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The Influence of Genetic Polymorphisms and Natural Health Products on Drug Metabolism

By

Bobby Chauhan

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Abstract

The activities of two major components involved in drug metabolism, P-glycoprotein (P-gp) and members of the cytochrome P450 oxidase (CYP) family were studied. This investigation specifically examined the influence of polymorphisms within the *ABCB1* gene on P-gp efflux activity and expression. Additionally, the role of natural health products (NHPs) on the activity of both P-gp and members of the CYP3A subfamily was also studied. Findings from this study suggest that the haplotype relationship of C3435T, G2677A/T and C1236T within the *ABCB1* gene does not influence P-gp efflux activity or expression. Furthermore, some commonly consumed NHPs, including trans-β-carotene, *Arctostaphylos uva-ursi L.* (Kinnikinnick), *Acorus americanus Raf.* and *Acorus calamus L.* (Acorus), *Curcuma longa L.* (turmeric powder), *Capsicum annuum L.* (chilli powder) and *Piper nigrum L.* (black pepper), are able to influence the activity of P-gp and members of the CYP3A subfamily *in vitro*. The results suggest that in order for conventional drug therapy to be effective, the genotype of an individual in relation to drug metabolism, as well as the influence of concomitantly consumed NHPs, should be considered in determining how the bioavailability of therapeutic drugs are influenced.

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Abbreviations

ABC	ATP binding cassette transporters
CAR	constitutive androstane receptor
CFTR	transmembrane conductance regulator
CYP	cytochrome P450 oxidase
DBF	dibenzylfluorescein
DEPC	diethylpyrocarbonate
dNTP	deoxyribonucleotide triphosphate
DR-3	direct repeat with three nucleotides
DR-4	direct repeat with four nucleotides
ER-6	everted repeat with six nucleotides
ER-8	everted repeat with eight nucleotides
FBS	fetal bovine serum
GSTs	glutathione-S-transferases
GSTP1	glutathione-S-transferase π
HDL	high density lipoprotein
HIV	human immunodeficiency virus
IKK	IKb Kinase
Keto	ketoconazole
LDL	low density lipoprotein
MRP1	multi-drug resistance associated protein 1
NAD	nicotinamide adenine dinucleotide
NHPs	natural health products
NR	nuclear receptor
NRP	Nutraceutical Research Programme
OATs	organic anion transporters
OATPs	organic anion transporting polypeptides
OCTs	organic cation transporters
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffer solution
PEPTs	peptide cotransporters
P-gp	p-glycoprotein
PXR	pregnane X receptor
Rh123	rhodamine 123
RXR	9-*cis* retinoic acid receptor
SNPs	single-nucleotide polymorphisms
UTIs	urinary tract infections
VR1	vanilloid receptor subtype 1

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1 Introduction

1.1 Introduction to Thesis

The metabolism of drugs is controlled by genetic and environmental factors that influence the enzymes and transporters involved in drug metabolism. Mutations in genes that express these enzymes and transporters can influence their level of expression within tissues such as the liver, kidneys or intestine, and may also influence their level of activity [1]. Another factor that may modulate drug efficacy is the influence of xenobiotics, such as natural health products (NHPs). In general, NHPs are widely available and frequently used, yet information is lacking on potential interactions with conventional drugs. As an increasing number of people use herbals, it is important that users and health care professionals be aware of any possible side effects involved with their use, particularly when used concomitantly with conventional medication. For this reason, the present thesis addresses the effect of NHPs on enzymes involved in drug metabolism.

1.2 Literature Review

A brief discussion concerning xenobiotic metabolism, the phases involved in metabolism, and the variety of enzymes and transporters that play a role in this process will be addressed. A more detailed understanding of the role of the cytochrome P450 oxidase (CYP) family, specifically the CYP3A subfamily, and P-glycoprotein (P-gp) will follow, with the goal of understanding how these two components function in a cooperative manner in the metabolism of xenobiotics. In addition, the presence of polymorphisms within the *ABCB1* gene, which expresses P-gp, will also be discussed to understand how

this may influence P-gp expression and activity. Finally, a discussion on NHPs will include how studies have suggested that some NHPs influence the activity and expression of CYP3A4 and P-gp, followed by a background of the NHPs that were used in this research project [2-4].

1.2.1 Xenobiotic Metabolism

Xenobiotics, which include therapeutic drugs, carcinogens or any other foreign compound that is not part of the human intermediary pathways, undergo metabolism through various mechanisms that generally involves three phases – Phase I, Phase II and Phase III. Phase I and Phase II metabolism occurs mainly in the liver, although some xenobiotics are metabolised extrahepatically in the plasma, lungs, the gut, and other tissues and organs [5]. Phase I metabolism involves modifying the xenobiotic by producing or revealing a functional group on the compound to allow for interaction with Phase II enzymes (Figure 1) [6]. Phase II metabolism entails the actual "detoxification" through a conjugation reaction with the functional group of the compound resulting in a water-soluble product that can be excreted [6]. The transport of the metabolized compounds for elimination is considered to be Phase III metabolism. A fourth process, known as Phase 0 metabolism has been described as a first line of defence against xenobiotics and involves the direct elimination of the xenobiotic, upon entrance within the cell, back into the gut lumen [7]. Although these phases may function in a cooperative manner as described above, in some cases the Phase I and II enzymes and Phase III transporters may compete in the metabolism and elimination of xenobiotics. The resultant effect on the metabolism of xenobiotics can vary and includes no change in

Figure 1: Phase I and Phase II Metabolism.
A chemical equation representing the Phase I oxidation of a xenobiotic (RH) by the common Phase I metabolising enzyme, CYP 3A4 in the presence of nicotinamide adenine dinucleotide phosphate (NADPH). The intermediate product (ROH), now with a functional group revealed, undergoes Phase II conjugation, as seen with the enzyme sulfotransferase and the high energy co-factor 3'-phosphoadenosine 5'-phosphosulfate (PAPS). The water soluble end product, $ROSO_3H$, is then eliminated via Phase III transport.

Phase I Metabolism:

$$RH + O_2 + NADPH + H^+ \xrightarrow{\text{CYP 3A4}} ROH + H_2O + NADP^+$$

Phase II Metabolism:

$$ROH + PAPS \xrightarrow{\text{Sulfotransferase}} ROSO_3H + \text{3-phosphoadenosine 5' phosphate}$$

↓

Elimination via Phase III
Metabolism

the activity of the xenobiotic, decreased activity of the xenobiotic or the formation of active metabolites. In the case of therapeutic drugs, a change in xenobiotic activity can also influence clinical response as well as drug toxicity.

1.2.2 Phase I Enzymes

Phase I enzymes produce or reveal a functional group on a xenobiotic generally through one of the following mechanisms – oxidation, reduction, hydrolysis or hydration [6]. This catabolic process sometimes leads to the production of a more chemically active and potentially toxic compound [5]. Some of the enzymes involved in the oxidation of xenobiotics include dehydrogenases, oxidases and aromatases. An example of Phase I activity can be seen in the metabolism of ethanol via oxidation by alcohol dehydrogenase in the presence of the coenzyme NAD+, resulting in an aldehyde product [6]. The most important and intensely studied Phase I oxidation enzymes are the cytochrome P450 oxidase (CYP) family, which will be discussed in further detail below. Reductive metabolism occurs less frequently than oxidative metabolism and involves some members of the CYP family, such as CYP2A6 which is able to metabolize warfarin, via the conversion of a ketone to a hydroxyl group [5]. Xenobiotics that are esters, amides, hydrazides or carbamates can undergo hydrolysis by various Phase I enzymes. For example, the local anesthetic procaine is known to be hydrolysed by pseudo-cholinesterase into para-aminobenzoic acid, which is excreted by the kidneys into the urine [6]. Furthermore hydration reactions can occur through hydrolases, as can be seen in the metabolism of epoxides by the enzyme epoxide hydrolase to form 1,2-diols or glycols [6, 8].

1.2.2.1 Cytochrome P450 Oxidases

Cytochrome P450 oxidases (CYP) form a large family of heme enzymes that play a significant role in the metabolism of drugs, carcinogens, steroids and fatty acids [9]. CYPs are found in a variety of organisms including, plants, micro-organisms, and mammals; the latter having this enzyme system within all tissues, with an abundance found within the liver [10]. Within humans, 18 CYP families and 43 subfamilies exist, with 57 sequenced genes and 58 pseudogenes identified [9]. CYPs are involved in the Phase I metabolism of xenobiotics, frequently via the addition of a hydroxyl group making the xenobiotic more water soluble, increasing the ability of the modified compound to be excreted and metabolised by Phase II enzymes [9]. This process requires the presence of NADPH cytochrome P450 reductase as a source of electrons and molecular oxygen. Within the human CYP family, CYP 3A4 represents the most important isoenzyme for the metabolism of a variety of xenobiotics by humans. It's content is highest in the liver but it is also present in the small intestine [11]. CYP 3A4 has a broad substrate specificity and is involved in the Phase I metabolism of approximately 50% of currently used drugs [12]. Other members within the CYP3A subfamily include, CYP 3A5, CYP 3A7 and CYP 3A43. Due to a common polymorphism within intron 3 of the *CYP3A5* gene, observed in 50% of African-Americans and 90% of Caucasians, CYP 3A5 has a lower expression and reduced function within the liver than CYP 3A4, however higher levels of CYP 3A5 have been observed within the gut, kidney, prostate and lungs [13]. The polymorphic expression of CYP 3A5 within the adult and fetal liver, and an increased substrate specificity in comparison to CYP 3A4, has been observed in previous studies [14-16].

Additional to differences in substrate binding, it has been suggested some inhibitors of CYP 3A4 such as erythromycin and verapamil, differ in their inhibitory effect on CYP 3A5 activity [17,18]. CYP 3A7 activity is mainly observed in the fetal liver, with some reports suggesting expression of CYP 3A7 within adults [19]. The CYP 3A43 protein is expressed in the kidneys, testis and prostate, with low levels present within the liver [13].

1.2.3 Phase II Enzymes

The functional group, either originally present or following Phase I metabolism, on the xenobiotic undergoes conjugation by Phase II enzymes. This anabolic process generally requires the presence of a high-energy co-factor, and results in the conversion of the xenobiotic into a hydrophilic product that can be excreted in the bile or urine [6]. Conjugation reactions include glucuronyl, glycosyl, methyl, acetyl, sulfate, amino acid, fatty acid and glutathione groups [5]. For example, the glucuronidation of morphine requires the presence of the high-energy co-factor UDP-glucuronic acid and the enzyme UDP-glucuronosyltransferase, resulting in the production of the water-soluble *O*-glucuronide products, morphine-3-*O*-glucuronide and morphine-6-*O*-glucuronide [6, 20]. Acetylation reactions require the co-factor acetyl-CoA and are involved in the metabolism of aromatic amines and sulfonamides. Isoniazid, used in the treatment and prevention of tuberculosis is metabolized into a more water-soluble form in the presence of acetyl-CoA and the enzyme N-acetyltransferase, allowing for excretion within the urine [6]. An additional example of Phase II metabolism can be seen with the conjugation of a synthetic derivative of noradrenaline, isoprenaline. Isoprenaline

undergoes sulfate conjugation in the presence of the co-factor 3'-phosphoadenosine-5'-phosphosulfate and the enzyme phenol sulfotransferase [6].

Phase II enzymes have also been associated with reducing carcinogenesis within humans, due to their reported role in neutralizing reactive electrophiles, which are believed to be involved in the activation of protooncogenes or inactivation of tumour suppressor genes [21]. One type of Phase II enzymes, glutathione-S-transferases (GSTs), have been shown to play a primary role in reducing carcinogenesis the kidney, liver, gut and other tissues [6]. Polymorphisms within GSTs have been associated with increases in the susceptibility to carcinogenesis, as observed with glutathione-S-transferase π (GSTP1), where an amino acid substitution within the *GSTP1* gene has been associated with an increase in bladder, testicular and prostate cancer [22]. Other studies have suggested that silencing of the *GSTP1* gene, which is commonly found within prostate cancer patients, can be countered via the induction of other Phase II enzymes, which are co-localized within the prostate, using the compound sulforaphane [23]. The importance of Phase II enzymes in xenobiotic metabolism is evident, when considering that many of the active electrophiles that cause carcinogenic effects are produced from the initial Phase I metabolism of non-reactive chemical compounds. Following Phase II metabolism of these carcinogenic substrates, transport proteins aid in the excretion of the conjugated compounds, mainly through the bile and urine.

1.2.4 Phase 0 and Phase III Transport Proteins

The two main groups of transporters that influence the metabolism and excretion of xenobiotics is the solute carrier family of proteins and the family of ATP binding cassette (ABC) transporters. The solute carrier family of proteins contains approximately 225 transporters making it the largest superfamily. It includes organic anion transporting polypeptides (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs) and peptide cotransporters (PEPTs) [24]. The 11 human OATPs members identified are expressed in various organs such as the brain, liver, testis and kidney. They are involved in the transport of endogenous compounds such as thyroid hormones and conjugated steroids as well as xenobiotics [25]. OATs are involved in anionic transport and are expressed in tissues including the liver, kidney and brain [24]. The three OCTs identified for transport within humans are OCT1 which is primarily expressed in the liver, intestine and kidney; OCT2 is found within the kidney and dopamine-rich areas of the brain; and OCT3 has a transport function within the heart and placenta [24]. Two humans PEPTs are involved in the hydrogen-dependent transport of di- and tri-peptides, where PEPT1 functions as an influx transporter to increase oral drug absorption within the small intestine and PEPT2 contributes to drug reabsorption within the kidneys [24].

The ABC family of active transporters transport a variety of substances, into (influx) or out (efflux) of cells, which include amino acids, inorganic ions, peptides, metals and xenobiotics. Within humans, there are currently 48 identified transporters that are involved in the transport of substrates, via the hydrolysis of ATP [26]. ABC transporters are expressed as full transporters consisting of two transmembrane domains and two

nucleotide-binding domains, as seen with ABCB1, or as half-transporters that form an active hetero-dimer, as seen within the ABCG family [27]. Within the seven subfamilies, ABCA to ABCG, each transporter has a specific function such as cholesterol and lipid transport by ABCA1 and iron transport by ABCB6 [27]. Mutations in the genes encoding these ABC transporters result in several disease states. A mutation within the *ABCC7* gene, which expresses the cystic fibrosis transmembrane conductance regulator (CFTR), acts as a chloride ion transporter and has been associated to the development of cystic fibrosis [26].

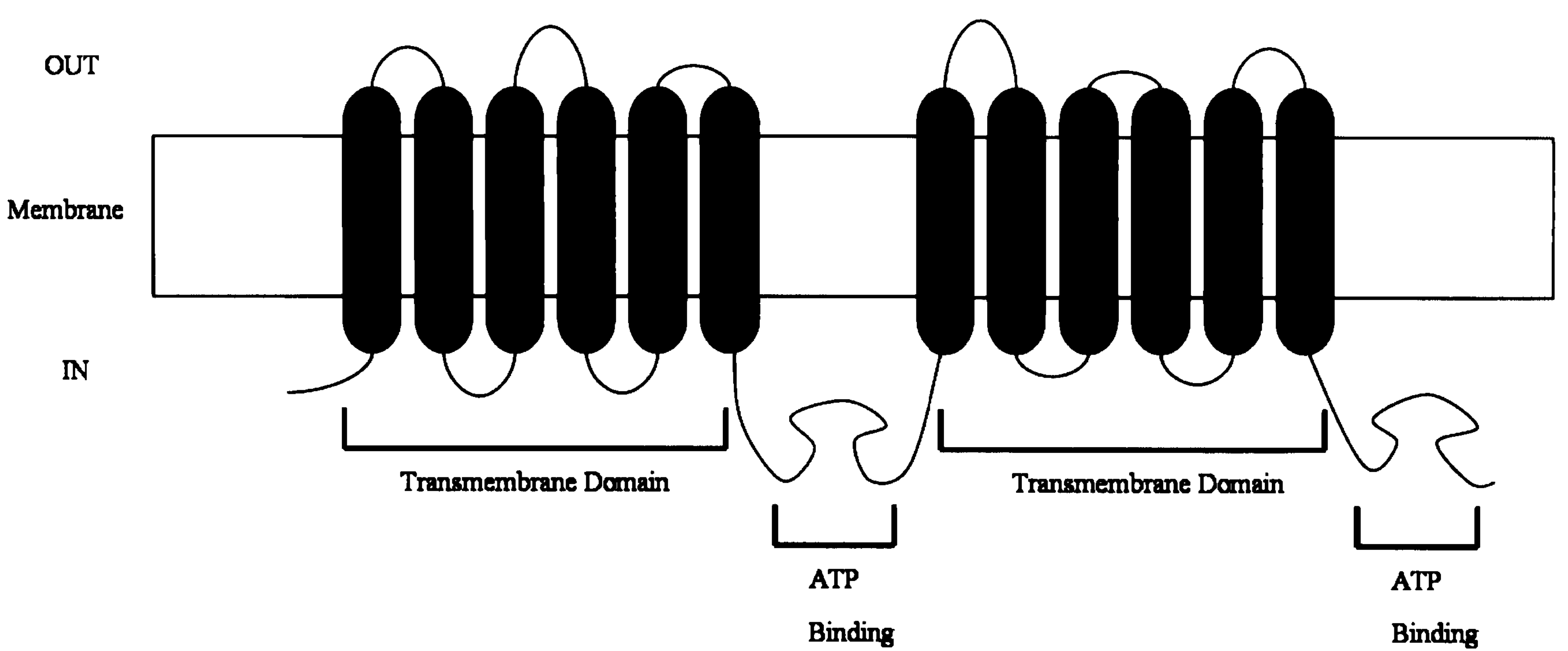
Four transporters – ABCB1, ABCC1, ABCC2 and ABCG2 – are believed to play a significant role in drug resistance [7]. Of these four, ABCB1 (P-glycoprotein, P-gp) plays the most significant role in drug resistance and will be discussed in further detail below. ABCC1, also known as the multi-drug resistance associated protein 1 (MRP1), is found throughout the body, with low levels within the liver [28,29]. It works in concert with P-gp to form the blood brain/placenta/testes barrier [30]. ABCC2, also known as the multi-drug resistance associated protein 2 (MRP2), is found mainly in the liver and kidney, and to a lesser extent in the intestine, placenta and blood-brain barrier [7]. Both ABCC1 and ABCC2 have been associated with the efflux of organic anions, conjugated compounds and amphipathic compounds [7,26]. ABCG2 is known for its resistance to chemotherapeutic agents such as mitoxantrone, doxorubicin and daunorubicin, and is also known as the breast cancer resistance protein [27,31]. Although expressed within a variety of tissues such as the breast, liver, small intestine and liver, ABCG2 has also been found to be highly expressed in placental tissue, where it is believed to transport

compounds between the blood-placental barrier [7, 26]. Transport proteins involved in drug metabolism transport Phase II metabolized products as discussed above, however also transport Phase I metabolized products or the original form of the xenobiotic via Phase 0 metabolism. Therefore there are several stages in which a xenobiotic or its metabolized product can undergo elimination. It should also be noted that the ABC transporters as well as other transporters involved in drug disposition may work in either conjunction or opposition to the actions of each other. For example, in the intestinal lumen ABCB1 and ABCC2 act as efflux pumps however ABCG2 and PEPT1 function as influx pumps for drugs which are substrates for these transporters [24]. An additional example is at the blood-brain barrier, where ABCB1, ABCC1, ABCC2 and ABCG2 work together as efflux transporters, whereas PEPT2 and members within the OATP and OAT families are involved in the influx of compounds at this site [24,32].

