7. As these plant

Drug	CYP	References
Antihyperglycaemic		
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
Thiazolidiones		
pioglitazone	2C8, 3A4	1 2
rosiglitazone	2C8, 2C9	1 2 3 6
α-glucosidase inhibitors		
acarbose	none	1
Hypertension or Nephropathies		
Angiotension-converting enzyme inhibitors		
captopril	2D6	1 3
enalapril	3A4	1 3
ramipril	3A4	1
perindopril	3A4	1
lisinopril	3A4	1
Angiotension-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

Drug	CYP	References
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		
B-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) Miller & 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*., 2004; 16) Garcia-Martin *et al*., 2004; 17) Grahramani *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.

Table 6: A list of the commonly used drugs by type 2 diabetics and the major CYP isoforms involved in their metabolism.

species are not common NHPs in Western society, there have been no studies conducted to investigate the inhibitory potency to CYPs.

4.5.1. Identifying the Inhibitory Potency of the Cree Plant Extracts

The majority of the extracts were low potent inhibitors of CYP1A2 and would not be expected to affect the metabolism of CYP1A2 substrates. The four extracts AD01, AD06, AD11, and W5 would 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 already associated with adverse effects - naproxen can damage the stomach lining and increase the risk of cardiac events (Adelman, 2001), whereas acetaminophen can cause liver toxicity (Laurent *et al*., 2007). By inhibiting their metabolism, the toxicity of these drugs can increase.

All of the extracts were low potent inhibitors of CYP2B6 and would not be expected to affect the metabolism of CYP2B6 substrates. None of the commonly used drugs for T2D are metabolized by CYP2B6 and therefore, these results are rather insignificant in the context of drug-NHP interactions in diabetics.

All of the extracts were low potent inhibitors of CYP2D6 except for AD01 which had moderate inhibitory potency. CYP2D6 metabolizes many of the 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 AD01 would have the greatest probability of interacting with the metabolism of these drugs. Inhibiting the drugs

involved with regulating 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 were low potent inhibitors of CYP2E1 and would not be expected to affect the metabolism of CYP2E1substrates. The four extracts AD01, AD03, W2, and W8 would have the greatest probability of interacting with CYP2E1 substrates because they had moderate inhibitory potencies. None of the commonly used drugs for T2D are metabolized by CYP2E1 and therefore, these results are rather insignificant in the context of drug-NHP interactions in diabetics. However, CYP2E1 activity can generate ROS (Cedarbaum, 2006) which are harmful because they can oxidize DNA, protein, carbohydrates, and lipids in the body (Cai & Harrison, 2000). In addition, ROS can inactivate a molecule called 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). CYP2E1 has been found to be induced in diabetes (Anzenbacher & Anzenbacherova, 2001). The lack of inhibition of CYP2E1 by the extracts can be viewed as a negative aspect due to their capability to produce ROS.

There was a broad range of inhibition against CYP2C8 by the 17 Cree plant extracts. The three extracts AD09, W8, and W9 had low potent inhibition and would not be expected to affect metabolism of CYP2C8 substrates. The two extracts AD07 and W6 had high potent inhibition and comparable inhibitory potency as the positive control ketoconazole (10 μM), and hence 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 moderate potent inhibitors and have a lower probability of affecting the metabolism of CYP2C8 substrates. CYP2C8 plays a major role in the metabolism of various drugs used by diabetics. CYP2C8 is the major metabolizer of the anti-hyperglycaemic thiazolidiones and the lipid-lowering statins. It also metabolizes the anti-hyperglycaemic drug repaglinide and the ß-blocker carvedilol. The extracts AD07 and W6 have a high potential to interact with these drugs, whereas the moderate potent inhibitors have a lower, but possible potential. The anti-hyperglycaemic 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.

There was also a broad range of inhibition against CYP2C9 by the 17 Cree plant extracts. The three extracts W5, W8, and W9 had low potent inhibition and would not be expected to affect metabolism of CYP2C9 substrates. The five extracts AD03, AD07, W3, W4, and W6 had high potent inhibition, and hence have the greatest probability of affecting the metabolism of CYP2C9 substrates. The other extracts (AD01, AD02, AD06, AD08, AD09, AD11, W1, W2, and W7) were moderate potent inhibitors and have a lower probability of affecting the metabolism of CYP2C9 substrates. CYP2C9 is extremely important in the metabolism of drug commonly used by diabetics. It is the major metabolizer of the anti-hyperglycaemic sulfonlyureas, nateglinide, and rosiglitazone; the angiotension-II type 1 receptor blockers which are used to treat hypertension; the diuretic torsemide; the lipid-lowering statin fluvastatin; the anti-coagulent warfarin; and several drugs used to treat neuropathies. The five extracts with

high potent inhibitory effects, AD03, AD07, W3, W4, and W6, have a great potential to interact with these drugs and should be used with caution. Several of the moderate potent inhibitors such as AD01 and AD02 have a potential to interact with the drug also. CYP2C9 is also 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 sodium in the kidney to decrease the blood volume (Sarkis *et al*., 2004). Hypertension is already a complication suffered by diabetics, and inhibition of EET production through the inhibition of CYP2C9, can increase its severity. Therefore, AD03, AD07, W3, W4, and W6 should be used with caution with drugs metabolized by CYP2C9, and also by diabetics suffering from hypetension.

CYP2C19 was the most inhibited CYP isoform studied. Only two extracts W5, W8, and W9 had low potent inhibitory effects against CYP2C19 and would be expected to not interact with drugs. The extracts AD01, AD02, AD03, AD07, AD11, W2, W4, and W6 were high potent inhibitors, whereas AD06, AD08, AD09, W1, W3, W5, and W7 were moderate potent inhibitors. The high potent inhibitors have a greater possibility of interacting with drugs, whereas the moderate potent inhibitors have a lower, possibility. However, CYP2C19 does not play a major role in the metabolism of drugs used by diabetics except for the anti-depressant nortriptyline used to treat pain. Therefore, even though CYP2C19 was the most inhibited CYP isoform by the Cree plant extracts, the clinical significance of this high inhibition is extremely low.