1.2.4.1 P-glycoprotein

P-glycoprotein (P-gp) is present within the cell membrane in several tissues and transports substances out of cells, by actively effluxing these substances into various excretory pathways, such as the bile, urine and the intestinal lumen (Figure 2). A consequence of P-gp's efflux action is the decreased cellular absorption of various therapeutic drugs. P-gp is found in various types of cells, such as hepatocytes, enterocytes, peripheral blood mononuclear cells (PBMCs), natural killer cells, and it is also expressed in tissues that have blood-tissue barriers such as the brain, testes, ovaries and placenta [33]. A broad spectrum of chemicals interacts with P-gp and a variety of

Figure 2: Illustration of P-glycoprotein (P-gp).
P-gp exists in the form of a dimer, consisting of two transmembrane domains and two nucleotide binding domains. The nucleotide binding domains are the site of ATP hydrolysis.


OUT
Membrane
IN
Transmembrane Domain
ATP
Binding
Transmembrane Domain
ATP
Binding

these substrates have an inhibitory effect on P-gp activity, with substrates such as valspodar or the surfactants Cremphor EL and Tween 80 acting as potent inhibitors of P-gp [34]. *In vitro* and animal model studies have shown that the antimycotic agent ketoconazole as well as the HIV protease inhibitors, in the following order of potency, saquinavir = nelfinavir > ritonavir = amprenavir > indinavir, have an inhibitory effect on P-gp activity [35-37]. Natural health products have also been shown to influence P-gp activity, as suggested in one study where St. John's Wort (*Hypericum perforatum*) increased expression and enhanced the drug efflux function of P-gp in PBMCs of healthy volunteers [2].

P-gp is encoded by the human *ABCB1* gene. Several studies have suggested that various single-nucleotide polymorphisms (SNPs) exist within the *ABCB1* gene [38-40]. Nonsynonymous SNPs result in a change in amino acids such as G2677T which causes a switch from alanine to threonine. Synonymous SNPs, such as C3435T and C1236T, do not result in a change in the encoded amino acid. However, these synonymous SNPs have been shown to influence P-gp expression and activity in some studies [38]. Of the 29 SNPs that have been reported, the polymorphisms C3435T and G2677T/A have been studied intensely and C1236T, to a lesser extent. Studies performed by Marzolini, have suggested that linkage disequilibrium exists between C3435T, G2677A/T and C1236T [41]. If a linkage disequilibrium exists, an SNP at C3435T for example would also result in an SNP at both G2677A/T and C1236T - leading to a haplotype relationship between all three loci. This haplotype relationship may provide an explanation for the increase in P-gp expression that some have observed when studying the synonymous C3435T

polymorphism [38,42]. Approximately 50% of the individuals studied contained this haplotype and it has also been characterized in a number of ethnic populations, including Caucasian, African American and Japanese [41].

1.2.5 Pregnane X Receptor

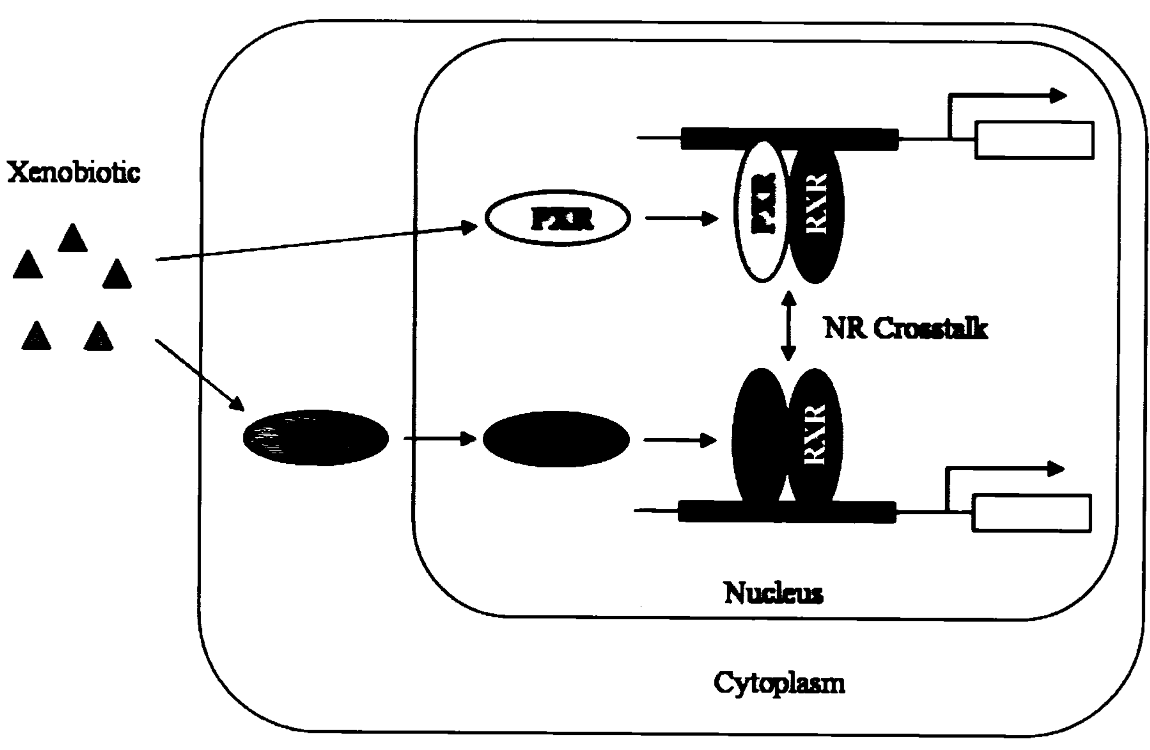
The pregnane X receptor (PXR), a member of the nuclear receptor family of ligand-activated transcription factors, is highly expressed within the liver and intestine. Similar to both CYP3A4 and P-gp, PXR binds to a wide range of hydrophobic compounds and upon activation forms a heterodimer with the 9-*cis* retinoic acid receptor (RXR) [43]. The formation of the PXR-RXR heterodimer complex, allows for regulation of the expression of various genes involved in xenobiotic metabolism, via the binding to response elements, such as *CYP 3A4, 3A5, 3A7, 3A23, 2B1, 2B6, 2C9, GSTs, sulfonotransferase, ABCB1* and *ABCC2* [43,44]. The PXR-RXR complex binds to one of four types of response elements which contain the half-site sequence AG(G/T)TCA either as a direct repeat with three (DR-3) or four (DR-4) nucleotides separating the response elements or an everted repeat with six (ER-6) or eight (ER-8) nucleotides separating the response elements [43]. For example, the PXR-RXR complex binds to the promoter region of the *CYP3A4* gene that contain DR-3 or ER-6 elements, whereas this heterodimer complex would bind to either DR-3, DR-4 or ER-6 elements within the *ABCB1* gene [43,44].

Although PXR is considered the primary nuclear receptor involved in increasing the expression of enzymes and transporters involved in xenobiotic metabolism, the

constitutive androstane receptor (CAR) has also been found to play a secondary role in this process [43,45]. The substrate specificity of CAR is not as broad as that observed with PXR, there is a 40% amino acid identity within their ligand-binding domains, however both nuclear receptors have been reported to bind to several xenobiotics and natural steroids [46]. Unlike PXR which is located within the nucleus, inactive CAR remains within the cytoplasm and upon ligand binding undergoes dephosphorylation allowing for translocation into the nucleus where it also forms a heterodimer with RXR (Figure 3) [46]. With both PXR and CAR are found in abundance within the liver, a major site of drug metabolism, and both of these receptors have been found to induce the expression of similar genes. Studies have suggested that CAR, like PXR, is able to induce the expression of *CYP2B* and *CYP3A* genes, binding to the same response elements, suggesting a cross-talk between the two receptors which would allow for compensation if the receptors malfunction [45,46]. Additional studies have suggested that CAR can also co-regulate other genes involved in drug metabolism including those that express members from the *CYP2C* family, *GSTs*, *sulfonotransferase* and the *ABCC2* gene [43].

Further to the fact that PXR and CAR have similar substrate specificities to CYP 3A4 and P-gp, many substrates that activate PXR and CAR also induce *CYP3A4* and *ABCB1* gene expression. The macrocyclic antibiotic, rifampician, is a known inducer of both CYP 3A4 and P-gp expression as well as an activator of PXR and CAR [43,44,46,47]. In other studies, the NHP St. John's Wort (*Hypericum perforatum*), has been suggested to activate PXR, and also induce the expression of both CYP3A4 and P-gp [4,48]. Both

Figure 3: Activation of PXR and CAR.
Upon the entrance of a xenobiotic into the cell via active or passive transport, activation of the constitutive androstane receptor (CAR) occurs through ligand binding allowing the nuclear receptor to translocate into the nucleus. Following entrance of the xenobiotic into the nucleus via active or passive transport, binding to PXR leads to activation of the nuclear receptor (NR). The activated PXR and CAR are able to heterodimerize with 9-*cis* retinoic acid receptor (RXR), and bind to their respective response elements on genes involved in drug metabolism allowing for the induction of gene expression. Since PXR and CAR bind to similar response elements, cross-talk between the two nuclear receptors exists.


Xenobiotic
PXR
PXR
RXR
NR Crosstalk
RXR
Nucleus
Cytoplasm

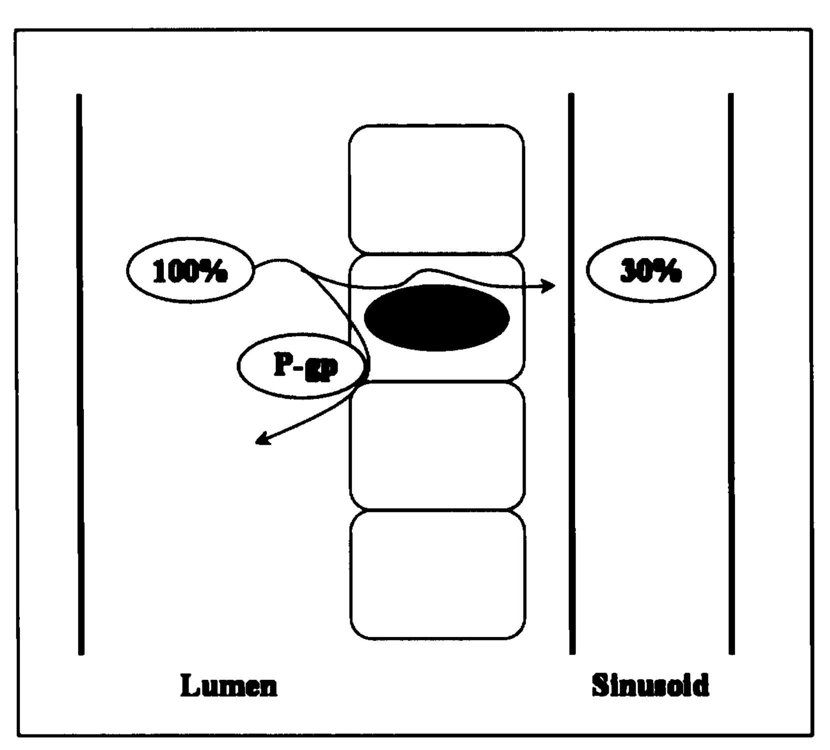
CYP 3A4 and P-gp, along with other Phase I and II enzymes and Phase III transporters, act collectively in the metabolism and excretion of xenobiotics (Figure 4), mainly within the liver but also within other tissues such as the intestinal tract [12]. Understanding the cooperative activity of Phase I and II enzymes and Phase III transporters, as well as the role of PXR and CAR in gene expression, provides a better appreciation of the various mechanisms involved in the metabolism of xenobiotics.

1.2.6 Natural Health Products

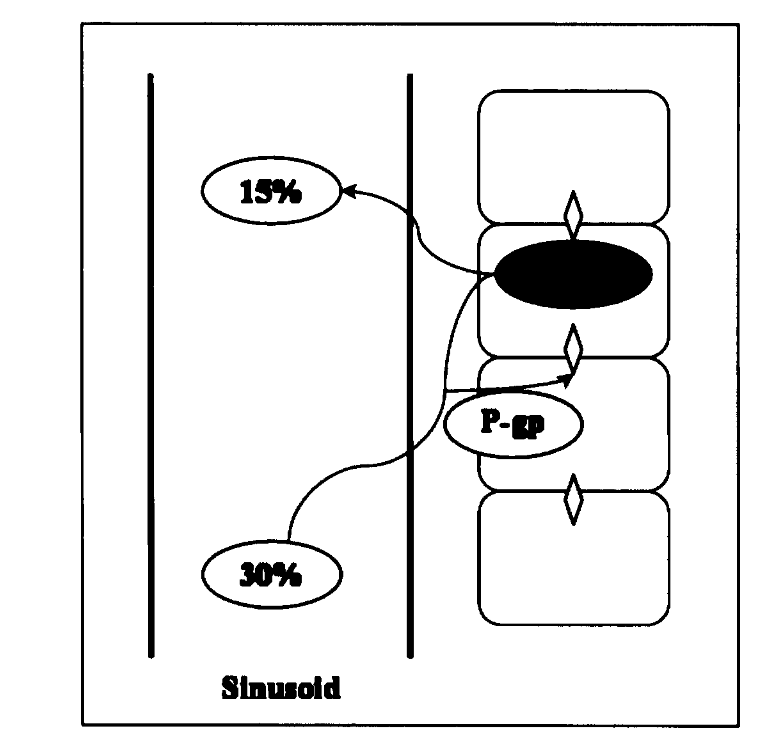
Natural health products (NHPs) include vitamins, minerals, herbal remedies, homeopathic and traditional medicines, amino acids, essential fatty acids and food products that are intended to produce a specific therapeutic effect. The effect of NHPs on drug metabolism varies with each product. One of the most intensely studied NHPs with respect to it's influence on drug metabolism has been St. John's Wort (*Hypericum perforatum*), which is commonly used in the treatment of depression [3]. The influence of St. John's Wort on drug metabolism was discovered after it was observed that patients taking conventional medication along with St. John's Wort, had a lower bioavailability of the conventional drug [49,50]. One study which considered the influence of co-administration of 900 mg daily of St. John's Wort for 14 days to healthy volunteers who were on steady-state indinavir, an HIV-protease inhibitor, observed a 57% decrease in plasma indinavir levels [51]. An additional study has suggested that healthy volunteers treated for 16 days with St. John's Wort, had increased P-gp expression and activity within their PBMCs [2], consistent with the possible role of St. John's Wort in activating PXR. The influence of St. John's Wort on drug metabolism can be further supported by a

Figure 4: Cooperative Action of P-gp and CYP3A4 in Drug Metabolism.
Illustration representing the mechanism of action of P-gp and CYP3A4 in the metabolism of a drug consumed orally. Metabolism in the small intestine, involves drug efflux on the apical side of enterocytes by P-gp. Drug absorbed by enterocytes are subject to metabolism by CYP 3A4, resulting in 30% of the drug remaining. Within the liver, drug transport into the bile for excretion is mediated by P-gp and CYP 3A4 metabolism within hepatocytes further reducing the bioavailability of the drug to 15%.

1) Enterocytes of small intestine



2) Hepatocytes of the liver



study performed within FVB mice, which were given 1250 mg/kg of St. John's Wort extract for 5 days and found to have a 3.7 fold and 3.5 fold increase in P-gp and CYP3A4 expression, respectively, within the small intestine compared to untreated mice [48]. Studies involving other NHPs such as grapefruit juice have been shown to have an inhibitory influence on P-gp similar to its known effect on CYP 3A4 [52]. An *in vitro* study which considered the influence of grapefruit juice on P-gp within HK-2 proximal tubular cells, found that following a 4 day exposure of grapefruit juice (0.05% to 5%) to the cells, a concentration dependent decrease in P-gp protein levels and mRNA expression was observed [52].

Studies performed within this lab have considered the influence of commercial extracts of NHPs, such as St. John's Wort and Echinacea, and found that between 1-2% of the full extract strength was able to significantly inhibit CYP 3A4 activity [53]. The influence of fresh garlic extracts on CYP 3A4, 3A5 and 3A7 activity was also tested within this lab, and demonstrated an inhibitory effect on all three CYP3A isoenzymes studied [54]. Although an inhibitory influence on P-gp efflux activity and CYP isoenzyme activity has been reported, as suggested in the studies mentioned above, long-term studies involving some NHPs, such as St. John's Wort, may result in the induction of P-gp and CYP activity [2-4]. One clinical study involving healthy male volunteers taking 300 mg daily of St. John's Wort for 14 days, observed increases in both P-gp and CYP 3A4 activity [3]. A second study observed a 4.2 fold increase in P-gp expression within isolated PBMCs of healthy volunteers, following a 16 day treatment of 600 mg of St. John's Wort three times daily [2]. The observed increase in P-gp expression following an extended

exposure to St. John's Wort is believed to be due to an activation of PXR, which in turn increases gene expression [4]. The biphasic effect observed suggests that consumption of a single dose, versus consumption of multiple doses over a period of time, of certain NHPs may have substantially different effects on the bioavailability of conventional medication taken concomitantly.

Preliminary experiments performed within this lab studied the possible influence that β-carotene may have on drug metabolism, and found a potential for interaction with some CYP isoenzymes. Therefore the *in vitro* influence of β-carotene as well other NHPs of interest, on P-gp and CYP3A isoenzyme activity was studied. Furthermore, the influence of β-carotene on P-gp expression in a clinical study was also examined. The NHPs that were studied and discussed for their influence on P-gp and CYP isoenzyme activity, included capsule formulations of β-carotene and two *Arctostaphylos uva-ursi* (Kinnikinnick) products, as well as raw materials which included three dried leaf varieties of Kinnikinnick, two dried varieties of *Acorus americanus* and *Acorus calamus* (Acorus), turmeric powder (*Curcuma longa*), chilli powder (*Capsicum annuum*) and black pepper (*Piper nigrum*) (Table 2, Appendix A).