There was a broad range of inhibition against CYP3A4 by the 17 Cree plant extracts. The three low potent inhibitors AD09, W7 and W9, would not be expected to have an affect on metabolism of CYP3A4 substrates. The seven moderately potent inhibitors AD02, AD03, AD08, W3, W4, W6 and W8, would have a greater probability of affect the metabolism of CYP3A4 substrates. The seven high potent inhibitors AD01, AD06, AD07, AD11, W1, W2 and W5, would have the greatest probability of affecting the metabolism of CYP3A4 substrates. 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, 2008). In addition, it metabolizes many drugs used by diabetics such as the angiotension-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-hyperglycaemic drugs. Therefore, similar to CYP2C9, CYP3A4 is an important CYP isoform for diabetics. The extracts AD01, AD02 AD06, AD07, AD11, W1, W2, and W5 should be used with caution with these drugs.

There was a broad range of inhibition against CYP3A5 by the 17 Cree plant extracts. The two low potent inhibitors W7 and W9, would not be expected to have an affect on metabolism of CYP3A4 substrates. The seven moderately potent inhibitors AD08, AD09, W3, W4, W5, W6 and W8, would have a greater probability of affect the metabolism of CYP3A4 substrates. The seven high potent inhibitors AD01, AD02, AD03, AD07, AD11, W2, W4 and W6, would have the greatest probability of affecting the metabolism of CYP3A4 substrates. CYP3A5 metabolizes the same drugs as CYP3A4 with the same activity or less (Williams *et al*., 2002). However, CYP3A5 contributes less

to drug metabolism because of its lower content in the liver and enterocytes (Lin & Lu, 1998), and therefore its inhibition is not as clinically relevant as the CYP3A4 inhibition results. Unlike CYP3A4, CYP3A5 is ubiquitously present in the kidney, (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 CYP3A7 by the 17 Cree plant extracts and was the second most inhibited isoform. There was only one low potent inhibitor W9 which is not expected to have an affect on metabolism of CYP3A7 substrates. The nine moderately potent inhibitors AD08, AD09, AD11, W1, W3, W5, W6, W7 and W8, would have a greater probability of affect the metabolism of CYP3A7 substrates The seven high potent inhibitors AD01, AD02, AD03, AD06, AD07, W2 and W4 would have the greatest probability of affecting the metabolism of CYP3A7 substrates. CYP3A7 metabolizes the same drugs as CYP3A4 but at a much lower degree (Williams *et al*., 2002). In addition, CYP3A7 is only expressed during the fetal stages of life (Lin & Lu, 1998) and hence, this isoform would only be relevant in gestational diabetes, which occurs when a woman develops T2D during her pregnancy. If women with gestational diabetes are taking anti-hyperglycaemic medication, the fetus may be exposed to the drug and hence, it would be important for CYP3A7 to be active in order to inactive the drug. The extracts AD01, AD02, AD03, AD06, AD07, W2 and W4 can potently inhibit CYP3A7 and therefore, can cause harmful effects to the fetus.

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. Unfortunately, these isoforms except for CYP2D6 were moderately or highly inhibited by many of the extracts and therefore, there is a great potential for drug interactions to occur from CYP inhibition. There was only one common high potent inhibitor for these three isoforms which was AD07. The extract W6 was a common high potent inhibitor for CYP2C8 and 2C9. There was also one extract that was a common low potent inhibitor for the three isoforms, W9. Each CYP isoform had different high potent inhibitors and therefore, a drug-extract interaction would depend on both the drug and the extract. For example, the extract W5 would interact with a CYP3A4 substrate such as diltiazem but not with a CYP2C9 substrate such as glyburide.

From **Table 3**, common high and low potent inhibitors were observed among the 10 CYP isoforms; however 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 extracts which were common high potent inhibitors (AD01, W4, W6), may be highly potent because they are rich in phytochemicals which may cause non-specific inhibition, or because they contain common inhibitory constituents. The common low potent inhibitors may be scarce in phytochemical content or contain constituents which are non-inhibitory towards CYPs. The phytochemical analysis for these Cree plant extracts are currently being conducted at Thor Arnason's lab. A manuscript with information on the phytochemicals present in four of the extracts is currently in press (Saleem *et al*., in

press). From the figures presented in this manuscript, possible phytochemicals which may possess high potent inhibitory capabilities of particular CYP isoforms can be deduced by comparing the concentrations of the phytochemicals in the four extracts to the inhibitory effect the extracts had on a CYP isoform. Possible 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 that I am aware of. Chlorogenic acid has been shown to not inhibit CYP1A2, 2C9, 2C19, 2D6, and 3A4 (Obach, 2000). In order to confirm if these phytochemicals are the potent inhibitors of the CYPs, each phytochemicals would need to be identified, quantified, isolated and tested in the CYP inhibition assays.

Similar inhibition trends were observed among members of the same subfamily especially CYP2C and 3A. This would be expected since members of the same subfamily are closely related and therefore would share similar enzyme conformation and substrate specificity. An interesting observation was observed with the CYP2C and 3A subfamilies - many of the extracts had opposite potencies towards the two subfamilies.The inhibitory values of the 17 extracts for CYP2C9 and 3A4 were plotted for a more visual comparison. Seven of the extracts had similar inhibitory potencies against CYP2C9 and 3A4, but the other ten extracts (AD01, AD06, AD11, W1, W3, W4, W5, W6, W7, and W8) had significant differences in inhibitory potencies for the two isoforms. In addition, six of the 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 is a specific constituent or several

constituents that are present in a particular extract that are more inhibitory against a particular CYP isoform.

The inhibitory ranking from the most inhibited to the least inhibited CYP isoform was 2C19 > 3A7 > 3A5 > 3A4 > 2C9 > 2C8 > 1A2 > 2E1 > 2D6 > 2B6, and their mean inhibition from the 17 Cree plant extracts ranged from 7.66% ± 1.62% to 68.40% ± 6.21% inhibition. A possible explanation for why CYP2C and 3A members are the most inhibited is because they have large active sites (Lewis *et al*., 2006) and therefore can accommodate a range of different sizes and shapes of inhibitors. The active site volumes of the same CYP isoform vary from study to study because of the difference in algorithms used to calculate these volumes (Otyepka *et al*., 2007). The largest calculated active site volumes of CYP2C8, 2C9, and 3A4 were 1386, 1137 and 1483 $Å^3$ respectively (Lewis *et al*., 2006; 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 as small, medium or large based on the size of their typical substrates: small-2E1; medium-1A2, 2B6; high- 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 can also accommodate several compounds simultaneously which can inhibit substrates from entering the active site to be metabolized. CYP2C9 and 3A4, two of the major drug-metabolizing members have also been found to have flexible active sites which increases 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 metabolism of substrates. CYP1A2 has been shown to possess an inflexible active site (Anzenbacher *et al*., 1998).