1.2.6.1 Trans-β-carotene (β-carotene)

Trans-β-carotene (β-carotene), a member of the carotenoid family, has been reported to have several health benefits which include a decrease in the development of lung, stomach, oral, colon, breast, prostate, cervical and skin cancers [55]. The antioxidant properties of β-carotene (Figure 5A) in the prevention of disease is thought to be the

underlying mechanism, as has been suggested with the redox induction of transcription factors such as NF-κB resulting in growth-inhibitory and pro-apoptotic effects within tumour cells [56]. Within HIV infected individuals, the oxygen stimulated NF-κB also plays a role by increasing HIV replication within infected T-lymphocyte cells [57]. Due to common symptoms associated with HIV infection, such as general malabsorption and diarrhoea, decreased levels of micronutrients and antioxidants develop. Therefore HIV infected individuals commonly use β-carotene supplements, due to their antioxidant properties. It is thought that antioxidants decrease HIV replication via NF-κB stimulation. An additional reason for the use of β-carotene supplementation, with an average dosage between 6 mg and 30 mg, is for its reported benefits in the prevention of cardiovascular disease, due to its role in the limiting oxidation of low-density lipoprotein [58]. However, several studies provide conflicting results on whether β-carotene supplementation does cause any significant reducution in the risk of development of atherosclerosis [59,60]. Additional to disease prevention, studies have suggested β-carotene's involvement in drug metabolism. A study examining the effect of cartenoids on the expression of *ABCB1* and *CYP3A* genes, via the activation of PXR, suggested that β-carotene is able to increase the gene expression of *CYP 3A4, 3A5* and *3A7* by 60% and increase *ABCB1* gene expression by 70% within HepG2 cells [61]. There is limited research addressing the influence of β-carotene on the dual effect of P-gp and CYP isoenzymes, such as CYP 3A4, 3A5 and 3A7. With the increased consumption of β-carotene by individuals who are in a diseased state or at risk of disease, and therefore likely on a medication regimen, it is of interest to determine the influence that β-carotene would have on components involved in drug metabolism, namely P-gp and the CYPs.

1.2.6.2 *Arctostaphylos uva-ursi L.* (Kinnikinnick)

Arctostaphylos uva-ursi L., synonyms include *Arbutus uva-ursi*, Bearberry and Kinnikinnick; is used to treat a variety of conditions including urinary tract infections, inflammatory conditions of the efferent urinary tract, cystitis, urethritis, diuresis, constipation, lithuria, dysuria, acidic urine, pyelonephritis, and bronchitis [62]. The main active constituents include arbutin (hydroquinone-O-ß-D-glucopyranoside, Figure 5B), tannins (such as corilagin and tellimagrandin I), and hydroquinone [63]. The typical dose of dried herb is 1.5 - 2.5 grams three to four times daily either as a tea (use of cold water to reduce tannin content) or in a commercial preparation. The main active ingredient in Kinnikinnick, arbutin, is absorbed from the gastrointestinal tract unchanged and is hydrolyzed to hydroquinone in alkaline urine. There it can exert antiseptic and astringent effects [64]. Crude Kinnikinnick extract can be more effective than the constituent arbutin as an astringent and antiseptic [64]. The hydroquinone is eliminated in urine as glucuronic and sulfuric acid conjugates [65]. Kinnikinnick is considered safe for short-term oral use but there is concern over long-term use, mainly due to the presence of hydroquinone which can have mutagenic and carcinogenic effects. Hydroquinone can also cause nausea, vomiting, sense of suffocation, shortness of breath, cyanosis, convulsions, delirium and collapse [66]. Large amounts of hydroquinone are reportedly oxytocic, increasing the rapidity of labour, and amounts equivalent to 30-100 grams of Kinnikinnick can cause death [67]. An observational case reported a 56-year-old woman who ingested Kinnikinnick for three years developed a typical bull's-eye maculopathy bilaterally [68]. The influence of Kinnikinnick with P-gp as well as CYPs (3A4/A5/A7)

was an area of study in this thesis as there is currently limited information on known interactions and possible roles it may play in drug metabolism.

1.2.6.3 *Acorus americanus Raf. and Acorus calamus L.* (Acorus)

Acorus americanus Raf. and *Acorus calamus L.*, other names include Acorus, Sweet Flag, Calamus, Flagroot and Myrtle Flag, has been used historically by First Nations populations as well as Asian societies for medicinal, ritualistic and cosmetic purposes [69]. The root/rhizome of the plant is the commonly used portion, which is ground into a fine powder. Some reported uses of Acorus include the remedy of appetite loss, bronchitis, chest pain, cramps, diarrhoea and indigestion [70]. A typical dosage of Acorus is 1 to 4 grams per day in the form of a tea. The oils within Acorus contain active ingredients which include sesquiterpenes, phenylpropanes, ketones, tannins, asarone and β-asarone (Figure 5C) [70]. Of the two asarones, β-asarone is a known carcinogen, found within the *Acorus calamus* variety, but not within the *Acorus americanus* variety [71]. There are three general types of Acorus, each containing different proportions of β-asarones. The essential oil of the North American Acorus (*Acorus americanus)* does not contain β-asarones, the European Acorus contains 10% β-asarones and the Asian variety can contain up to 96% β-asarones [71].

Aside from medicinal purposes, Acorus has also been used for recreation drug purposes due to the ability of β-asarones to induce hallucinations [72]. A recent study investigating the use of herbal supplements for recreational purposes, in order to alter mood, perception or behaviour, discovered that *Acorus calamus* is an ingredient in 10%

of the products used [73]. With the increased use of Acorus for both medicinal and recreational purposes, further studies need to be performed to develop a better understanding of the influence that this product may have on drug metabolism. Currently, there are no known publications available which consider the possible influence that Acorus may have on P-gp and CYP activity, and whether any differences may exist between the *Acorus americanus* and *Acorus calamus* varieties on drug metabolism.

1.2.6.4 *Curcuma longa L.* (Turmeric Powder)

Turmeric Powder, obtained from the dried rhizome of the *Curcuma longa L.* plant, is a spice commonly used in a variety of Asian dishes [74]. A family of polyphenolic pigments known as curcuminoids are responsible for the yellow colour associated with this product. The major curcuminoids and their relative proportions are curcumin (77%), demethoxycurcumin (17%) and bisdemethoxycurcumin (3%) [75]. Curcuminoids, and specifically curcumin (Figure 5D), has been reported to be involved in the treatment and prevention of several diseases [76-79]. Several studies have associated the influence of curcumin supplementation with reductions in various forms of cancers, through a series of mechanisms which include acting as an antioxidant to reduce free radical production, inhibiting the activity of lipooxgenase as well as decreasing the expression of cyclooxgenase-2 [79-81]. A recent study has associated the use of curcumin supplementation to the suppression of NF-κB and IKK activities, resulting in anti-proliferation and pro-apoptotic effects within melanoma cells [77]. Curcumin supplementation has also been shown to have a positive influence in reducing cholesterol

levels and decreasing the risk in the development of atherosclerosis. A study involving Swiss albino rats on a cholesterol rich diet, observed lower levels of serum total cholesterol and LDL, and higher levels of HDL in rats whose diet included curcumin [78]. Other recent studies have also associated curcumin to the prevention of cystic fibrosis caused by the ΔF508 mutation within the CFTR [76]. It is believed that the mutated ΔF508 CFTR is functional, however undergoes degradation by proteasomes, resulting in a decrease in chloride permeability and an increase in sodium absorption [82]. The presence of curcumin has been shown to prevent the degradation of the ΔF508 CFTR, possibly by blocking calcium transport, thereby preventing calcium-dependent degradation of the mutated CFTR [76].

Whether or not turmeric powder, and specifically the more effective active ingredient curcumin in the form of supplements, provides any significantly positive clinical effects is still not agreed upon. However, reports suggesting benefits, strongly influences the decisions of patients looking for alternatives to relieve symptoms and diseases that are difficult to treat using conventional medicine. Aside from the potential clinical benefits, the possible impact that curcumin may have on drug metabolism also needs to be considered. Some studies have considered the influence of curcuminoids on drug metabolism, with a focus on the influence on P-gp activity and expression. One study which considered the *in vitro* influence of curcumin (50 μM) on P-gp activity within KB-C2 cells, found that following a 2 h incubation with curcumin, the intracellular levels of the P-gp substrate daunorubicin increased 2.5 fold, relative to untreated cells [83]. Another study which considered the influence of curcumin on P-gp activity and

expression within the KB-V1 carcinoma cell line discovered a dose-dependent, between 1 μM and 10 μM curcuminoid mixture, decrease in P-gp levels following a 72 h incubation [75]. The same study also found that bisdemethoxycurcumin was able to reduce P-gp levels by 88% and decrease *ABCB1* expression by 51%, compared to untreated cells, following a 72 h incubation [75]. If curcumin does have the ability to influence P-gp activity and expression as suggested above, a potential for adverse drug reactions may occur for individuals taking this NHP concomitant with conventional medication. Further studies need to consider the role that curcumin, and the more commonly consumed turmeric powder, may have on P-gp and CYP isoenzyme activity.

1.2.6.5 *Capsicum annuum L.* (Chilli Powder)

Chilli powder, obtained from ground chilli pepper fruits (*Capsicum annuum L.)*, contains a family of active compounds known as capsaicinoids, which provides the spicy sensation associated with the consumption of foods containing this product [84]. Capsaicinoids are produced within the glands of the white placenta found inside the fruit [84]. Within this family, the major capsaicinoids, and percent proportions are capsaicin (69%), dihydrocapsaicin (22%), nordihydrocapsaicin (7%), homocapsaicin (1%) and homodihydrocapsaicin (1%) [85]. Due to the large percentage within chilli powder, capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is considered the main active ingredient. Capsaicin (Figure 5E) is commonly found in peppers prepared in a variety of ways for human consumption however this compound can also be found and used in pepper spray for riot control, as well as for medicinal purposes in the relief of pain. The ability of capsaicin to relieve pain is related to it's capability to bind and activate the vanilloid

receptor subtype 1 (VR1) located within sensory neurons [86]. The activation of VR1 causes a depolarization of the neuron, resulting in a burning signal to the brain. Treatment of a painful site with a topical cream containing capsaicin, over stimulates the sensory neurons and results in a desensitization of the pain [87]. There are a limited number of studies that consider the influence of capsaicin on drug metabolism enzymes and transporters such as CYP and P-gp. One study that considered the *in vitro* influence of capsaicin on P-gp activity within KB-C2 cells, found an over three-fold increase in the intracellular concentration of the P-gp substrate daunorubicin, when treated with capsaicin at a concentration 100 μM, compared to untreated cells [83]. The same study furthermore presented the inhibitory influence of capsaicin on P-gp activity, with data suggesting a 75% decrease in the growth of KB-C2 cells in the presence of the cytotoxic chemical and P-gp substrate vinblastine (0.5 μM) when treated with 50 μM capsaicin, compared to untreated cells [83]. Further studies determining the influence of capsaicin on drug metabolism should be considered, as well as how the more commonly consumed forms containing capsaicin, such as chilli powder, would influence the activity of drug transporters such as P-gp and metabolizing enzymes which include members within the CYP3A subfamily.

1.2.6.6 *Piper nigrum L.* (Black Pepper)

Piperine, the main active ingredient found and responsible for the spicy taste within black pepper (*Piper nigrum L.*), is a member of the Piperaceae family (Figure 5F) [88]. Studies have suggested the capability of piperine to manipulate the bioavailability of therapeutic drugs through its influence on drug metabolizing enzymes such as those within the CYP

family and drug transporters including P-gp [89,90]. Other studies have suggested piperine's influence on bioavailability may be due to increasing the thermogenic properties, an increase in metabolic rate and xenobiotic absorption, of the epithelial cells involved in absorption within the gastrointestinal tract [91]. Through these mechanisms of action, piperine supplementation is considered useful in the co-administration with other supplements that are reported to aid in the prevention and treatment of diseases, which would have a reduced bioavailability if administered alone. For example, a clinical study has suggested that the co-administration of 20 mg of piperine with 2 g curcumin, increased the bioavailability of curcumin by 2000% [92]. An additional clinical study considered the influence of 5 mg piperine co-administration with 15 mg of β-carotene taken orally for 15 days, and discovered a 60% increase in serum β-carotene levels, which were suggested to be due to an increase in the thermogenic activity of epithelial cells [91]. Piperine supplementation has also been associated with the prevention of benzo(*a*)pyrene induced lung cancer in studies involving Swiss albino mice [93]. In the aforementioned study, mice initially exposed to 50 mg/kg benzo(*a*)pyrene for 4 weeks and then treated with 50 mg/kg piperine for 16 weeks, showed to have between a 35-40% decrease in levels of lipid peroxidation and protein carbonyls and increased levels of the anti-oxidant enzyme glutathione by 50% within lung tissue, compared to mice only administered 50 mg/kg benzo(*a*)pyrene for 4 weeks and maintained under normal conditions for 16 weeks [93]

Figure 5: Illustration of Active Constituents within Natural Health Products Studied.
Chemical structures of active constituents found within the natural health products (italics); **A** β-Carotene (*β-Carotenes supplements*), **B** Arbutin (*Arctostaphylos uva-ursi L.*), **C** β-Asarone (*Acorus calamus L.*), **D** Curcumin (*Curcuma longa L.*), **E** Capsaicin (*Capsicum annuum L.)* and **F** Piperine (*Piper nigrum L.*).

A

B

C

D

E

F

1.3 Rationale, Hypothesis and Objectives

1.3.1 Rationale

Some studies have suggested that the synonymous C3435T polymorphism within the *ABCB1* gene influences the activity of P-gp [38,42]. However other studies have suggested that the haplotype relationship between C3435T and G2677T/A, influences P-gp activity, mainly due to the possible role that the non-synonymous G2677T/A polymorphism may play [41]. The synonymous C1236T polymorphism is also believed to be linked to C3435T and G2677T/A, and may play a role in influencing the activity of P-gp [94,95]. A key question is whether a C3435T, G2677T/A, C1236T haplotype would affect drug disposition, and the potential role it may have on P-gp activity and expression.

The use of NHPs by individuals is increasing due to the reported potential benefits stated above. Patient populations that have few treatment alternatives to relieve symptoms associated with a disease may use NHPs more frequently. Carotenoids are commonly used within HIV infected populations, due to the claimed benefits such as immune modulation and as antioxidants. A study performed by Kim found that P-gp limits the oral bioavailability and penetration of the HIV protease inhibitors, indinavir, nelfinavir, and saquinavir into the brain [35]. Thus, in HIV infected individuals, P-gp's prevention of antiretroviral drugs from entering infected cells and tissues contributes to the development of sanctuary sites for HIV. P-gp's action in diminishing the amounts of drug entering HIV infected cells, also results in the undesirable development of viral

resistance to antiretroviral drugs. However, the clinical influence of β-carotene supplementation on P-gp expression and activity has not been determined.

Although published reports are available that consider the influence that the main active constituents within NHPs may have on P-gp and CYP activity, there is limited information available on the effect that the commonly consumed forms containing these active constituents may components involved in drug metabolism [75,83,90]. This represents the underlying rationale for this research project. For the NHPs that will be studied and discussed, active constituents comprise between 0.01% - 0.7% of the commonly consumed food source. Therefore, a better understanding of the influence the more commonly consumed food sources may have on the activities of P-gp and CYPs needs to be developed.

1.3.2 Hypotheses

1. It is hypothesized that the C3435T, G2677T/A, C1236T haplotype within the *ABCB1* gene of healthy male Caucasians, affects P-gp efflux activity and expression.

2. A 28 day β-carotene supplementation in HIV infected patients would influence P-gp expression levels.

3. The NHPs - β-carotene, Kinnikinnick, Acorus, turmeric powder, chilli powder and black pepper, will influence the efflux activity of P-gp and the isoenzyme activity of CYP 3A4, 3A5 and 3A7.

1.3.3 Objectives

1. To determine the C3435T, G2677T/A, C1236T haplotype in 80 healthy male Caucasian individuals. Following identification of the haplotype, P-gp efflux activity and mRNA expression within isolated PBMCs will be determined, and differences are to be compared between CC3435 and TT3435 individuals.
2. To determine the influence of a 28 day β-carotene supplementation on P-gp expression through a clinical study involving 14 HIV infected patients with a carotene deficiency.

3. To assess the influence of β-carotene, Kinnikinnick, Acorus, turmeric powder, chilli powder and black pepper on P-gp efflux activity, an *in vitro* study was performed within Caco-2 human intestinal carcinoma cells.

4. To test whether the metabolic activity of the Phase I enzymes, CYP3A4, 3A5 and 3A7 is influenced by the presence of β-carotene, Kinnikinnick, Acorus, turmeric powder, chilli powder and black pepper, *in vitro*.

In addition to the studies outlined above, an internship was performed through the APOGEE-Net program, which provided training in policy development related to genetic testing.

2 Materials and Methods

2.1 Substrates and Reference Compounds

The trans-β-carotene (β-carotene) and two *Arctostaphylos uva-ursi* (Kinnikinnick) products, in the form of single-entity capsules, and the bulk variety of *Curcuma longa* (turmeric powder), *Capsicum annuum* (chilli powder) and *Piper nigrum* (black pepper) studied, were purchased at local outlets. The dried, ground, bulk preparations of the three Kinnikinnick and two *Acorus americanus and Acorus calamus* (Acorus) products were obtained from suppliers. All tested substrates were assigned Nutraceutical Research Programme (NRP) accession numbers and vouchers stored in the herbarium, University of Ottawa (Table 2, Appendix A). Rhodamine 123 (Rh123) was obtained from Molecular Probes and dibenzylfluorescein (DBF) was obtained from BD Gentest. All other chemicals and solvents were of analytical grade.

2.2 Statistical Analysis

Statistical analysis for P-gp activity studies involving TT3435 and CC3435 patients and P-gp mRNA expression studies were performed using the Wilcoxon matched-pairs test or two sample test. Studies involving the determination of P-gp efflux activity and CYP activity in the presence of NHPs *in vitro* involved the paired t-test for statistical analysis. A p-value ≤ 0.05 would indicate that the null hypothesis; that there is no difference in between the two populations studied; is rejected, suggesting a statistically significant difference between the two populations.

2.3 Determination of C3435T, G2677T/A, and C1236T Haplotype

2.3.1 DNA isolation from human blood samples

Blood was collected in 5 ml Vacutainer EDTA tubes (Beckton-Dickinson) from patients. Genomic DNA was isolated using the QIAamp DNA Blood Midi Kit (Qiagen Inc.). Briefly, 2 ml of blood was incubated with 200 μl Qiagen protease and 2.4 ml Buffer AL for 10 min at 70°C. Following incubation, 2 ml of 100% ethanol was added and the sample was transferred to a QIAamp Midi column and centrifuged for 3 min at 1850 RCF. Following centrifugation, the sample was washed with buffers AW1 and AW2 separately for 1 min at 4500 RCF. Finally, the isolated DNA sample was eluted out of the QIAamp Midi column using 150 μl of distilled water. Samples were stored at -80°C until required for sequencing.