The extracts had different mean inhibitory potencies for the 10 CYP isoforms ranging from 5.09% ± 3.09% to 60.43% ± 8.18% mean 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 how much phytochemicals are present in the extracts or if they contain high inhibitory phytochemicals. In addition, substrates have greater access to an active site of a CYP if there are more lipophilic. Lipophilicity has been shown to play a role in inhibitory potency in CYP2C9 and 2C19 (Lewis *et al*., 2006). It is possible that the high potent inhibitory extracts contain more inhibitory phytochemicals that are more lipophilic in nature.

4.6 Mechanism-Based Inhibition of CYP3A4

4.6.1 Identifying Mechanism-Based Inhibitors

MBI is more harmful than reversible inhibition such as competitive or non-competitive inhibition because it is irreversible and requires *de novo* synthesis of CYPs to replace the inactivated ones. If any of the extracts are mechanism-based inhibitors, these extracts should be used with extreme caution because chronic and daily use of the extract can inhibit a large amount of CYP and not provide enough time to synthesis new CYP and restore CYP levels required for normal drug metabolism. The inhibitions observed in the previous experiments could be a result of MBI because the assay conditions for those studies do not differentiate between types of inhibition. The MBI of CYP3A4 from 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. Many of the extracts had similar inhibitory trends observed with the negative control ketoconazole in both assays. Several of the extracts showed greater 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.

The time-dependent assay for MBI has been used by Yamamoto *et al.*, to identify inhibitors that are also substrates from the same CYP isoforms 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, and the activity of the CYPs would be greater than CYPs that have been pre-incubated with the inhibitor for a short pre-incubation time where less inactive metabolites would be present. This trend was observed with the extract AD01, indicating that inhibitory constituents of AD01 are substrates for CYP3A4. As mentioned in Chapter 1, pharmaceuticals can be administered as prodrugs requiring their metabolism before they are pharmacologically active. It can be possible that the metabolites of phytochemicals in the extracts are inhibitory towards CYPs. If a phytochemical was a substrate for a CYP isoform, and its metabolite was inhibitory towards the same CYP, it can be identified using the same logic that was used to identify inhibitory substrates used by Yamamoto *et al*. After a long pre-incubation time, there would be more inhibitory metabolites present compared to a short pre-incubation time, and less activity of the CYP. The extract AD02 appears to contain these inhibitory metabolites. In addition, since NADPH is required for the metabolism reaction, these predications were further validated using **Figure 24A**. The time-dependent assay may be

used for future studies to identify which CYPs are involved in the metabolism of the Cree plants, as well as to identify inhibitory metabolites.

4.6.2. Confirmation of Mechanism-Based Inhibitors

A possible reason for why none of the 17 Cree plant extracts were detected as mechanism-based inhibitors was because the concentration tested (10 µg/mL) was too low for inhibition to occur. Further MBI tests were performed with AD02, AD09, and using a higher concentration of 100 µg/mL. Overall, the results indicate that AD09 and W3 are not mechanism-based inhibitors. However, minor MBI trends were observed with AD02 in both the NADPH- and time-dependent assays using both concentrations. 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, none of the extracts expect for possibly AD02 are mechanism-based inhibitors of CYP3A4. Even though high potent inhibition of CYP3A4 was observed in the earlier assay, confirming that none of the extracts (expect for AD02) are mechanism-based inhibitors is a positive discovery as this type of inhibition is irreversible. However, it is still undetermined whether the extracts are mechanism-based inhibitors of the other CYP isoforms.

4.7 Drug Interaction Study with Enalapril

A drug interaction study was performed with enalapril which is a drug used to treat hypertension. 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. A single concentration of 0.3 μM was chosen to be tested for numerous reasons: 1) this is the mean concentration of enalapril in the blood (Noormohamed *et al*., 1990; Weisser *et* al., 1992; Song & White, 2002); 2) many diabetics in the CEI suffer from hypertension (Kuzmina & Dannenbaum, 2004); 3) enalapril is metabolized by CYP3A4 and may compete with DBF or inhibitors present in the extract for active site binding (Triplitt, 2006; Siest, 2007); and 4) the metabolite of enalapril (enalaprate) may react with constituents of the extract which can affect CYP activity. Experiments which study the interaction between two compounds are typically performed *in vivo* or clinical, but not *in vitro* and therefore only a few extracts were chosen for this study because it was unsure of the study's success: AD01, AD08, and AD09. The IC_{50} values of the extracts were tested to maximize the ability to observe deviations between the CYP activity with the extract compared to the combination of the extract and enalapril. As mentioned previously, three different assays were used because it was unsure what were the optimal assay conditions. In the 2nd and 3rd assay, the extract and enalapril are allowed to interact with each other under different conditions before the addition of the enzyme. These experiments also did not use any positive or negative controls. In all three assays, the metabolism of DBF was not affected by enalapril which was expected to decrease because DBF and enalapril would both 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*., 1999; Kenworthy *et al*., 2001), and that CYP3A4 has multiple active sites (Kenworthy *et al*., 1999), which may account for the lack of inhibition of the metabolism of DBF. It also may be possible

that DBF is being metabolized by the enzyme, and is inhibiting the metabolism of enalapril which cannot be detected in this assay. In a study completed by Wang *et al*., they showed that when the two CYP3A4 substrates nifedipine and testosterone were present with CYP3A4, nifedipine inhibited the metabolism of testosterone, but testosterone did not inhibit the metabolism of nifedipine (Wang *et al*., 1999). The presence of enalapril did not affect the inhibitory potency of the extracts in all three assays. Very similar inhibitory results were observed with the extract and the combination of the extract with enalapril. The inhibitory effect of the AD08 with or without enalapril was not significantly different than the MeOH control in the 2nd and 3rd assay. There was less inhibition with AD08 compared to the others even though 50% of the activity of CYP3A4 was expected for all extracts, indicating that the IC_{50} values were not that accurate in the assays.