2.3.2 Sequencing

Sequencing was performed by the Ottawa Genome Centre. The purified amplicons, developed via PCR, were sequenced on a 3730 DNA Analyzer (Applied Biosystems) using Big Dye Terminator v3.1 according to the protocol recommended by the supplier. Removal of unincorporated fluorescent dyes was achieved by the use of CleanSeq Magnetic Bead Purification (AgenCourt). Elution of the purified sequencing products was performed in water and the products were loaded directly onto the 3730 DNA Analyzer for sequence detection. Basecalling and sequence analysis was performed with Seq Analysis 5.1 (Applied Biosystems). The resulting sequences were uploaded into Vector NTI and alignments to ABCB1 sequence NM_000927 were performed to generate the single nucleotide polymorphism calls. Primer sequences used for

sequencing the 3435, 2677 and 1236 loci are stated below, and all primer design was performed by the use of Vector NTI and synthesized by Invitrogen.

ABCB1 3435 Forward primer: 5'-TGCTTGATGGCAAACAAATAAA-3'
ABCB1 3435 Reverse primer: 5'-AGTGACTCGATGAAGGCATGTA-3'

ABCB1 2677 Forward primer: 5'-TTAGTTTGACTCACCTTCCCAG-3'
ABCB1 2677 Reverse primer: 5'-CAGGCTATAGGTTCCAGGCT-3'

ABCB1 1236 Forward primer: 5'-CCTCTGTGGGGTCATAGAGC-3'
ABCB1 1236 Reverse primer: 5'-TCCTGTGTCTGTGAATTGCC-3'

2.3.3 P-gp Transport Study with Isolated PBMCs

The following method has been adopted from Oselin et al., 2003 [96]. Blood was collected in 8 ml BD Vacutainer CPT with Sodium Heparin tubes, and centrifuged at room temperature in a horizontal rotor (swing-out head) for 20 min at 1800 RCF (IEC Centra MP4R). Following centrifugation, the buffy coat was transferred to a 15 ml polypropylene tube (Falcon) and fresh phosphate buffer solution (PBS; 0.5 M, pH 7.4) was added to a final volume of 15 ml. The tube was centrifuged for 15 min at 700 RCF followed by removal of the supernatant, and washed with an additional 10 ml of PBS involving a 10 min centrifugation. Following removal of the supernatant, the cell pellet was re-suspended in 2.5 ml of Dulbecco's modified Eagle media (DMEM, Gibco) supplemented with 10% FBS (Gibco) and the cells counted via a microscope (Leica DMIL) using a haemocytometer. A volume of 1×10^6 cells/ 988 µl of cell suspension was added to two 1.5 ml microfuge tubes each (control and test). To the control 12 µl of PBS was added and to the test 12 µl of Rh123 (final concentration 150 ng/ml) was added, followed by a 30 min incubation for both microfuge tubes at 37°C and 5% CO_2.

Following incubation, control and test microfuge tubes were centrifuged at 700 RCF for 5 min, supernatant removed, and washed twice with 1 ml of fresh PBS under the same centrifugation conditions. Following the final wash and centrifugation, the supernatant was removed and 1 ml of fresh PBS added to re-suspend the cells. A 300 μl aliquot of the cell suspension, in triplicate, was measured on a 96 well plate at 37°C for reading using a fluorometric microtiter plate assay (CytoFluor Series 4000 Multi-Well Plate Reader). Fluorescence was measured at 485 nm excitation and 530 nm emission, with a gain of 60.

2.4 Influence of trans-β-carotene on P-gp expression

2.4.1 Total RNA isolation from human blood samples

Five HIV-infected patients with a carotene deficiency were given 25,000 units of oral β-carotene (Exact), twice a day for 28 days. Prior to the β-carotene supplementation (day 0) and following the 28 day period, blood was collected in 8 ml BD Vacutainer CPT with Sodium Heparin tubes (Beckton-Dickinson) from patients and centrifuged at room temperature in a horizontal rotor (swing-out head) for 20 min at 1,800 RCF. Following centrifugation, the buffy coat was collected as a 2 ml sample and mRNA isolation was performed using the QIAamp RNA Blood Mini Kit (Qiagen Inc.). Briefly, 2 ml of buffy coat was incubated with 10 ml of Buffer EL for 15 min on ice and centrifuged at 400 RCF for 10 min at 4°C. Following removal of supernatant, the cell pellet was washed with another 4 ml of Buffer EL and centrifuged as described above. After discarding the supernatant, 600 μl of Buffer RLT was added to the pellet sample and the mixed solution was homogenized in a QIAshredder spin column for 2 min at 16,000 RCF. The

homogenized lysate was mixed with 600 µl of 70% ethanol and the total sample was placed in a QIAamp spin column and centrifuged for 15 sec at 8,000 RCF. Following centrifugation, the QIAamp spin column was washed with buffers RW1 (700 µl) and RPE (500 µl) for 15 sec at 8,000 RCF. Following washes, an additional 500 µl of Buffer RPE was added to the QIAamp spin column and centrifuged at 16,000 RCF for 3 min. The isolated mRNA sample was eluted out of the QIAamp spin column using 30 µl of supplied RNase-free water. Samples, both pre and post β-carotene, were stored at -80°C until further use.

2.4.2 Reverse Transcriptase-Polymerase Chain Reaction

The initial step of the reverse transcription reaction was performed containing a final concentration of 1.0 mM dNTP mix, 50 ng/µl Oligo$(dT)_{12-18}$, and DEPC-treated water with a final volume of 10 µl. The reaction mixture was incubated at 65°C for 15 min, and then cooled on ice for 5 min. The second step involved the addition of 10X RT buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 5 mM $MgCl_2$, 10 mM DTT, and 2 units/µl RNaseOUT recombinant ribonuclease inhibitor. Following an incubation at 42°C for 2 min, 2.5 units/µl of SuperScript II RT was added for a final volume of 20 µl. The reaction mixture was incubated at 42°C for 50 min, and the reaction was terminated at 70°C for 15 min. The cDNA samples were stored at -20°C until use for amplification. All components used were obtained from Invitrogen and reaction mixtures were incubated in a Mastercycler gradient (Eppendorf).

Amplification was performed with 3 μl of cDNA, containing a final concentration of 10X RT buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 mM of each primer, 0.1 units/μl of Taq DNA polymerase, and autoclaved, distilled water for a total volume of 50 μl. All components used are products of Invitrogen. Primer sequences were determined from published sequences of the *ABCB1* gene, and synthesized by Invitrogen [97].

ABCB1 Forward primer: 5'-GCAAATCTTGGGACAGGAAT-3'
ABCB1 Reverse primer: 5'-AGTAGCGATCTTCCCAGCAC-3'

DNA amplification was carried out in a Mastercycler gradient (Eppendorf). Following an initial denaturation step at 94°C for 2 min, 40 cycles were performed, including a denaturation at 94°C for 45 sec, followed by primer annealing at 60°C for 30 sec, and fragment extension at 72°C for 90 sec, with a terminal elongation at 72°C for 5 min. The expected size of the amplified *ABCB1* gene sequence was 174 bp.

2.4.3 Gel Electrophoresis of PCR Products

PCR products were run on a 7.5% polyacrylamide gel electrophoresis system (BioRad Laboratories) for 2 h at 100 volts. A 25 base pair DNA ladder and a 10% glycerol, bromophenol blue/xylene cyanol running dye were used. Following 30 min staining with 0.5 μg/ml ethidium bromide (Boehringer Mannheim) in PBS, DNA bands were visualised and recorded using a gel scanning system (Image Station 440-CF, Kodak).

2.5 Influence of NHPs on P-gp and P450 Activity

2.5.1 Aqueous and Organic Extractions

Stock solutions of the aqueous and organic extraction samples of the NHPs were prepared at room temperature under reduced lighting conditions. For extractions, samples were dissolved in either de-ionized water (aqueous) or pure methanol (organic) to the desired stock solution concentration in a 1.5 ml microfuge tube. The solution was centrifuged (Eppendorf Centrifuge 5415R) for 15 min at 15,000 RCF and supernatant collected for use.

2.5.2 Cell Culture and Rh123 Assay Procedure

2.5.2.1 Caco-2 Cell Line

Human adenocarcinoma colon C2BBe1 cells (ATCC # CRL-2102) were routinely sub-cultured, grown, and maintained horizontally in 25 cm^2 cell culture flasks (Nunclon) in Dulbecco's modified Eagle media (Invitrogen) supplemented with 20% FBS and 0.5% penicillin-streptomycin (Invitrogen). Cell culture health was routinely assessed by direct observation of cultured cells via microscopy (Leica DMIL) for contamination and cell density count comparisons. Confluent cells (8.0×10^5 cells/ml) were treated with 0.25% trypsin/EDTA (Invitrogen) and plated into a 24 well cell culture plate (Falcon) at a concentration of 4.5×10^5 cells/ml. Following a 48 h incubation at 37°C and 5% CO_2, cells were provided 938 μl of fresh media and incubated for an additional 24 h to reach 100% confluence which was determined by direct observation via microscopy.

The Rh-123 assay was carried out in the following manner. Briefly, following 100% confluence within 24 well cell culture plate, 12 μl Rh123 (final concentration 150 ng/ml) and 50 μl sample extraction was added to each well. Cell mixture was incubated at three different time points (15 min, 60 min and 18 h) at 37°C and 5% CO_2. Following incubation, cell wells were washed twice with PBS (0.5M, pH 7.4) and treated with 250 μl trypsin/EDTA. Trypsin/EDTA treated cells were incubated for 10 min at 37°C and 5% CO_2, followed by the addition of 750 μl of PBS to each well. A 300 μl aliquot of the cell suspension, in triplicate, was measured on a 96 well plate at 37°C for reading using a fluorometric microtiter plate assay (CytoFluor Series 4000 Multi-Well Plate Reader). Fluorescence was measured at 485nm excitation and 530nm emission, with a gain of 60.

2.5.2.2 THP-1 Cell Line

Human peripheral blood monocytes from patients with acute monocytic leukemia (ATCC# TIB-202) were routinely sub-cultured, grown, and maintained horizontally in 80 cm^2 cell culture flasks (Nunclon) in a Fisher Scientific 605 incubator set to 37°C and 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% penicillin-streptomycin (the above supplied by Invitrogen) and 1% β-mercaptoethanol (Sigma). Cell culture health was routinely assessed by direct observation of cultured cells via microscopy (Leica DMIL) for contamination and cell density count comparisons. Suspended cells in culture medium are centrifuged (Hettich Zentrifugen, Rotofix 32) at 300 RCF at room temperature for 10 min, then re-suspended in the fresh culture medium at the concentration of 1 x 10^6 cells/ 938 μl for experiments.

The Rh123 assay was carried out in the following manner. Briefly, 938 μl of THP-1 cells were plated into a 24 well cell culture plate (Falcon), along with 12 μl Rh123 (final concentration 150 ng/ml) and 50 μl of the sample extraction. Following incubation at the three different time points (15 min, 60 min and 18 h), cells were collected in 1.5 ml microfuge tubes and washed twice with PBS (0.5 M, pH 7.4) and centrifuged at 500 RCF (Eppendorf Centrifuge 5415R) for 5 min each. Following the final wash and centrifuge at 500 RCF the supernatant was discarded and the cell pellet was resuspended in 1 ml of fresh PBS. A 300 μl aliquot, in triplicate, of the cell suspension was read using a fluorometric microtiter plate assay as described for the Caco-2 cells.

2.5.3 Cytochrome P450 Assay Procedures

Aliquots (2 μl) of stock solutions from the NHP extracts were screened for their ability to inhibit CYP 3A4, 3A5 and 3A7 (Gentest) metabolism of dibenzylfluorescein (DBF) to fluorescein using an *in vitro* fluorometric microtiter plate assay (CytoFluor Series 4000 Multi-Well Plate Reader). Briefly, assays were performed with 2 μl of extract in clear-bottom, opaque-welled microtiter plates (96 well, Corning Costar). Control and control blank wells contained distilled water or 20% methanol, and test and test blank wells contained the aqueous or organic extract. All wells tested contained distilled water, β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH; Sigma Chemicals), and the enzyme substrate DBF (final concentration of 1 μM per well), for a total reaction volume of 200 μl. Control and test wells also contained live isoenzyme, within human liver microsomes, (1 μl for CYP 3A4, 3A5 and 3A7) in phosphate buffer solution (0.5 M, pH 7.4), and control blank and test blank wells contained denatured

isoenzyme in PBS. Comparative testing of CYP 3A4, 3A5 and 3A7 was previously done with balanced amounts of specific activity and protein content using a Gentest insect control [54]. For all assays, microsomes were rapidly thawed and mixed gently with the substrate solution. All microsomes were stored at -80°C until used and were not subjected to more than two freeze-thaw cycles. All samples were prepared in triplicate with the resultant percent inhibition calculations based on the mathematical combinations for the differences in fluorescence between the test/test-blank wells and the mean difference between each control and blank well. A positive control using the CYP inhibitor ketoconazole at a final concentration of 0.5 μg/ml, was run with every assay. All assays were performed under reduced lighting conditions, at 37°C, and fluorescence was measured at 485 nm excitation and 530 nm emission, with a gain of 50.

3 Results

3.1 Influence of *ABCB1* Polymorphisms on P-gp Activity and Expression

3.1.1 Identification of a C3435T, G2677T/A, and C1236T Haplotype

To determine the C3435T, G2677T/A, and C1236T haplotype, initially 80 healthy male Caucasian individuals were screened for the C3435T polymorphism. This was followed by screening of the 19 participating individuals for G2677T/A, and C1236T, resulting in 9 individuals being homozygous TT3435 and 10 individuals wild-type CC3435 (Table 1). Within the TT3435 population, 3 out of 9 individuals were also homozygous TT2677 and TT1236, whereas 5 out of 9 individuals were heterozygous GT2677 and CT1236. One individual was observed to be GT2677 and TT1236. Of the 10 individuals within the wild-type CC3435 population, 7 individuals were also homozygous wild-type GG2677 and CC1236, with two individuals being GA2677 and CC1236 and one individual being heterozygous GT2677 and CT1236.

3.1.2 Influence of C3435T Polymorphism on P-gp Efflux Activity

In order to determine whether any difference in P-gp activity is observed between the TT3435 and CC3435 populations, PBMCs were isolated from the 19 participating healthy male Caucasian individuals mentioned above, and treated with Rh123 (final concentration of 150 ng/ml). A quantification of the amount of Rh123 accumulated within the cells following a 30 min incubation was measured. The amount of Rh123 accumulated within the cells provides an inverse relationship to the efflux activity of P-gp. The percent increase in Rh123 within the isolated PBMCs, relative to individual controls, ranged between the TT3435 and CC3435 populations (Figure 6). Within the

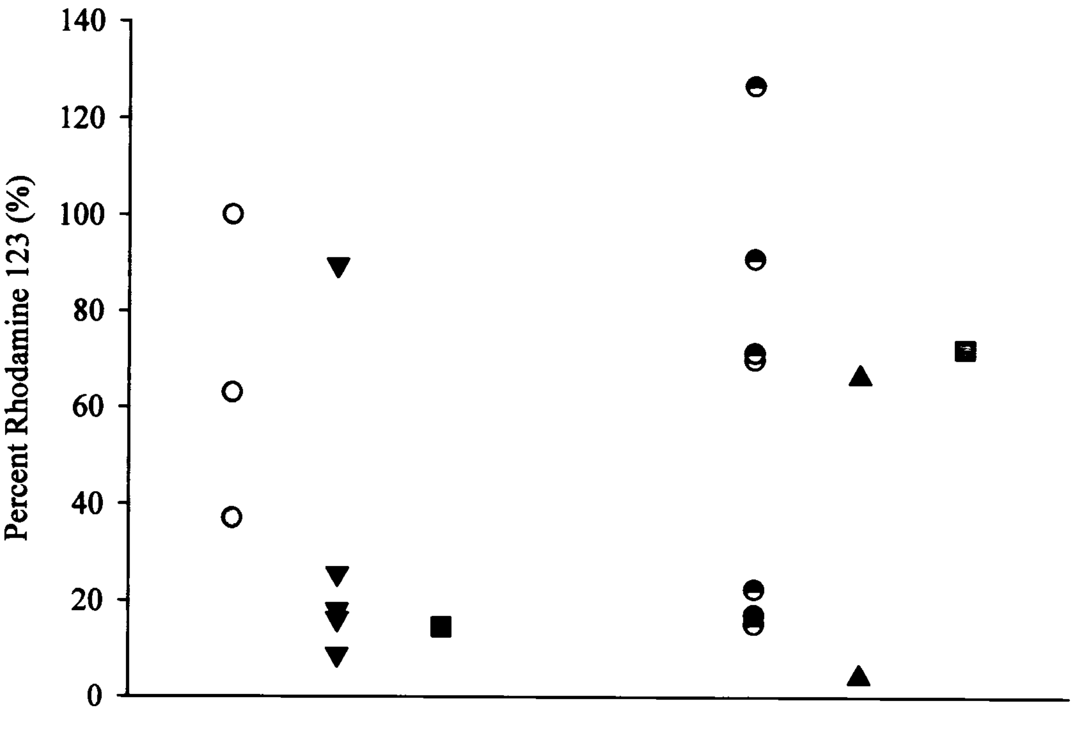
Table 1: Haplotype determination of TT3435 and CC3435 populations.

The 3435, 2677 and 1236 haplotype within the *ABCB1* gene was determined within TT3435 and CC3435 healthy male Caucasian individuals, n = 19, from isolated peripheral blood mononuclear cells (PBMC), using DNA sequencing.

TT3435 Individual Identification Number	Haplotype		CC3435 Individual Identification Number	Haplotype	
	G2677T/A	C1236T		G2677T/A	C1236T
1	TT	TT	24	GG	CC
6	TT	TT	26	GG	CC
128	TT	TT	37	GG	CC
9	GT	TT	51	GG	CC
10	GT	CT	53	GG	CC
33	GT	CT	113	GG	CC
52	GT	CT	151	GG	CC
75	GT	CT	136	GA	CC
140	GT	CT	150	GA	CC
			14	GT	CT

Figure 6: Scatter plot comparing P-glycoprotein (P-gp) efflux activity between TT3435 and CC3435 populations.

P-gp efflux activity was measured from isolated peripheral blood mononuclear cells (PBMC) of healthy male Caucasian individuals containing a TT3435 or CC3435 genotype and one of six haplotype variations, with G2677T/A and C1236T, as stated within the legend. Following a 30 min incubation at 37°C and 5% CO_2, the amount of rhodamine 123 (Rh123) accumulated within the PBMCs was measured using a CytoFluor Series 4000 Multi-Well Plate Reader. (% Rh123, n = 19 individuals. Each individual value represents triplicate readings. The percent Rh123 was calculated relative to individual Rh123-free PBMC controls.) A p-value of 0.44 between the TT3435 and CC3435 populations was obtained using the two sample Wilcoxon test, indicating no statistically significant difference between the two populations.



○ TT3435, TT2677, TT1236
▼ TT3435, GT2677, CT1236
■ TT3435, GT2677, TT1236
◒ CC3435, GG2677, CC1236
▲ CC3435, GA2677, CC1236
▣ CC3435, GT2677, CT1236

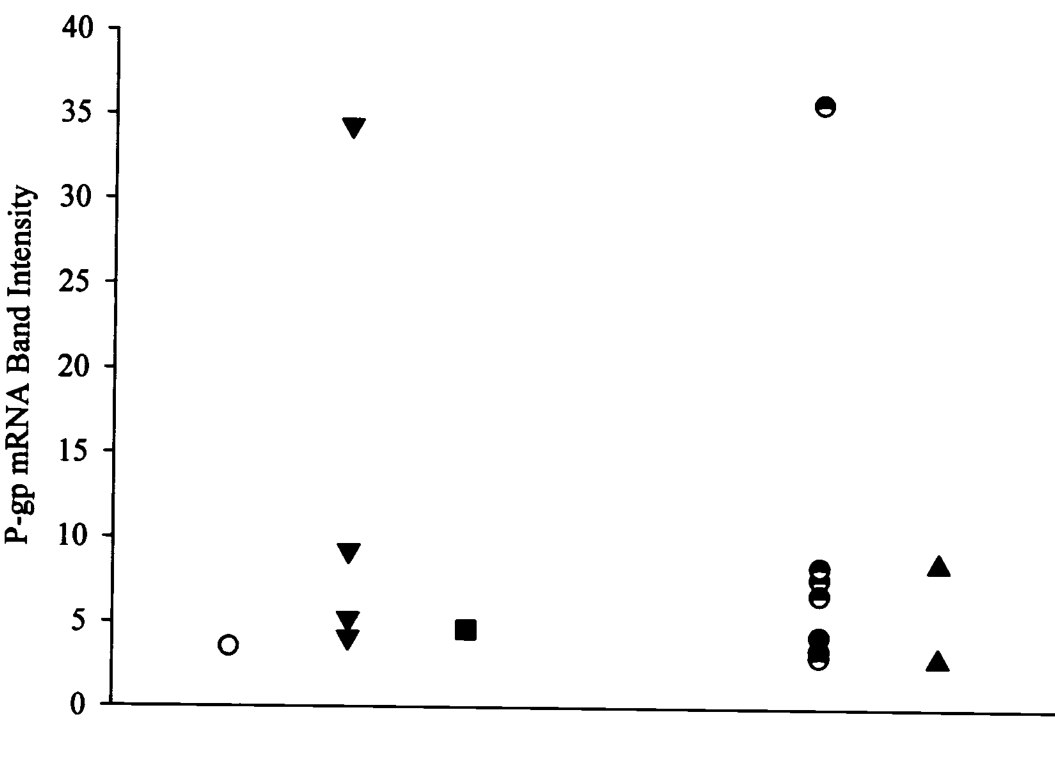
TT3435, TT2677 and TT1236 sub-population Rh123 levels were between 36.9% ± 6.6% and 99.8% ± 7.3%, and the single TT3435, GT2677 and TT1236 individual resulted in Rh123 levels at 14.6% ± 9.3%. Isolated PBMCs from the TT3435, GT2677 and CT1236 sub-population resulted in Rh123 levels between 8.8% ± 8.6% and 89.5% ± 14.6%. Within the CC3435 population Rh123 values ranged between 15.0% ± 11.8% and 126.6% ± 85.4% for PBMCs isolated from CC3435, GG2677 and CC1236 individuals. The two CC3435, GA2677 and CC1236 individuals were observed to have Rh123 values of 4.1% ± 2.5% and 66.3% ± 2.0% and an Rh123 value of 72.1% ± 25.2% was observed for the single CC3435, GT2677 and CT1236 individual. Statistical analysis between the TT3435 and CC3435 populations was obtained using the two sample Wilcoxon test, and provided a p-value of 0.44, indicating no statistically significant difference between the two populations.

3.1.3 Influence of C3435T Polymorphism on P-gp mRNA Expression

In order to determine whether any difference in P-gp mRNA expression exists between the TT3435 and CC3435 populations, PBMCs were isolated and collected from the 15 participating healthy male Caucasian TT3435 and CC3435 individuals. Following isolation of total RNA, amplification and gel electrophoresis of P-gp mRNA, data from band intensities were analysed and adjusted based on total RNA concentrations, in order to obtain a P-gp mRNA value representing a band intensity per total RNA concentration (Figure 7). The six individuals from the TT3435 population exhibited a range of P-gp mRNA values. Isolated PBMCs from the TT3435, GT2677 and TT1236 individual and TT3435, TT2677 and TT1236 individual, consisted of P-gp mRNA values of 4.6 and 3.5,

Figure 7: Scatter plot comparing P-glycoprotein (P-gp) mRNA expression between TT3435 and CC3435 populations.

P-gp mRNA levels were measured from isolated peripheral blood mononuclear cells (PBMC) of healthy male Caucasian individuals containing a TT3435 or CC3435 genotype and one of five haplotype variations, with G2677T/A and C1236T, as stated within the legend, within the *ABCB1* gene. P-gp mRNA expression levels were determined using reverse-transcriptase followed by cDNA amplification using PCR methodology. Samples were analysed using a polyacrylamide gel electrophoresis system and band intensity measured using a Kodak 440-CF band analysis system. (Average mRNA band intensity, adjusted based on total RNA concentration within sample, n = 15 patients. Each patient value represents triplicate readings). A p-value of 0.78 between the TT3435 and CC3435 populations was obtained using the two sample Wilcoxon test, indicating no statistically significant difference between the two populations.



40
35
30
25
20
15
10
5
0
P-gp mRNA Band Intensity
Haplotype
TT3435, TT2677, TT1236
TT3435, GT2677, CT1236
TT3435, GT2677, TT1236
CC3435, GG2677, CC1236
CC3435, GA2677, CC1236

respectively. The four individuals within the TT3435, GT2677 and CT1236 sub-population exhibited P-gp mRNA values between 4.1 and 34.3. Within the CC3435 population, the 9 individuals tested included two sub-populations. Isolated PBMCs from the CC3435, GG2677 and CC1236 sub-population comprised of P-gp mRNA values between 3.0 and 35.6. The two individuals within the CC3435, GA2677 and CC1236 sub-population had P-gp mRNA values of 2.9 and 8.5. This data was also presented in table form (Tables 3A and 3B, Appendix A). Statistical analysis between the TT3435 and CC3435 populations was obtained using the two sample Wilcoxon test, and provided a p-value of 0.78, suggesting no statistically significant difference in P-gp mRNA expression between the two populations.

3.2 Influence of Trans-β-carotene (β-carotene) on P-gp Expression

The initial study planned for 14 HIV infected patients, with a carotene deficiency, to be provided with 25,000 units of β-carotene twice daily, in order to determine if in any influence on P-gp expression occurs following supplementation for 28 days. The results presented from this study, is based on data obtained from 5 HIV infected patients willing to participate. The effect of a 28-day β-carotene supplementation regimen on P-gp expression levels within HIV infected patients was measured from isolated PBMCs and compared to expression levels prior to β-carotene supplementation. The average P-gp mRNA band intensity following β-carotene supplementation was adjusted based on the ratio of RNA concentrations between the samples collected from each patient pre and post supplementation. From the five patients tested, the increase in P-gp mRNA expression following β-carotene supplementation ranged between -27.1% ± 7.7% and

49.1% ± 15.0% (Figure 8). A representation of this data is also presented in table form (Table 4, Appendix A). Statistical analysis between the pre- and post- β-carotene treatment P-gp mRNA samples was obtained using the matched-pairs Wilcoxon test, and provided a p-value of 0.31, meaning no statistically significant difference in P-gp mRNA expression.

3.3 Influence of NHPs on P-gp and CYP Activity

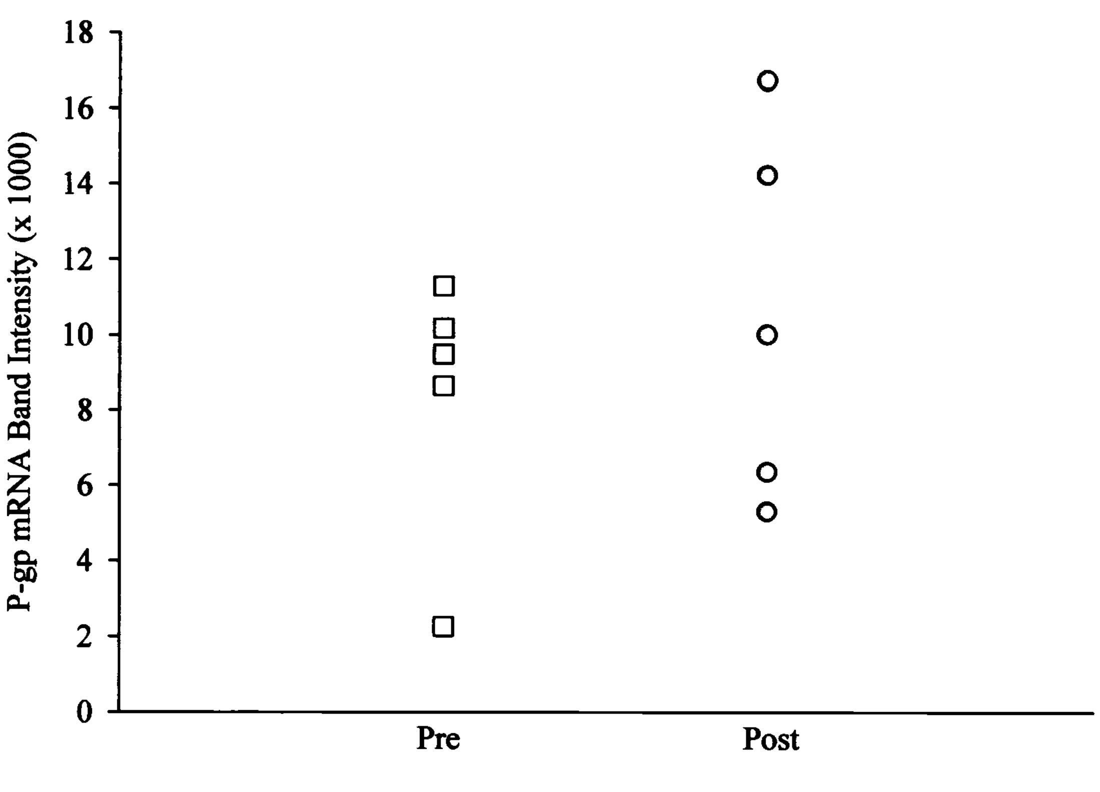
3.3.1 Trans-β-carotene (β-carotene)

The influence of aqueous and methanolic extracts of β-carotene (NRP 157) supplements on the efflux activity of P-gp using Caco-2 cells *in vitro* was determined. The percent of Rh123 accumulated in the cell provides an inverse relationship to P-gp efflux activity. Aqueous and methanolic extracts of β-carotene (5 mg/ml) did not influence P-gp efflux activity at 15 min, whereas a slight decrease, 10.4% ± 1.9%, in Rh123 levels was observed for the methanolic extract of β-carotene at 60 min relative to the control (Figure 9). At 18 h, neither aqueous and methanolic extracts of β-carotene produced noticeable differences in the levels of Rh123 accumulation.

An *in vitro* study was performed to test whether the metabolic activity of the Phase I enzymes, CYP 3A4, 3A5 and 3A7, is influenced by extracts of β-carotene. The metabolic activity of these CYPs was measured via the metabolism of DBF to the metabolite fluorescein [98]. Neither aqueous or methanolic extracts of β-carotene inhibited CYP 3A4 metabolic activity, but instead resulted in a 93.4% ± 5.1% enhancement in the metabolism of DBF by the 1.7 mg/ml aqueous extract of β-carotene and 154.7% ± 2.8%

Figure 8: Scatter plot representing the effect of β-carotene supplementation on P-glycoprotein (P-gp) mRNA expression.

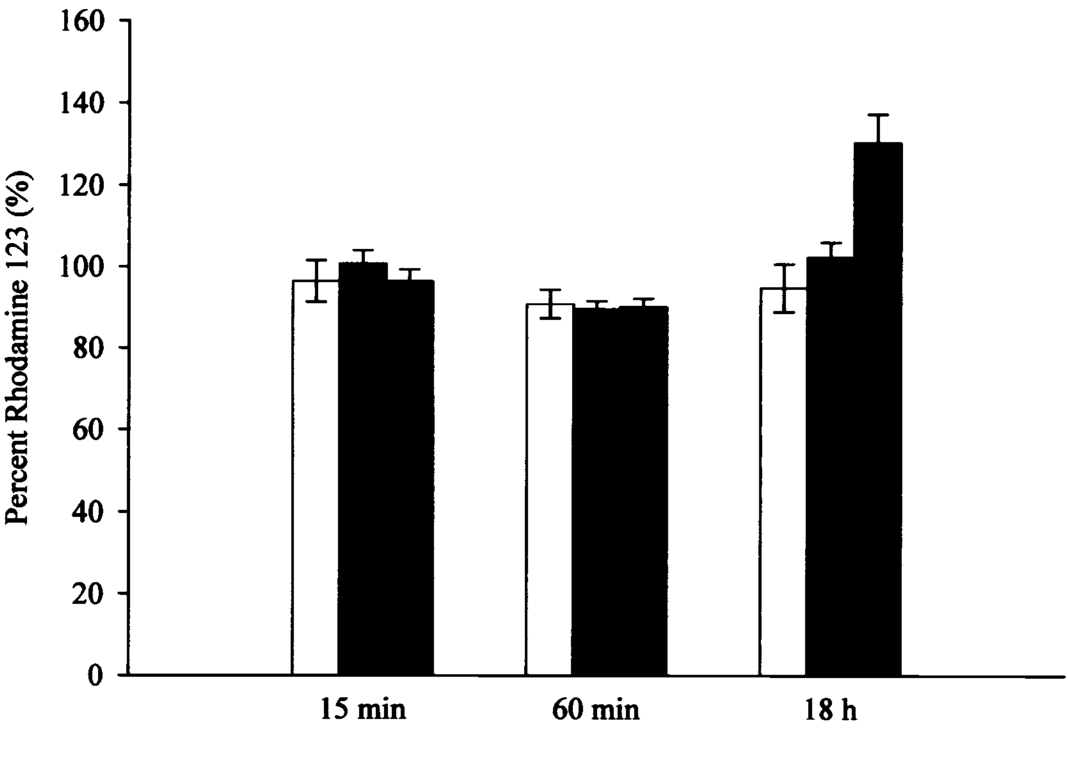
P-gp mRNA levels were measured from isolated peripheral blood mononuclear cells (PBMC) pre- and post- β-carotene (NRP 157) supplementation of HIV infected patients. P-gp mRNA expression levels were determined using reverse-transcriptase followed by cDNA amplification using PCR methodology. Samples were analysed using a polyacrylamide gel electrophoresis system and band intensity measured using a Kodak 440-CF band analysis system. (Average mRNA band intensity ± SD, adjusted based on total RNA concentration within sample, n = 5 patients. Each patient value represents triplicate readings). A p-value of 0.31 between the pre- and post- β-carotene samples was obtained using the matched-pairs Wilcoxon test, indicating no statistically significant difference in P-gp mRNA expression between the pre- and post- β-carotene samples.



P-gp mRNA Band Intensity (x 1000)
18
16
14
12
10
8
6
4
2
0
Pre
Post
β-Carotene Supplementation

Figure 9: Influence of aqueous and methanolic extracts of β-carotene on P-glycoprotein (P-gp) efflux activity within Caco-2 cells.

The efflux activity of P-gp within Caco-2 cells was determined using the fluorescent substrate rhodamine 123 (Rh123; 150 ng/ml) in the presence of freshly prepared aqueous and 55% methanolic extracts of β-carotene (NRP 157). Following 15 min, 60 min and 18 h incubations at 37°C and 5% CO_2, the amount of Rh123 accumulated within the Caco-2 cells was measured using a CytoFluor Series 4000 Multi-Well Plate Reader. (Control = 100%, % Rh123 increase ± SD; 100 mg/ml aqueous and methanolic stock extract, n = 6. Ritonavir is the positive control. The final concentration of aqueous and methanolic extracts of β-carotene in the reaction medium is equivalent to 5 mg/ml). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in P-gp efflux activity in Caco-2 cells treated with β-carotene compared to untreated controls.


Percent Rhodamine 123 (%)
160
140
120
100
80
60
40
20
0
15 min
60 min
18 h
Aqueous
Methanolic
Ritonavir (27.5 μg/ml)

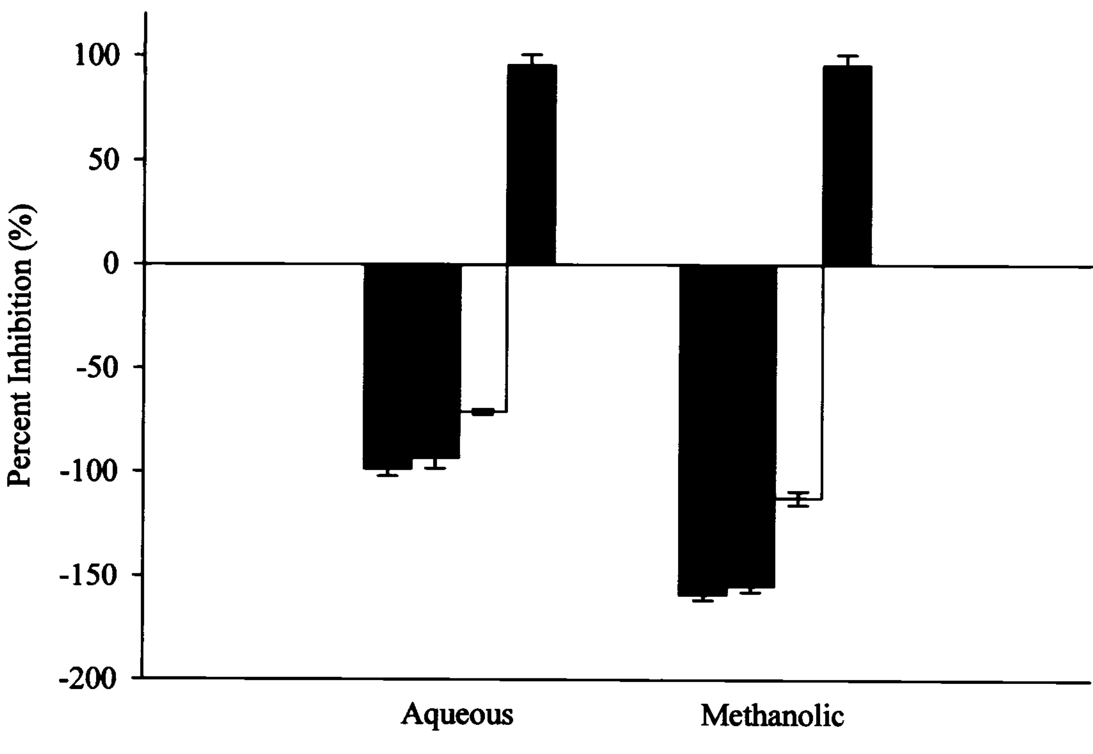
enhancement in DBF metabolism by the 1.7 mg/ml methanolic extract of β-carotene (Figure 10), with similar values observed at 3.3 mg/ml and 1.1 mg/ml. The influence of the aqueous extracts of β-carotene on CYP 3A5 activity was not similar to the results mentioned with CYP 3A4, where no influence on CYP 3A5 activity was observed (Figure 11). However, the presence of the methanolic extracts of β-carotene strongly influenced CYP 3A5 metabolic activity resulting in a 51.4% ± 1.3%, 76.9% ± 2.7% and 107.3% ± 1.6% enhancement in DBF metabolism at 3.3 mg/ml, 1.7 mg/ml and 1.1 mg/ml, respectively. The influence of the aqueous extracts of β-carotene on CYP 3A7 activity between the three concentrations tested resulted in no inhibition at 3.3 mg/ml and 1.1 mg/ml and a weak, -12.1% ± 0.8%, enhancement in DBF metabolism at 1.7 mg/ml (Figure 12). The methanolic extracts of β-carotene influenced the activity of CYP 3A7 with 55.9% ± 4.5%, 41.7% ± 6.6% and 21.2% ± 3.8% inhibition at 3.3 mg/ml, 1.7 mg/ml and 1.1 mg/ml, respectively.