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 limited in their physiological setting. 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 and N-acetyltransferase, may have an impact on their interactions which cannot be determined in the assay used. In addition, it was not verified whether enalapril was being metabolized in the assay, which does alter the perspective of the results, as they were based on the enalapril being metabolized by CYP3A4. Confirmation of the metabolism of enalapril in the assay can be confirmed by high-pressure liquid chromatography or gas chromatography.

Overall, based on the results enalapril does not affect the inhibitory potency of the extracts AD01, AD08, and AD09 against CYP3A4 activity, and drug-NHP interactions are not expected to occur with these xenobiotics.

4.8 Comparison of Inhibitory Potencies

Typically in the literature, comparisons of inhibitory compounds of CYPs are performed by comparing IC_{50} values, which allows for cross-comparison and analysis with other published studies. However, determining these IC_{50} values is time-consuming and for the task of determining the inhibitory potency of the 17 Cree plant extracts for 10 CYP isoforms, this would be infeasible for the time provided. The original screening assays for the CYP isoforms tested one concentration of 10 µg/mL of extract which allowed comparisons to be made based on potency to categorize extracts as low, moderate, and high potent inhibitors. From the IC_{50} values that were obtained in preparation of the drug interaction study with enalapril, a ranking of their potency was obtained and compared to the ranking of their potency obtained from the original CYP3A4 inhibition study. Similar inhibition rankings between the two studies were obtained based on their potency. The extracts with low potency had identical rankings for both assays: AD09 < W3 < AD08. However, the moderate and high potent inhibitors, AD01, AD11, W1, and W5 did not have identical rankings. This indicates that in the original screening assay, there was saturation of the CYPs and therefore differentiation of the inhibitions was not possible. The original screening assay was able to categorize low, moderate and high inhibitors, but it was not able to show the exact inhibitory potencies of the extracts due to saturation of the CYPs; 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 low, moderate, and high potent inhibitors which was sufficient for the studies for this thesis.

4.9 Overall Summary

The Cree plant extracts were tested for their 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 *in vitro*. The experiments were designed to study the effects of the extracts as if they were taken as alternative or complementary medicine.

The extracts W2, W4, W5, and W9 did not have an effect on the contraction rate of cardiomyocytes, and therefore are not expected to have an effect on the heart rate clinically. W9 was found to be toxic in cardiomyocytes at a low concentration (10 µg/mL) that can be obtained physiologically in humans, and should be used with caution. Even at very high doses, W2 and W4 will not have a toxic effect with cardiomyocytes. Very high doses of W9 (greater than 100 µg/mL) with a high plasma concentration of metformin (50 µM) was toxic to the cardiomyocytes and decreased their contraction rate.

Many of the extracts potently inhibited the different CYP isoforms posing a risk for developing drug-NHP interactions and consequent adverse effects. Several extracts including AD09, W7, and W9 had an overall low potent inhibition against all CYP isoforms and would be the safest to take concomitantly with drugs. Enalapril did not potentiate the inhibitory effects of AD01, AD08, and AD09. Several extracts such as AD03, AD07, W2, W4, and W6 were high and/or moderate potent inhibitor of CYP2C8 and 2C9, and have a great potential to interact with the metabolism of many of the anti-hyperglycaemic drugs, the angiotension-II type 1 receptor blockers, the statins, torsemide, and warfarin. In addition, the extracts AD01, AD06, AD07, AD11, W1, W2, W4, and

W5 were high and/or moderate potent inhibitors of CY3A4 and 3A5, and have a great potential to interact with the metabolism of the calcium-channel blockers, the HMG-CoA reductase inhibitors, and the angiotension-converting enzyme inhibitors. Interestingly, W9 was found to be toxic to cardiomyocytes at the same concentration that had low inhibitory potency against the CYPs (10 µg/mL). The extracts W2 and W4 also displayed a similar contradiction because W2 and W4 were not toxic to cardiomyocytes at high concentrations, yet at a low concentration of 10 µg/mL, they were found to inhibit important drug-metabolizing CYPs with moderate or high potency. In fact, W2 was the second most potent inhibitor of all the CYP isoforms after AD01. However, if AD01 and W2 were used as alternative medicine, these extracts can have a beneficial effect by reducing the production of ROS through the inhibition of CYP2E1. Overall, the extract AD01 was found to be the most unsafe extract with respect to its ability to inhibit CYPs and cause adverse drug effects. It not only was the most potent in causing CYP inhibition, it also appeared to be metabolized by CYP3A4 and therefore, can compete with the metabolism of concomitant drugs that are metabolized by CYP3A4 increasing the risk of developing adverse drug reactions. The extract AD02 was found to be a possible mechanism-based inhibitor of CYP3A4, and repeated, chronic dosing of this plant should be avoided to maintain active levels of CYP3A4.

4.10 Conclusion

Our studies suggest that at physiological plasma concentrations, several of the Cree medicinal plants can have a harmful effect with diabetics by affecting the viability of cardiomyocytes, or by affecting the metabolism of drugs commonly used by diabetics through the inhibition of numerous drug-metabolizing CYPs. On the other hand, the

plants (that were studied) are not expected to have an effect on the heart rate, and should not interact with metformin or enalapril under physiological conditions. Several of the plant species investigated displayed more noteworthy results than others: at 10 μg/mL, W9 was found to be toxic to cardiomyocytes but had low inhibitory potency against all CYP isoforms; W2 and W4 were found to be non-toxic to cardiomyocytes, but had moderate or high inhibitory potency against the important CYP isoforms; AD01 had the greatest potential to cause adverse drug effects; AD02 inhibited CYP3A4 with moderate potency, but may be of the irreversible type; and AD09, W7 and W9 were shown to have the least potential to interact with drug metabolism. It is important to keep in mind, that determining which of the Cree plants would be the most safest or harmful to use is difficult to ascertain due to the different effects the plants had in the assorted studies. Furthermore, their harmfulness also depends on whether the plants are used as alternative or complementary medicine, and which pharmaceuticals are concomitantly administered.

4.11 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 S7B, Yap & Camm, 2003). In addition, it would be important to test the extracts on diabetic cardiomyocytes and should be a study to greatly focus on.

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 CYP 2C8, 2C9 and 3A5 should also be performed. In addition, identifying whether enalapril is being metabolized by CYP3A4 in our drug interaction study should be performed using high-pressure liquid chromatography or gas chromatography.

APPENDIX

A1 Determination of the Exact 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 it cannot be determined what concentration of phytochemicals have been extracted from the bulk material. To determine the final extract concentrations, the phytochemicals in the extract solutions were quantified by evaporating out 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 true extract concentration produced. For each determination of extract concentration, three separate samples were used and averaged.

A1.2 Results

The actual 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 actual 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 %.

(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

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

A2 Identifying an 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 the cardiomyocytes can undergo changes in their cellular and electrophysiological processes 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 their 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 recording from a MEA, 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) using Ascensia elite test strips.

A.2.2 Results

A time period of 5 to 7 DIV was ultimately chosen for numerous reasons. Four separate cultures of cardiomyocytes grown under identical conditions and their contractions rates and ISI deviations from 1 to 9 DIV were studied **(Figure A1)**. Detectable FP activity was observed after three or four 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 observed ($r2 = 0.630$, $p < 0.001$) indicating that an inverse relationship existed between the contraction rate and 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 separate 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

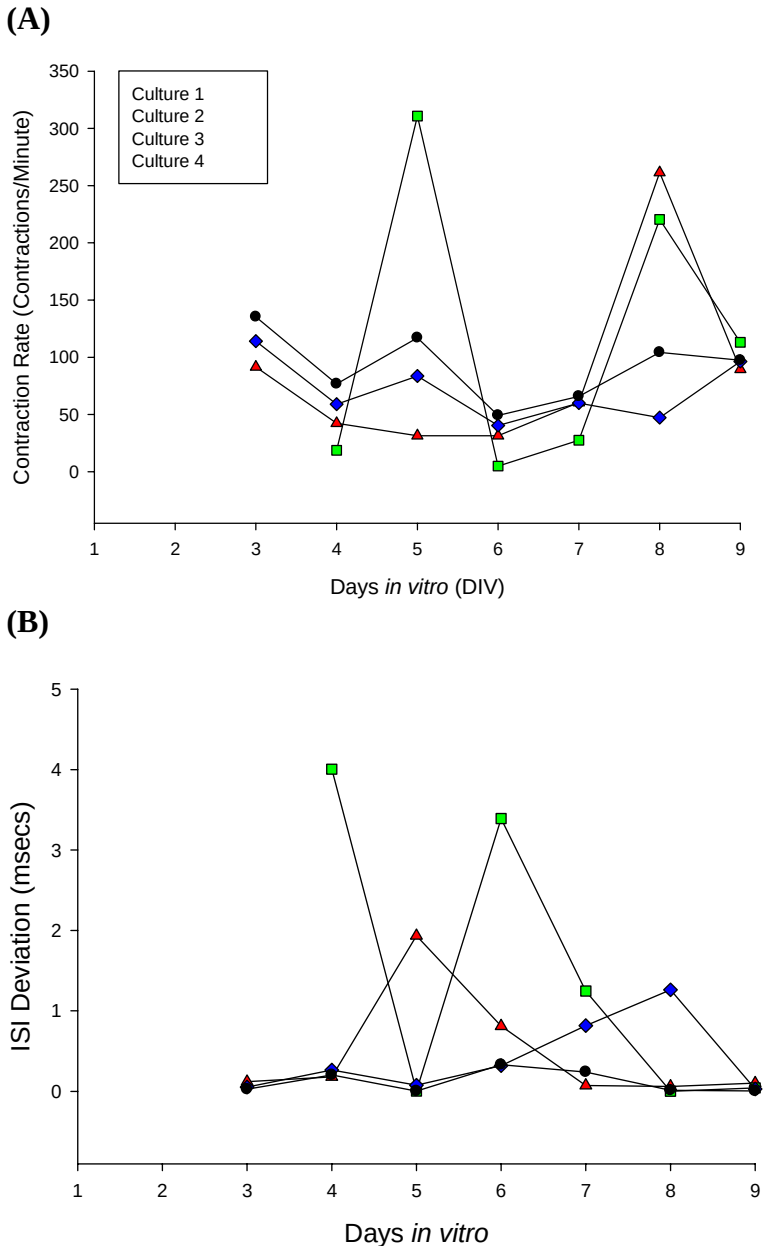


Figure A1: The contraction rate and inter-spike interval deviations of four cultures of neonatal rat cardiomyocytes. The electrical activity characteristics of four separate cultures of neonatal rat cardiomyocytes from 1 to 9 DIV based on the parameters: **(A)** contraction rate and **(B)** inter-spike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