3.3.2 *Arctostaphylos uva-ursi* (Kinnikinnick)

Aliquots of 5 extracts of 2 single-entity capsules and 3 bulk products of *Arctostaphylos uva-ursi* (Kinnikinnick) were tested for their effects on P-gp efflux of Rh123 *in vitro*, in Caco-2 cells. The influence of each aqueous extract of Kinnikinnick (5 mg/ml) on Rh123 levels differed at the three time points studied (Figure 13). At the 18 h time point, all extracts resulted in substantially lower Rh123 levels within the Caco-2 cells, relative to controls. A considerable range in Rh123 levels was observed for the aqueous extract of NRP 247A at the three time points studied, with a 44.9% ± 11.6% increase in Rh123 at

Figure 10: Influence of aqueous and methanolic extracts of β-carotene on CYP 3A4 isoenzyme activity *in vitro*.

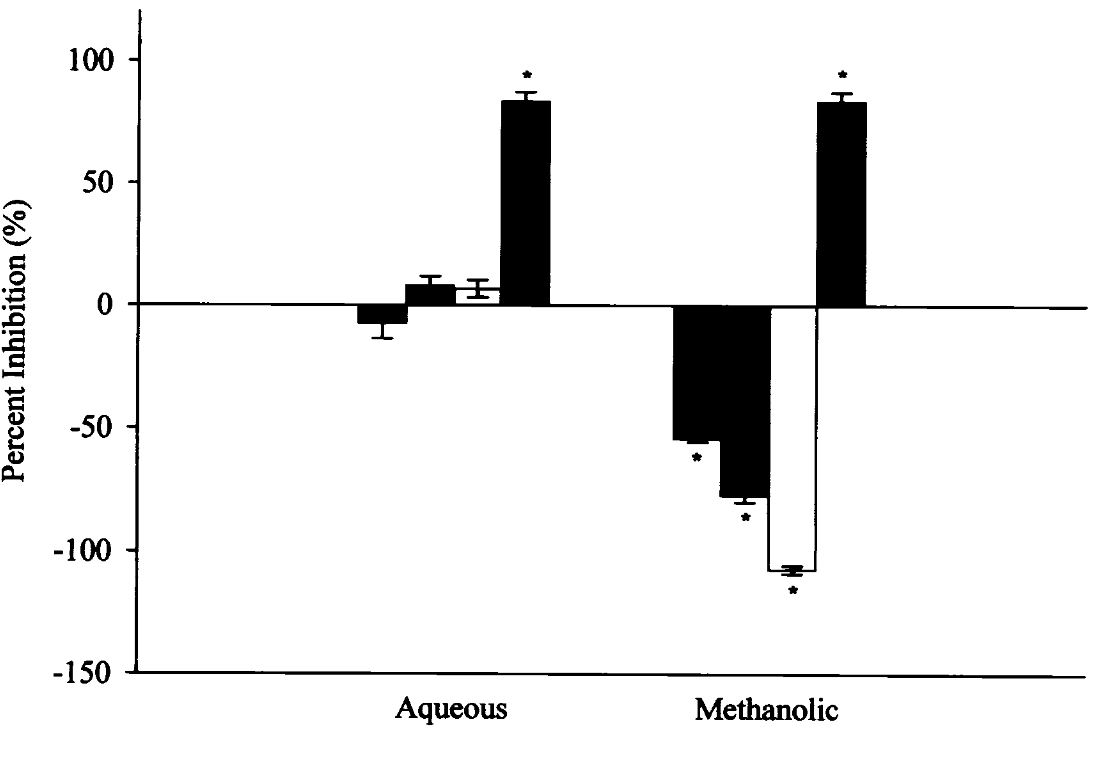
The enzymatic activity of CYP 3A4 in the metabolic conversion of 200 μM dibenzylfluorescein to fluorescein was assessed in the presence of freshly prepared aqueous and methanolic extracts of β-carotene (NRP 157). Following a 20 min incubation at 37°C, enzymatic activity was measured based on fluorescence produced using a CytoFluor Series 4000 Multi-Well Plate Reader. (% Inhibition ± SD, n = 6. Ketoconazole is the positive control. The final concentrations of extract in the reaction medium are equivalent to what is stated within the legend). All data presented have p-values ≤ 0.05 using the paired t-test, indicating a statistically significant difference in CYP 3A4 isoenzyme activity when treated with β-carotene compared to untreated controls.



Percent Inhibition (%)
100
50
0
-50
-100
-150
-200
Aqueous
Methanolic
3.3 mg/ml
1.7 mg/ml
1.1 mg/ml
Ketoconazole (0.5 μg/ml)

Figure 11: Influence of aqueous and methanolic extracts of β-carotene on CYP 3A5 isoenzyme activity *in vitro*.

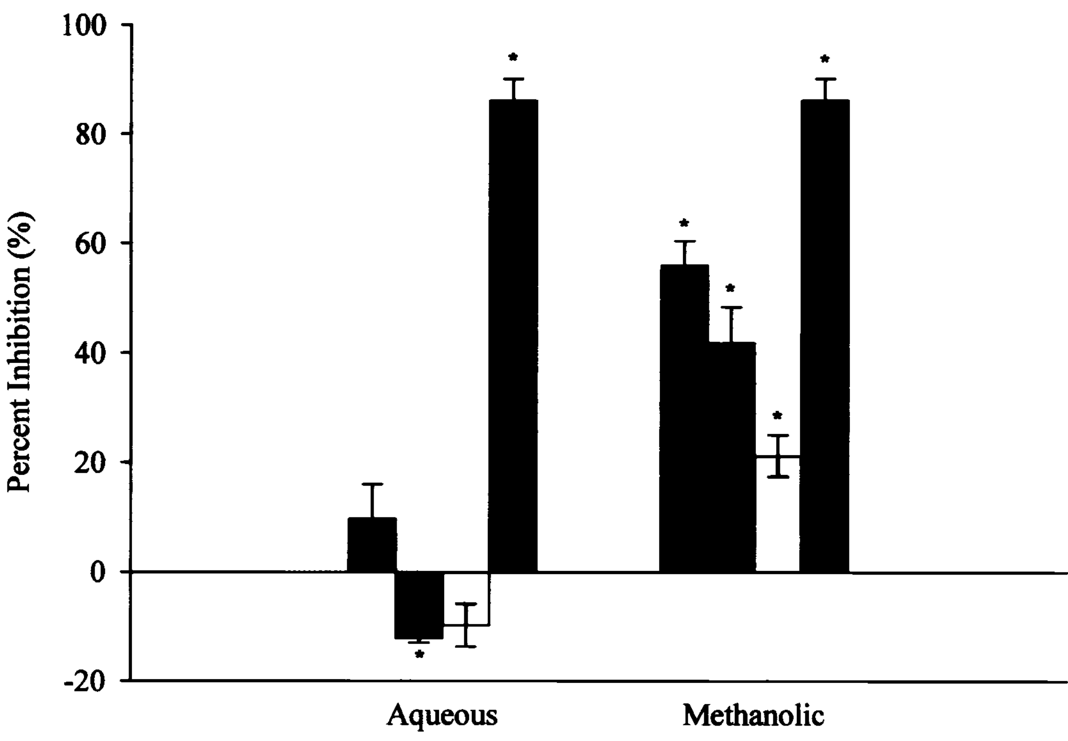
The enzymatic activity of CYP 3A5 in the metabolic conversion of 200 μM dibenzylfluorescein to fluorescein was assessed in the presence of freshly prepared aqueous and methanolic extracts of β-carotene (NRP 157). Following a 20 min incubation at 37°C, enzymatic activity was measured based on fluorescence produced using a CytoFluor Series 4000 Multi-Well Plate Reader. (% Inhibition ± SD, n = 6. Ketoconazole is the positive control. The final concentrations of extract in the reaction medium are equivalent to what is stated within the legend). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in CYP 3A5 isoenzyme activity when treated with β-carotene compared to untreated controls.



Percent Inhibition (%)
100
50
0
-50
-100
-150
Aqueous
Methanolic
3.3 mg/ml
1.7 mg/ml
1.1 mg/ml
Ketoconazole (0.5 μg/ml)

Figure 12: Influence of aqueous and methanolic extracts of β-carotene on CYP 3A7 isoenzyme activity *in vitro*.

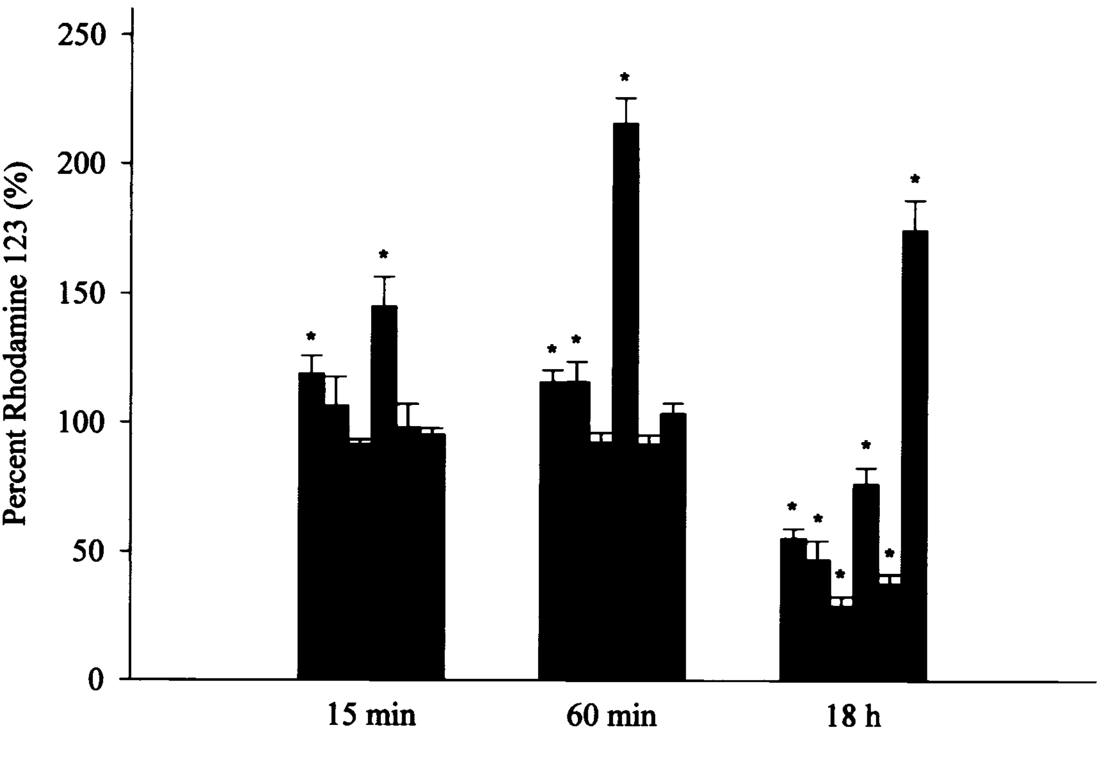
The enzymatic activity of CYP 3A7 in the metabolic conversion of 200 μM dibenzylfluorescein to fluorescein was assessed in the presence of freshly prepared aqueous and methanolic extracts of β-carotene (NRP 157). Following a 40 min incubation at 37°C, enzymatic activity was measured based on fluorescence produced using a CytoFluor Series 4000 Multi-Well Plate Reader. (% Inhibition ± SD, n = 6. Ketoconazole is the positive control. The final concentrations of extract in the reaction medium are equivalent to what is stated within the legend). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in CYP 3A7 isoenzyme activity when treated with β-carotene compared to untreated controls.



100
80
60
40
20
0
-20
Percent Inhibition (%)
Aqueous
Methanolic
3.3 mg/ml
1.7 mg/ml
1.1 mg/ml
Ketoconazole (0.5 µg/ml)

Figure 13: Influence of aqueous extracts of *Arctostaphylos uva-ursi* (Kinnikinnick) on P-glycoprotein (P-gp) efflux activity within Caco-2 cells.

The efflux activity of P-gp within Caco-2 cells was determined using the fluorescent substrate rhodamine 123 (Rh123; 150 ng/ml) in the presence of freshly prepared aqueous extracts of Kinnikinnick (NRP – 191, 192, 193, 247A and 247B, respectively). Following 15 min, 60 min and 18 h incubations at 37°C and 5% CO_2, the amount of Rh123 accumulated within the Caco-2 cells was measured using a CytoFluor Series 4000 Multi-Well Plate Reader. (Control = 100%, % Rh123 increase ± SD, n = 6. Ritonavir is the positive control. The final concentration of extract in the reaction medium is equivalent to 5 mg/ml). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in P-gp efflux activity in Caco-2 cells treated with Kinnikinnick compared to untreated controls.



NRP 191
NRP 192
NRP 193
NRP 247A
NRP 247B
Ritonavir (55 μg/ml)

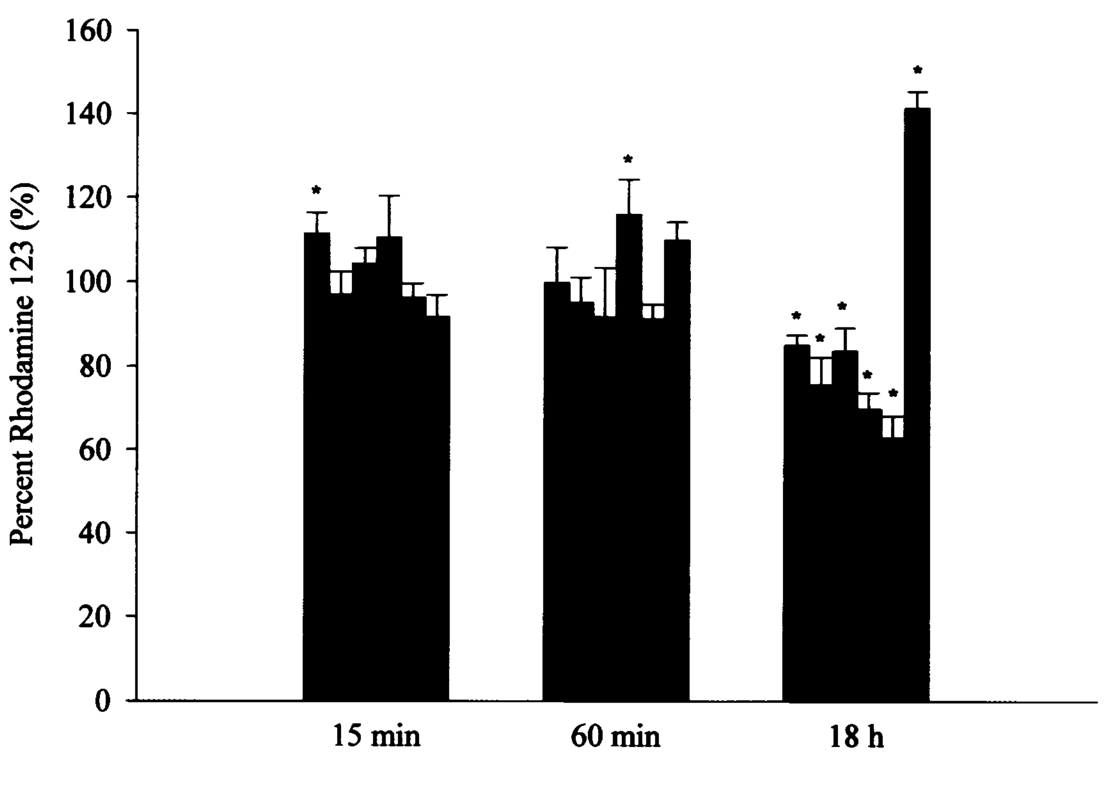
15 min, followed by a 115.6% ± 9.9% increase at 60 min and a 23.6% ± 6.2% decrease at 18 h, relative to the control. The methanolic extracts (1 mg/ml) of Kinnikinnick resulted in a range of Rh123 levels at the 15 min and 60 min time points; however like the aqueous extracts lower Rh123 levels at 18 h were observed (Figure 14). The methanolic extract of NRP 247A resulted in a similar biphasic effect observed with the aqueous extract, with a 15.8% ± 8.4% increase at 60 min followed by a 30.4% ± 4.0% decrease at 18 h.

Preliminary studies were performed within human THP-1 cells using aqueous and methanolic extracts of Kinnikinnick (Figures 15 and 16). Results obtained with all five aqueous extracts (5 mg/ml) of Kinnikinnick indicate an accumulation of Rh123 within THP-1 cells at 60 min, relative to controls. Following an 18 h incubation, all five aqueous extracts decreased Rh123 levels between 3.8% ± 2.9% (NRP 247B) and 46.8% ± 2.3% (NRP 247A), relative to controls. All five methanolic extracts (1 mg/ml) of Kinnikinnick decreased P-gp efflux activity at 15 min, resulting in increased Rh123 levels between 22.8% ± 1.0% (NRP 191) and 48.7% ± 10.1% (NRP 247A), relative to controls. Subsequently, at 60 min all five methanolic extracts further increased Rh123 accumulation within THP-1 cells. Similar to the aqueous extracts of Kinnikinnick, all five methanolic extracts exhibited lower levels of Rh123 at 18 h, with a decrease between 19.9% ± 5.3% (NRP 247B) and 53.6% ± 1.6% (NRP 193), relative to controls.