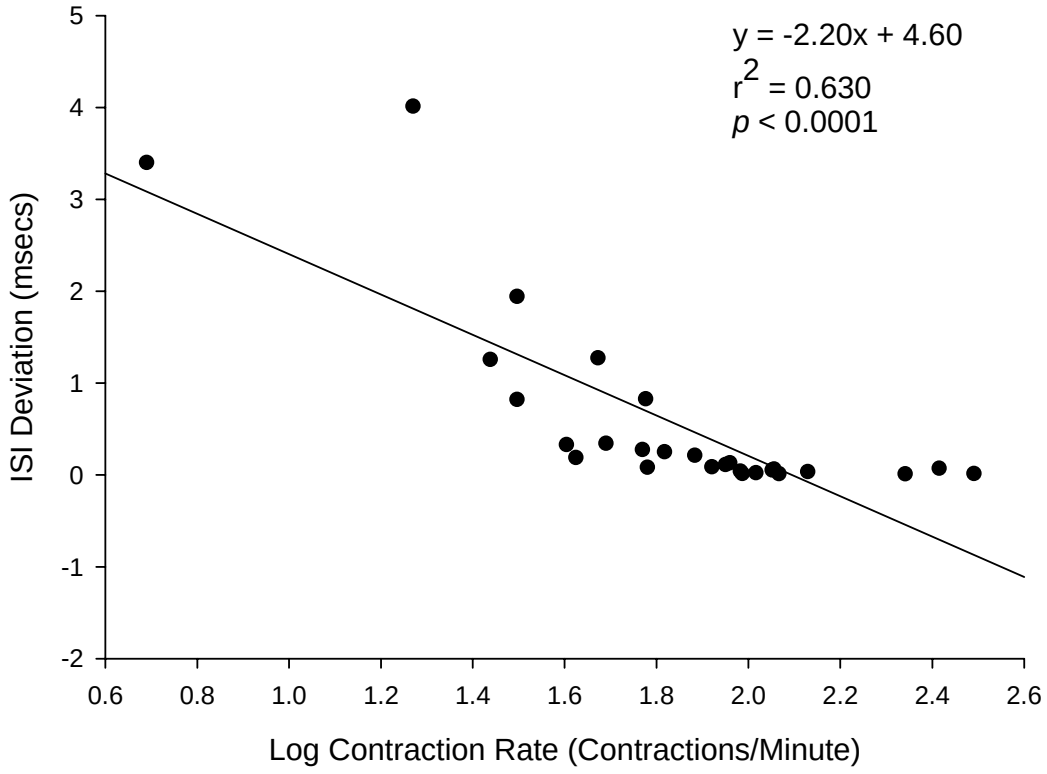


Figure A2: A Pearson correlation of neonatal rat cardiomyocyte contraction rate to inter-spike interval deviation. The log contraction rate was plotted as a function of inter-spike interval deviation. A line of best fit was obtained using linear regression. The equation ($y = -2.20x + 4.60$), r^2 value (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.

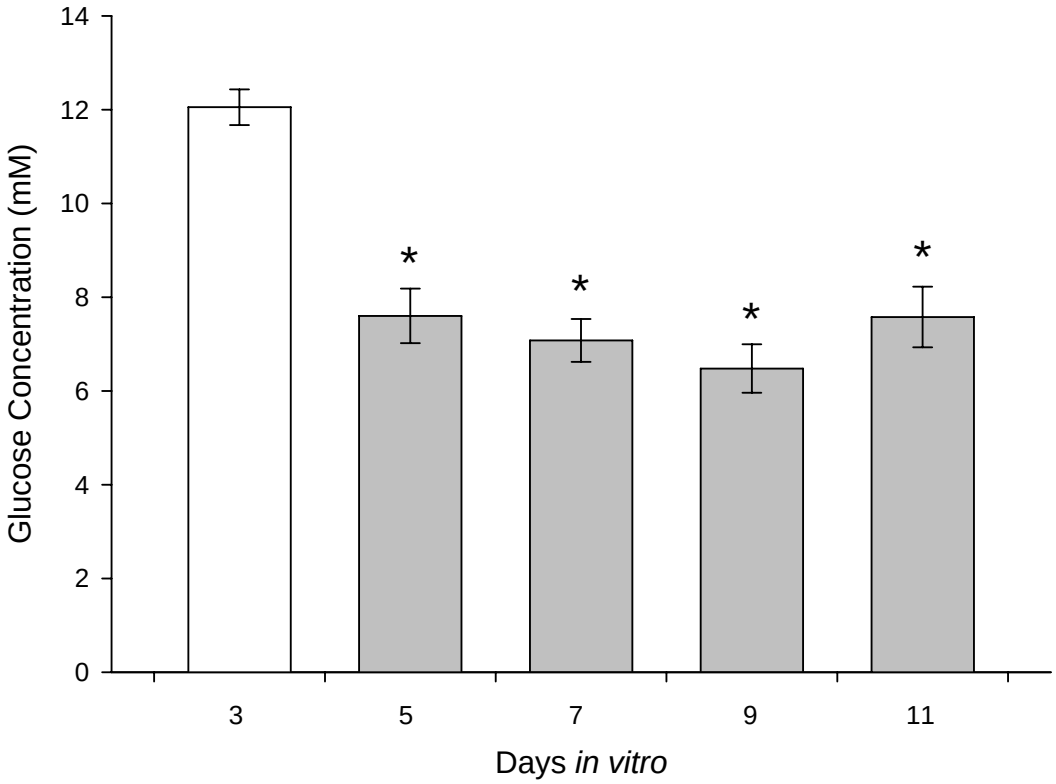


Figure A3: The glucose concentrations of used media collected during media changes from 3 to 11 DIV. After media changes, the used media was saved and their glucose concentrations were measured using as Ascensia Elite XL Glucose Meter. The results were expressed as the mean glucose concentration (mM)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

concentrations remained relatively constant and significant differences were not observed 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 5 to 7 DIV, the FP activity is detectable and stable, the cardiomyocytes should not have undergone extensive dedifferentiation **(Figure ??)**, 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 Identifying the 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 switch 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 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 (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 experiment does affect the experimental results and all the experiments should be completed in a set amount of time.

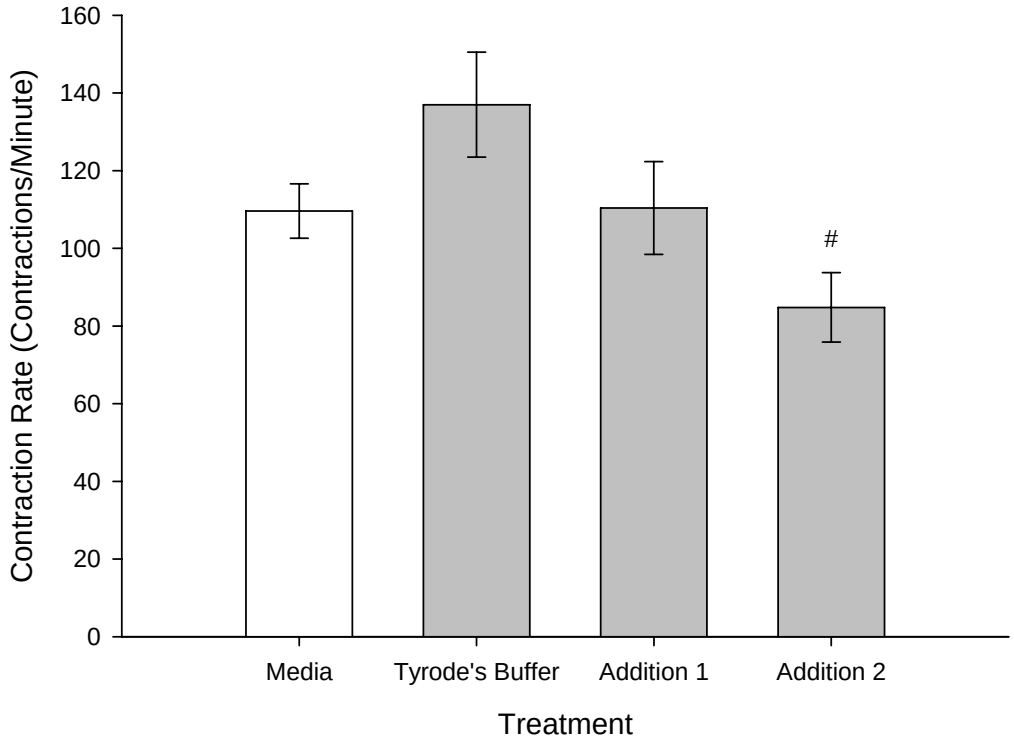


Figure A4: The chronotropic effect of the experimental technique with neonatal rat cardiomyocytes. The experimental technique for drug or extract testing was mimicked using Tyrode's buffer without drugs or extracts to determine if the technique itself affected the contraction rate of the cardiomyocytes. The Tyrode's buffer represents the initial switch from 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 ($n = 5$). $^{*}p \leq 0.05$ 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.

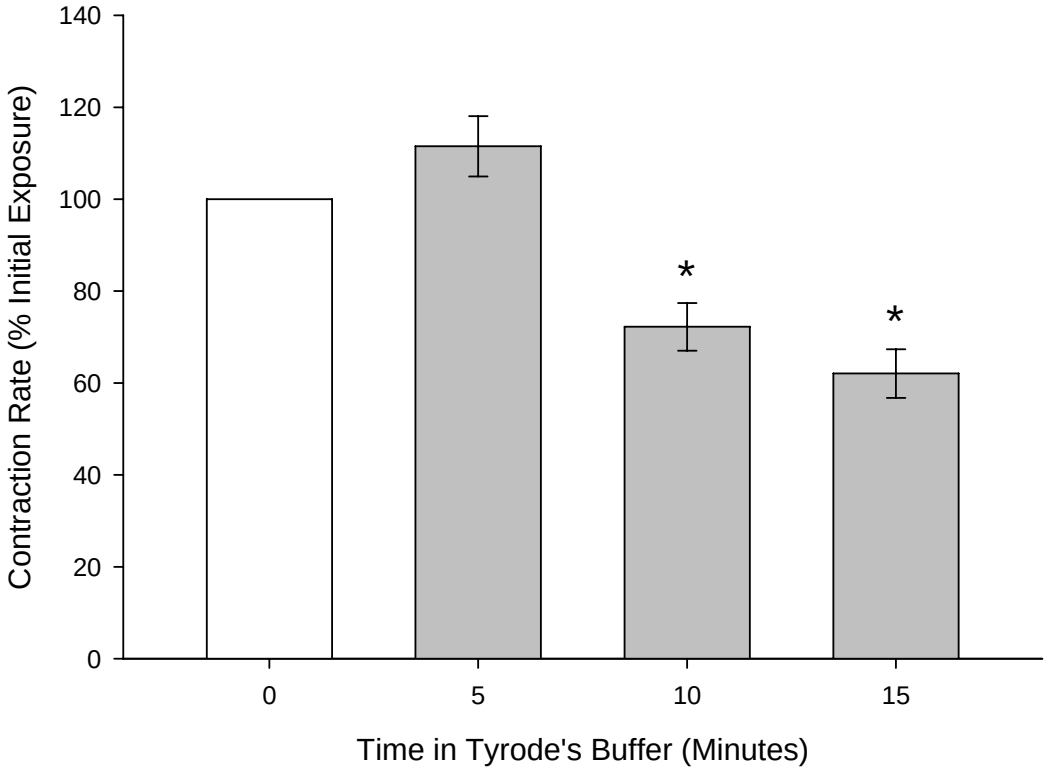


Figure A5: The changes in contraction rate that occur upon 15 minutes of exposure to Tyrode's buffer. The contraction rate of neonatal rat cardiomyocytes after 0 to 15 minutes of exposure to Tyrode's buffer was measured using the MEA system. The contraction rates at 5, 10 and 15 minutes were expressed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

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 determined when the metabolism of CYP substrates follows a linear fashion to ensure that none of the assay reagents are limiting the metabolism of the substrates. 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 our 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