The influence of the five Kinnikinnick extracts on the metabolic activity of the Phase I enzymes, CYP3A4, 3A5 and 3A7, was determined by measuring the metabolism of the

Figure 14: Influence of methanolic extracts of *Arctostaphylos uva-ursi* (Kinnikinnick) on P-glycoprotein (P-gp) efflux activity within Caco-2 cells.

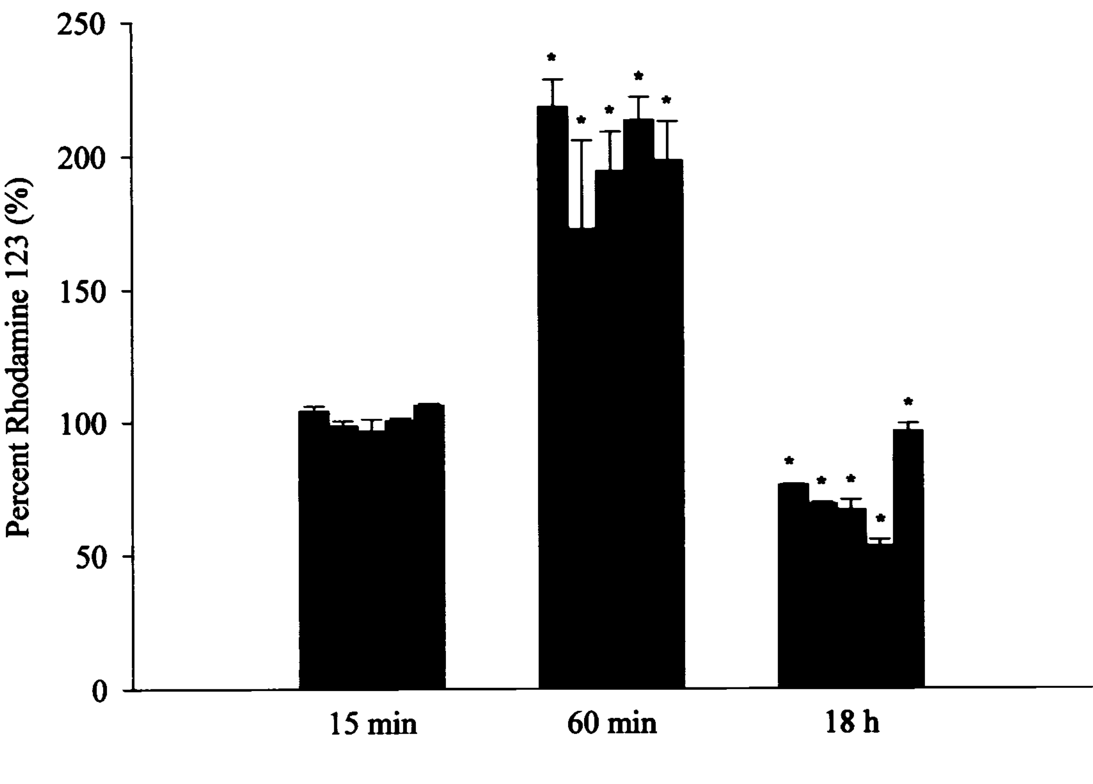
The efflux activity of P-gp within Caco-2 cells was determined using the fluorescent substrate rhodamine 123 (Rh123; 150 ng/ml) in the presence of freshly prepared 55% methanolic extracts of Kinnikinnick (NRP – 191, 192, 193, 247A and 247B, respectively). Following 15 min, 60 min and 18 h incubations at 37°C and 5% CO_2, the amount of Rh123 accumulated within the Caco-2 cells was measured using a CytoFluor Series 4000 Multi-Well Plate Reader. (Control = 100%, % Rh123 increase ± SD, n = 6. Ritonavir is the positive control. The final concentration of extract in the reaction medium is equivalent to 1 mg/ml). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in P-gp efflux activity in Caco-2 cells treated with Kinnikinnick compared to untreated controls.



Percent Rhodamine 123 (%)
160
140
120
100
80
60
40
20
0
15 min
60 min
18 h
NRP 191
NRP 192
NRP 193
NRP 247A
NRP 247B
Ritonavir (55 µg/ml)

Figure 15: Influence of aqueous extracts of *Arctostaphylos uva-ursi* (Kinnikinnick) on P-gp efflux activity within THP-1 cells.

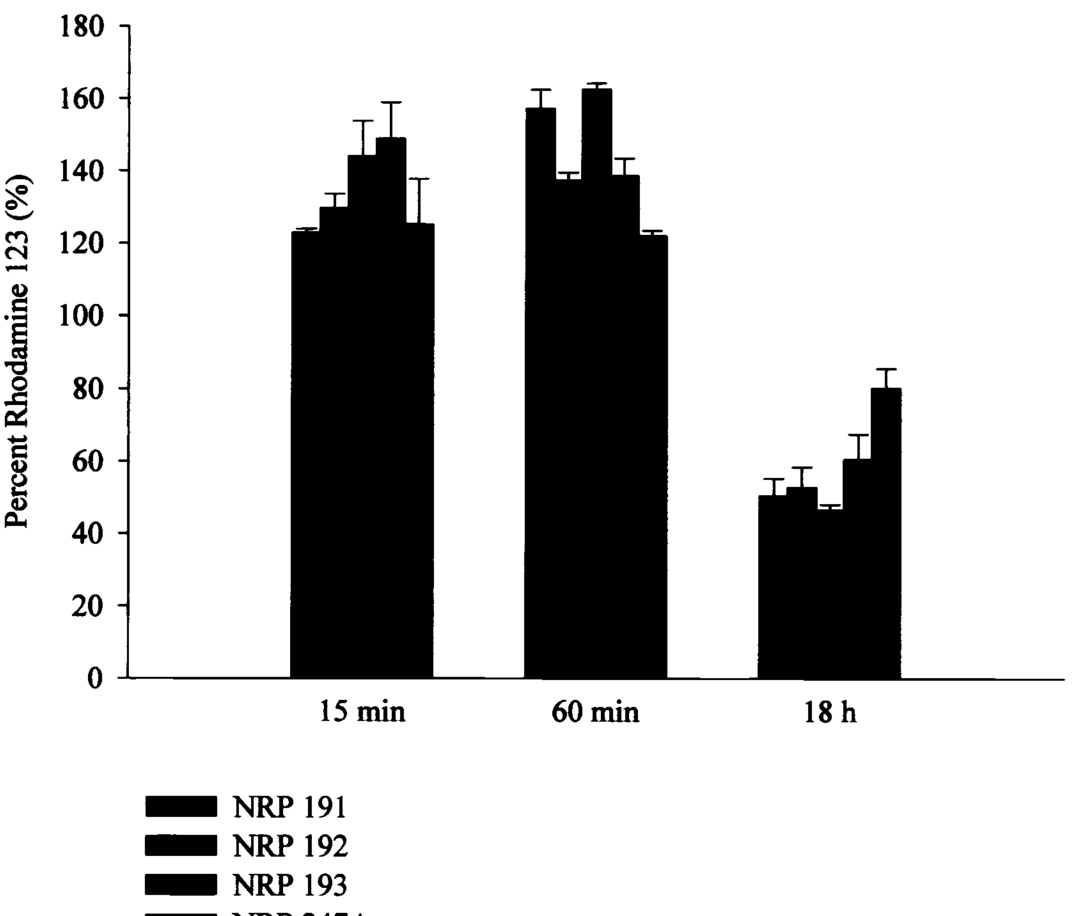
The efflux activity of P-gp within THP-1 cells was determined using the fluorescent substrate Rh123 (150 ng/ml) in the presence of freshly prepared aqueous extracts of Kinnikinnick (NRP – 191, 192, 193, 247A and 247B, respectively). Following 15 min, 60 min and 18 h incubations at 37°C and 5% CO_2, the amount of Rh123 remaining within the THP-1 cells was measured using a CytoFluor Series 4000 Multi-Well Plate Reader. (Control = 100%, % Rh123 Increase ± SD, n = 6. The final concentration of extract in the reaction medium is equivalent to 5 mg/ml). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in P-gp efflux activity in THP-1 cells treated with Kinnikinnick compared to untreated controls.



Percent Rhodamine 123 (%)
250
200
150
100
50
0
15 min
60 min
18 h
NRP 191
NRP 192
NRP 193
NRP 247A
NRP 247B

Figure 16: Influence of methanolic extracts of *Arctostaphylos uva-ursi* (Kinnikinnick) on P-gp efflux activity within THP-1 cells.

The efflux activity of P-gp within THP-1 cells was determined using the fluorescent substrate Rh123 (150 ng/ml) in the presence of freshly prepared 55% methanolic extracts of Kinnikinnick (NRP – 191, 192, 193, 247A and 247B, respectively). Following 15 min, 60 min and 18 h incubations at 37°C and 5% CO_2, the amount of Rh123 remaining within the THP-1 cells was measured using a CytoFluor Series 4000 Multi-Well Plate Reader. (Control = 100%, % Rh123 Increase ± SD, n = 6. The final concentration of extract in the reaction medium is equivalent to 1 mg/ml). All data presented have p-values ≤ 0.05 using the paired t-test, indicating a statistically significant difference in P-gp efflux activity in THP-1 cells treated with Kinnikinnick compared to untreated controls.



180
160
140
120
100
80
60
40
20
0
Percent Rhodamine 123 (%)
15 min
60 min
18 h
NRP 191
NRP 192
NRP 193
NRP 247A
NRP 247B

CYP substrate DBF to the metabolite fluorescein. The aqueous extracts of Kinnikinnick inhibited substrate metabolism for all three isoenzymes at the 1.25 mg/ml concentration tested (Figure 17). The aqueous extracts had a consistently high inhibitory effect on CYP 3A5 and 3A7, with percent inhibitions between 87.1% ± 2.4% to 100.3 % ± 2.8% for CYP 3A5 and 89.3% ± 9.8% and 96.7% ± 4.1% for CYP3A7. However, the percent inhibitions for CYP3A4 ranged between 49.8% ± 2.4% and 89.7% ± 8.8% among the five aqueous extracts tested. The influence of the methanolic extracts of the five Kinnikinnick products on the CYP3A isoenzymes studied, resulted in strong inhibition of DBF metabolism at the 50 μg/ml concentration tested (Figure 18). The methanolic extract of NRP 247A, had the highest inhibitory effect on CYP 3A4, with an 86.1% ± 0.8% inhibition and NRP 193 had the weakest inhibitory effect with a 14.2% ± 3.4% inhibition. The inhibitory influence of all five methanolic extracts of Kinnikinnick slightly ranged for the other two isoenzymes, with values between 68.6% ± 13.1% and 94.7% ± 1.0% for CYP 3A5 and between 49.4% ± 4.2% and 74.8% ± 9.1% for CYP 3A7.

3.3.3 *Acorus americanus and Acorus calamus* (Acorus)

The *in vitro* influence of *Acorus americanus* and *Acorus calamus* on P-gp efflux activity, using the fluorescent substrate Rh123, was determined within Caco-2 cells. The two bulk Acorus samples studied within Caco-2 cells did not influence the efflux activity of P-gp. The aqueous extracts of Acorus (5 mg/ml) did not influence the levels of Rh123 within the Caco-2 cells noticeably at the first two time points, as observed with Rh123 levels of 93.5% ± 3.5% and 95.5% ± 1.7% for NRP 205 and NRP 240, respectively, at 60 min.

Figure 17: Influence of aqueous extracts of *Arctostaphylos uva-ursi* (Kinnikinnick) on CYP3A isoenzyme activity *in vitro*.

The enzymatic activity of CYP 3A4, 3A5 and 3A7 in the metabolic conversion of 200 μM dibenzylfluorescein to fluorescein was assessed in the presence of freshly prepared aqueous extracts of Kinnikinnick (NRP – 191, 192, 193, 247A and 247B, respectively). Following a 20 min (3A4 and 3A5) or 40 min (3A7) incubation at 37°C, enzymatic activity was measured based on fluorescence produced using a CytoFluor Series 4000 Multi-Well Plate Reader. (% Inhibition ± SD, n = 6. Ketoconazole (Keto.) is the positive control. The final concentration of extract in the reaction medium is equivalent to 1.25 mg/ml). All data presented have p-values ≤ 0.05 using the paired t-test, indicating a statistically significant difference in CYP3A isoenzyme activity when treated with Kinnikinnick compared to untreated controls.

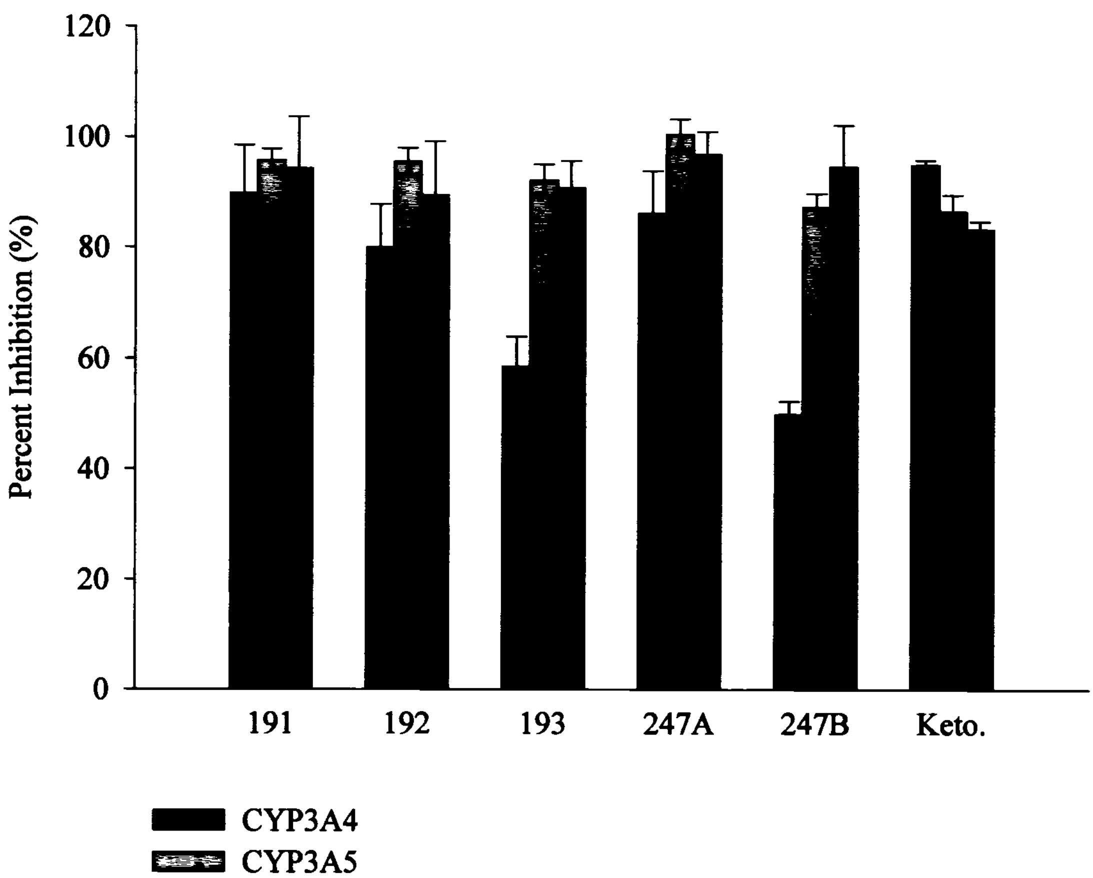
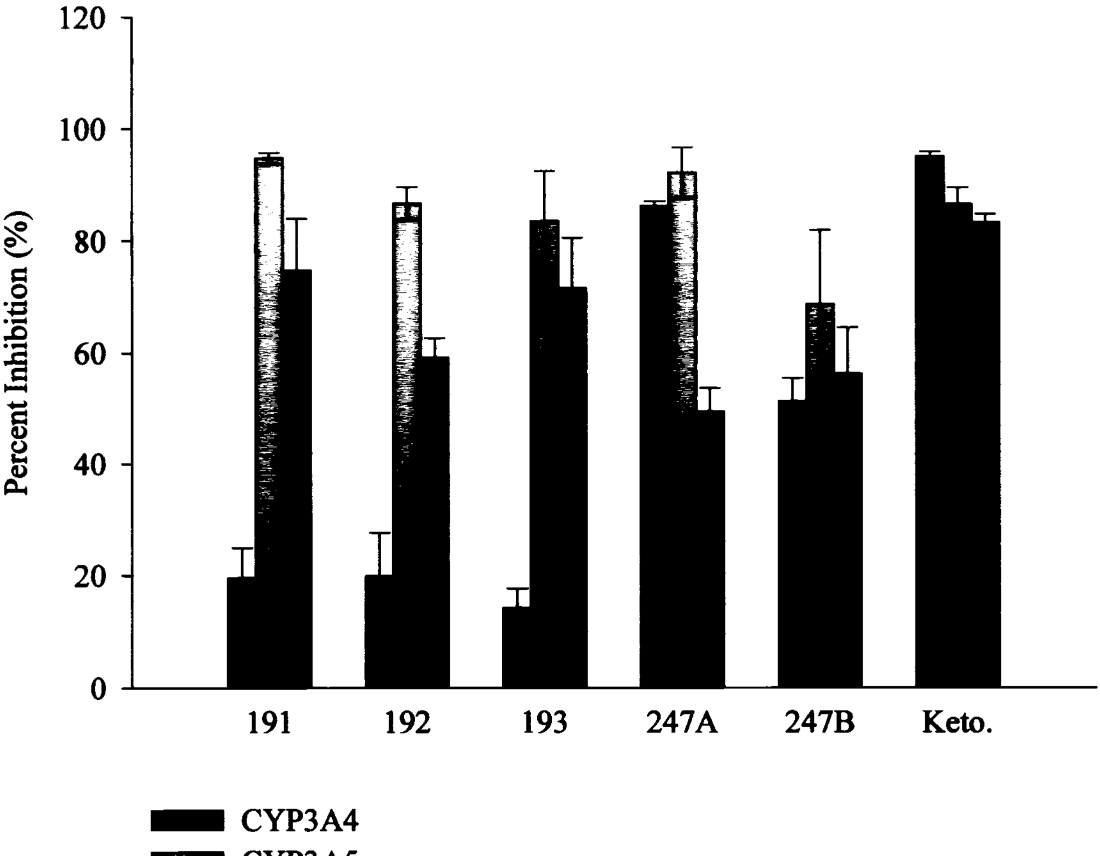

120
100
80
60
40
20
0
Percent Inhibition (%)
191
192
193
247A
247B
Keto.
CYP3A4
CYP3A5
CYP3A7

Figure 18: Influence of methanolic extracts of *Arctostaphylos uva-ursi* (Kinnikinnick) on CYP3A isoenzyme activity *in vitro*.