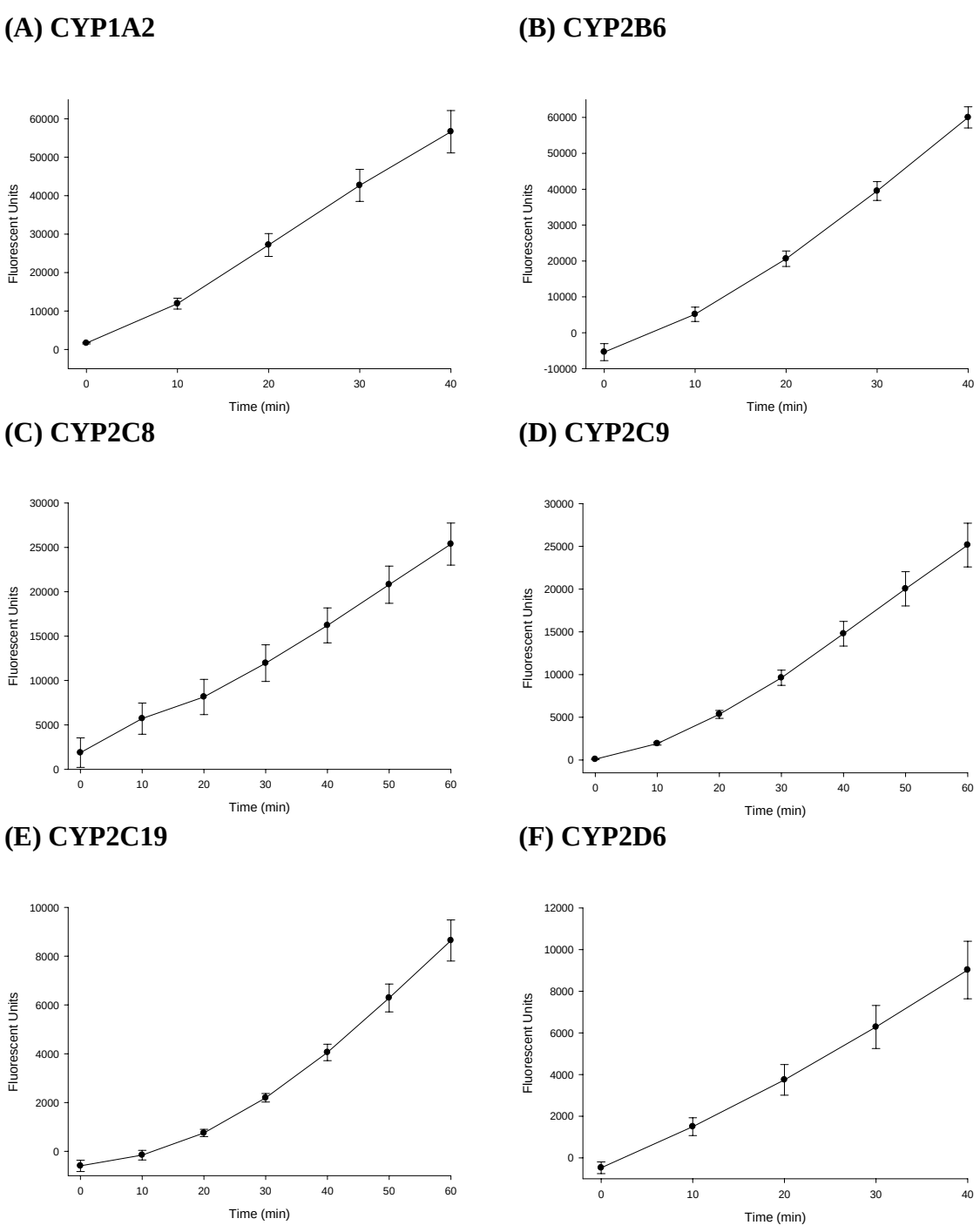


Figure A6: The activity curves of the CYP isoforms 1A2, 2B6, 2C8, 2C9, 2C19, and 2D6. Activity curves for the CYP isoforms **(A)** 1A2, **(B)** 2B6, **(C)** 2C8, **(D)** 2C9, **(E)** 2C19, and **(F)** 2D6 were plotted based on the fluorescence released from metabolized substrates of the CYPs at various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

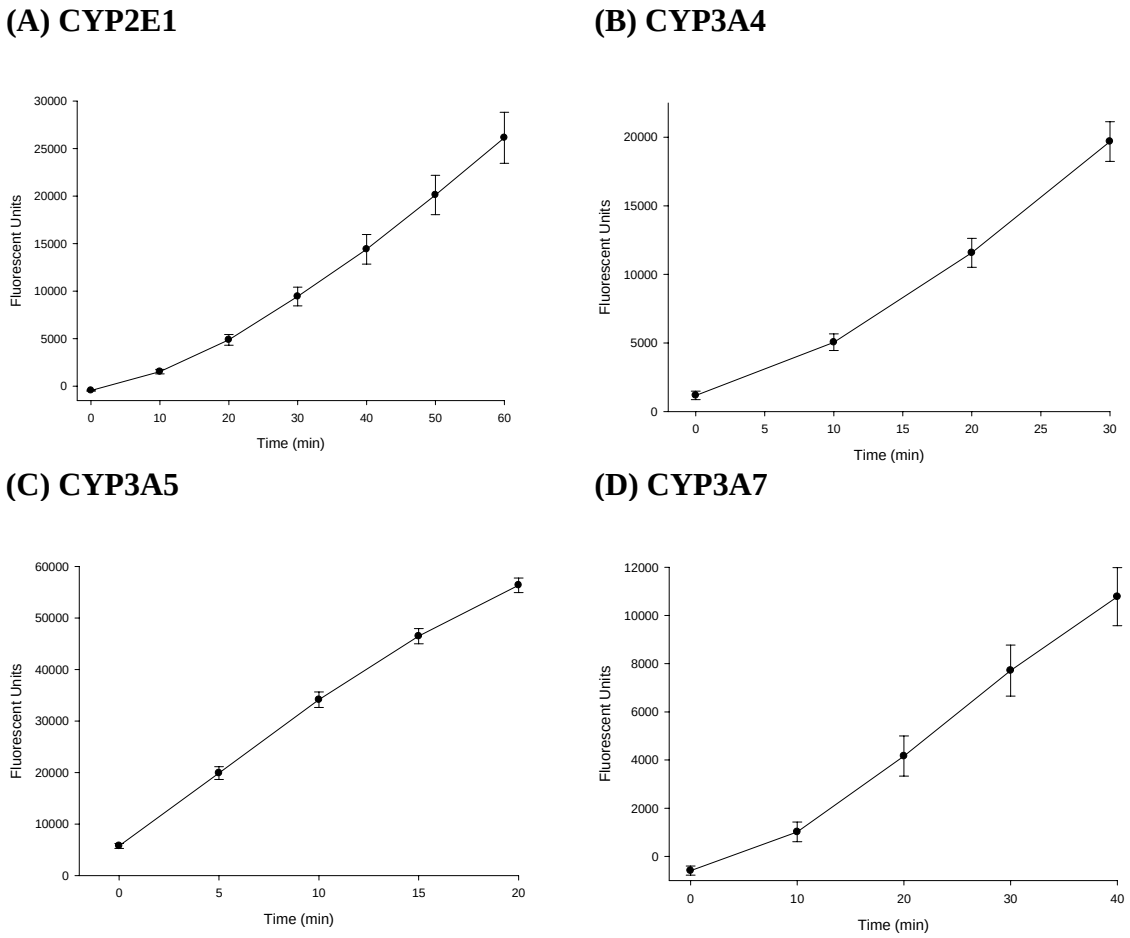


Figure A7: The activity curves of the CYP isoforms 2E1, 3A4, 3A5, and 3A7. Activity curves for the CYP isoforms **(A)** 2E1, **(B)** 3A4, **(C)** 3A5, and **(D)** 3A7 were plotted based on the fluorescence released from metabolized substrates of the CYPs at various time points. The results are expressed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

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 of the large amount of extracts to be tested. 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.

A5 Summary of the Cytochrome P450 Inhibition Results

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

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	17.18 ± 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	22.75 ± 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	27.21 ± 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	47.1 ± 4.6	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	12.00 ± 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	10.43 ± 0.8	4.3 ± 2.1	77.1 ± 400	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	15.25 ± 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	

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 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: low (<30%) – green; moderate (31-74%) - yellow; high (>75%) – red.