The enzymatic activity of CYP 3A4, 3A5 and 3A7 in the metabolic conversion of 200 μM dibenzylfluorescein to fluorescein was assessed in the presence of freshly prepared methanolic extracts of Kinnikinnick (NRP – 191, 192, 193, 247A and 247B, respectively). Following a 20 min (3A4 and 3A5) or 40 min (3A7) incubation at 37°C, enzymatic activity was measured based on fluorescence produced using a CytoFluor Series 4000 Multi-Well Plate Reader. (% Inhibition ± SD, n = 6. Ketoconazole (Keto.) is the positive control. The final concentration of extract in the reaction medium is equivalent to 50 μg/ml). All data presented have p-values ≤ 0.05 using the paired t-test, indicating a statistically significant difference in CYP3A isoenzyme activity when treated with Kinnikinnick compared to untreated controls.

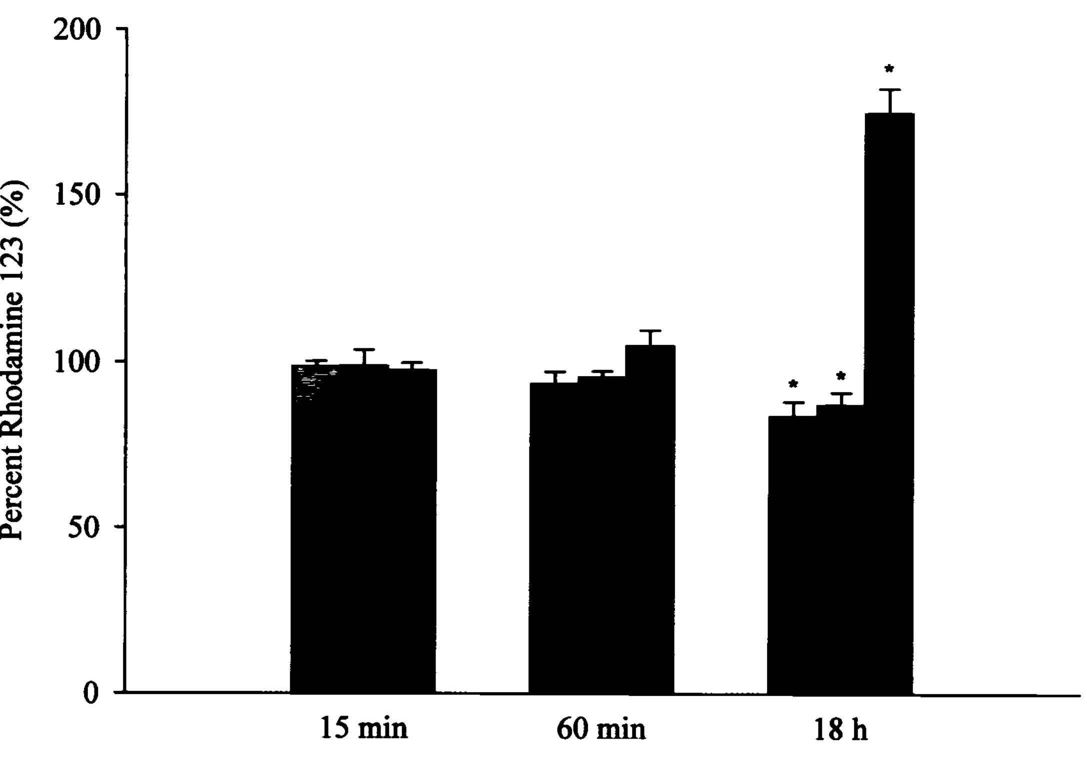

120
100
80
60
40
20
0
Percent Inhibition (%)
191
192
193
247A
247B
Keto.
CYP3A4
CYP3A5
CYP3A7

(Figure 19). At 18 h, slightly lower levels of Rh123 were observed for both aqueous extracts, with NRP 205 and NRP 240 resulting in a 16.3% ± 4.3% and 13.0% ± 3.7% decrease, respectively, relative to the control. Similarly, the methanolic extracts of Acorus (250 μg/ml) were not able to influence Rh123 levels within the Caco-2 cells at the three time points tested (Figure 20). At 60 min, Rh123 levels for NRP 205 and NRP 240 were 106.4% ± 5.0% and 106.6% ± 2.4%, and at the 18 h point 94.6% ± 4.2% and 106.8% ± 2.1%, respectively.

In order to test whether the activity of the Phase I enzymes, CYP 3A4, 3A5 and 3A7 is influenced in the presence of *Acorus americanus* and *Acorus calamus*, an *in vitro* study was performed which measured the metabolism of DBF to the metabolite fluorescein. The influence of the aqueous extracts of Acorus (5 mg/ml) on CYP 3A4, 3A5 and 3A7 differed between each isoenzyme tested (Figure 21). The inhibitory influence of the two extracts was the least on CYP 3A4, with no inhibition by NRP 205 and a slight 28.9% ± 5.5% inhibition by NRP 240. There was also no substantial influence from the aqueous extract of NRP 205 on CYP 3A5 activity, and a moderate 72.8% ± 1.0% inhibition in the presence of the 5 mg/ml aqueous extract of NRP 240. Both aqueous extracts were also able to moderately inhibit CYP 3A7 activity, with 73.5% ± 3.4% and 93.8% ± 1.6% inhibition by NRP 205 and NRP 240, respectively. The methanolic extract of NRP 205 (250 μg/ml) influenced CYP 3A4 activity in an opposite manner compared to the aqueous counterpart, producing a 77.3% ± 5.8% enhancement in DBF metabolism (Figure 22). However, the influence of the 250 μg/ml methanolic extract of NRP 240 moderately inhibited CYP 3A4 activity by 25.5% ± 4.5%. The effect of the two methanolic extracts

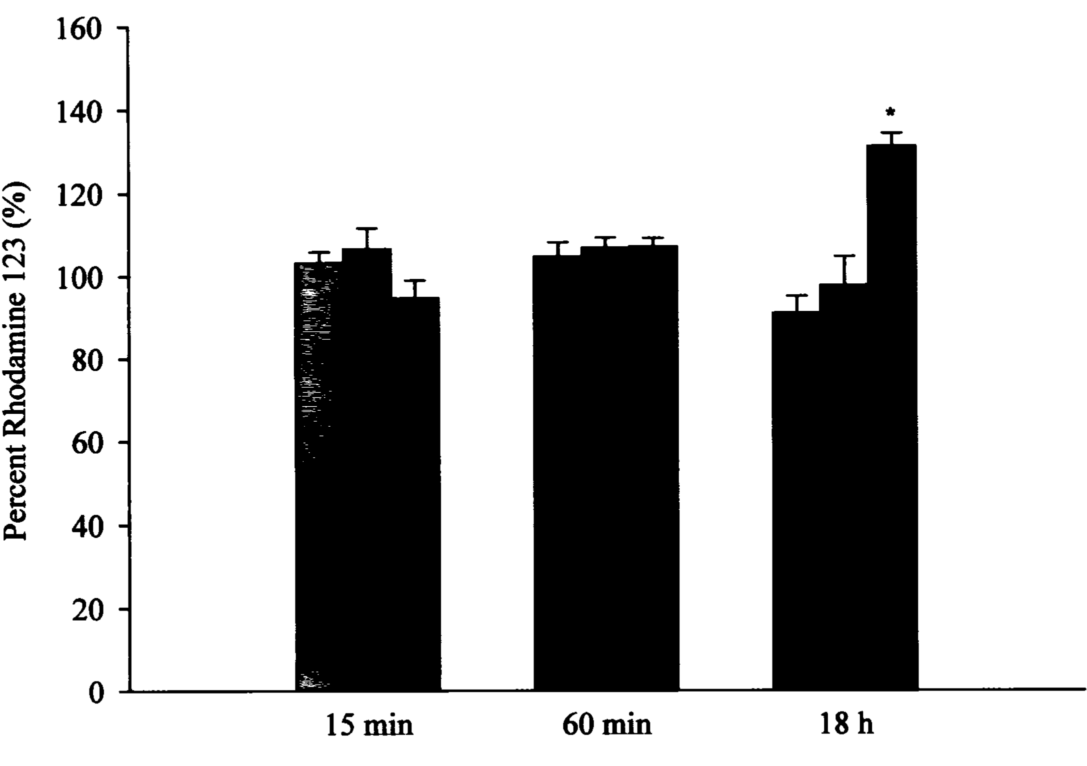
Figure 19: Influence of aqueous extracts of *Acorus americanus* and *Acorus calamus* (Acorus) on P-glycoprotein (P-gp) efflux activity within Caco-2 cells.

The efflux activity of P-gp within Caco-2 cells was determined using the fluorescent substrate rhodamine 123 (Rh123; 150 ng/ml) in the presence of freshly prepared aqueous extracts of Acorus (NRP – 205 and 240, respectively). Following 15 min, 60 min and 18 h incubations at 37°C and 5% CO_2, the amount of Rh123 accumulated within the Caco-2 cells was measured using a CytoFluor Series 4000 Multi-Well Plate Reader. (Control = 100%, % Rh123 increase ± SD, n = 6. Ritonavir is the positive control. The final concentration of extract in the reaction medium is equivalent to 5 mg/ml). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in P-gp efflux activity in Caco-2 cells treated with Acorus compared to untreated controls.



200
150
100
50
0
Percent Rhodamine 123 (%)
*
*
*
15 min
60 min
18 h
NRP 205
NRP 240
Ritonavir (55 μg/ml)

Figure 20: Influence of methanolic extracts of *Acorus americanus* and *Acorus calamus* (Acorus) on P-glycoprotein (P-gp) efflux activity within Caco-2 cells. The efflux activity of P-gp within Caco-2 cells was determined using the fluorescent substrate rhodamine 123 (Rh123; 150 ng/ml) in the presence of freshly prepared 55% methanolic extracts of Acorus (NRP – 205 and 240, respectively). Following 15 min, 60 min and 18 h incubations at 37°C and 5% CO_2, the amount of Rh123 accumulated within the Caco-2 cells was measured using a CytoFluor Series 4000 Multi-Well Plate Reader. (Control = 100%, % Rh123 increase ± SD, n = 6. Ritonavir is the positive control. The final concentration of extract in the reaction medium is equivalent to 250 μg/ml). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in P-gp efflux activity in Caco-2 cells treated with Acorus compared to untreated controls.



160
140
120
100
80
60
40
20
0
Percent Rhodamine 123 (%)
*
15 min
60 min
18 h
NRP 205
NRP 240
Ritonavir (27.5 μg/ml)

Figure 21: Influence of aqueous extracts of *Acorus americanus* and *Acorus calamus* (Acorus) on CYP3A isoenzyme activity *in vitro*.

The enzymatic activity of CYP 3A4, 3A5 and 3A7 in the metabolic conversion of 200 μM dibenzylfluorescein to fluorescein was assessed in the presence of freshly prepared aqueous extracts of Acorus (NRP – 205 and 240, respectively). Following a 20 min (3A4 and 3A5) or 40 min (3A7) incubation at 37°C, enzymatic activity was measured based on fluorescence produced using a CytoFluor Series 4000 Multi-Well Plate Reader. (% Inhibition ± SD, n = 6. Ketoconazole is the positive control. The final concentration of extract in the reaction medium is equivalent to 5 mg/ml). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in CYP3A isoenzyme activity when treated with Acorus compared to untreated controls.

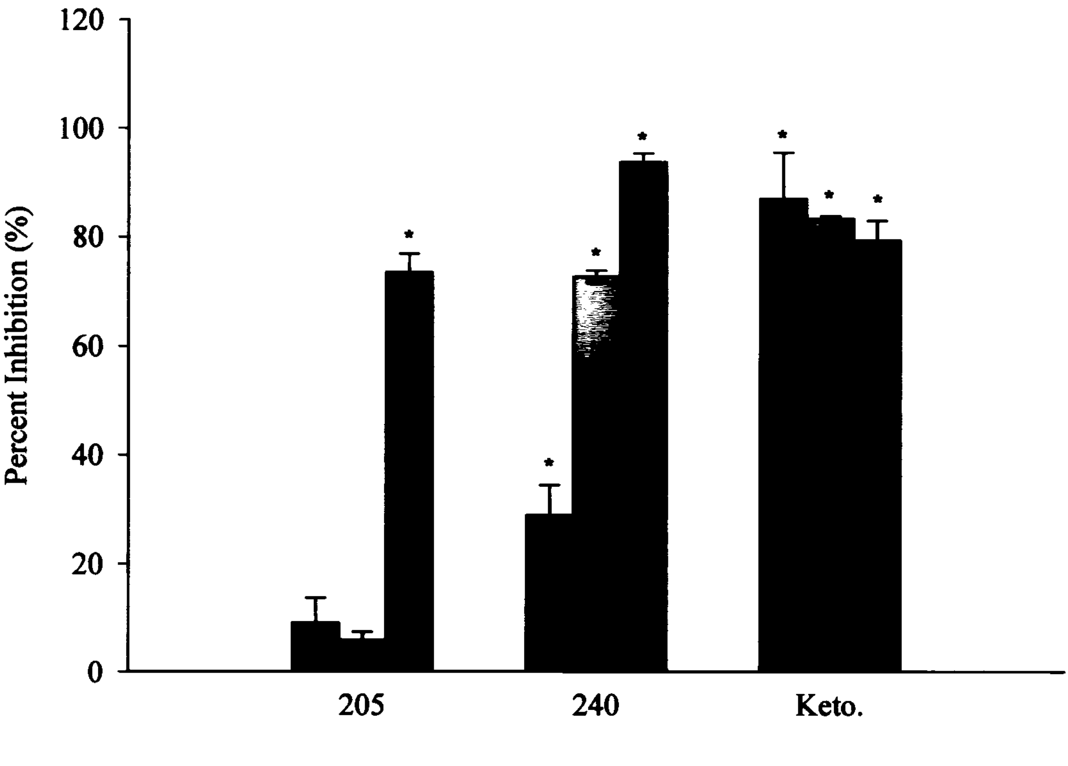
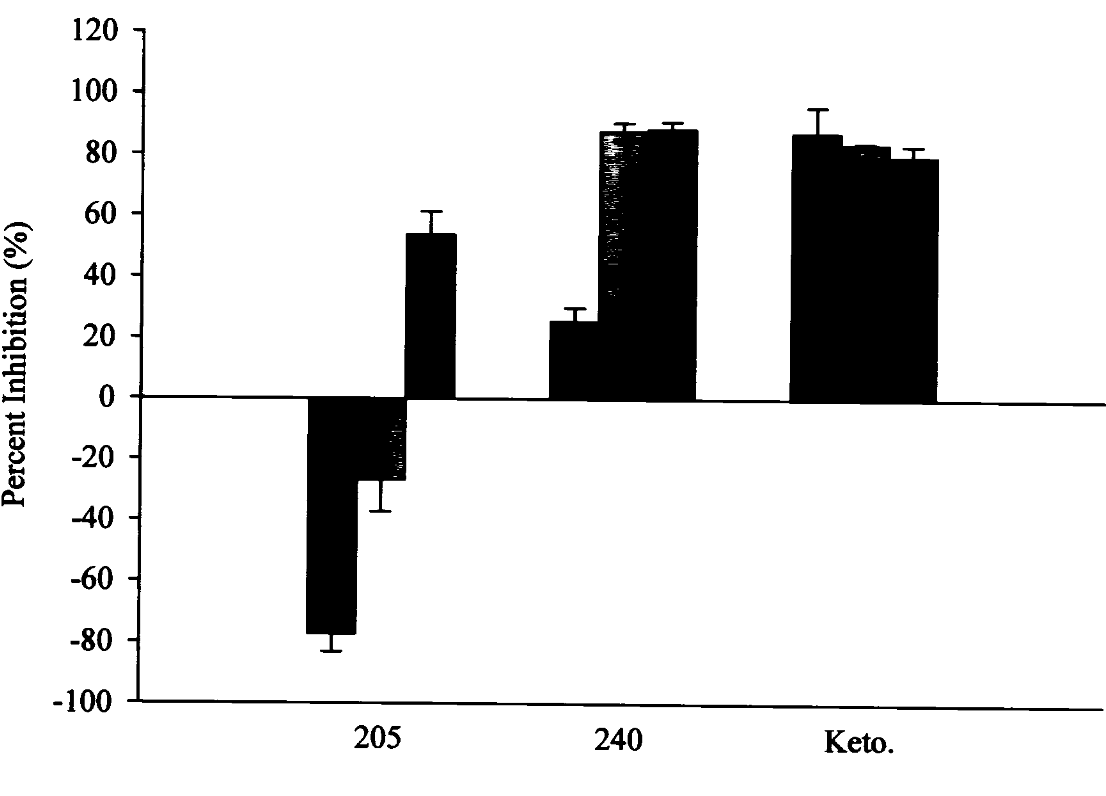

120
100
80
60
40
20
0
Percent Inhibition (%)
205
240
Keto.
CYP 3A4
CYP 3A5
CYP 3A7

Figure 22: Influence of methanolic extracts of *Acorus americanus* and *Acorus calamus* (Acorus) on CYP3A isoenzyme activity *in vitro*.

The enzymatic activity of CYP 3A4, 3A5 and 3A7 in the metabolic conversion of 200 μM dibenzylfluorescein to fluorescein was assessed in the presence of freshly prepared methanolic extracts of Acorus (NRP – 205 and 240, respectively). Following a 20 min (3A4 and 3A5) or 40 min (3A7) incubation at 37°C, enzymatic activity was measured based on fluorescence produced using a CytoFluor Series 4000 Multi-Well Plate Reader. (% Inhibition ± SD, n = 6. Ketoconazole is the positive control. The final concentration of extract in the reaction medium is equivalent to 250 μg/ml). All data presented have p-values ≤ 0.05 using the paired t-test, indicating a statistically significant difference in CYP3A isoenzyme activity when treated with Acorus compared to untreated controls.


120
100
80
60
40
20
0
-20
-40
-60
-80
-100
Percent Inhibition (%)
205
240
Keto.
CYP 3A4
CYP 3A5
CYP 3A7

of Acorus on CYP 3A5 were similar to CYP 3A4, with NRP 205 moderately enhancing DBF metabolism by 27.0% ± 10.4% and NRP 240 strongly inhibiting activity by 87.6% ± 2.8%, respectively. The inhibitory effect of the methanolic extracts on CYP 3A7 activity was similar to that observed with the aqueous extracts, with a considerable inhibition in isoenzyme activity of 53.7% ± 7.5% and 88.4% ± 2.3% by NRP 205 and NRP 240, respectively.

3.3.4 Spices (*Curcuma longa, Capsicum annuum, Piper nigrum*)

The influence of extracts of three bulk product spices – *Curcuma longa* (turmeric powder, *Capsicum annuum* (chilli powder) and *Piper nigrum* (black pepper), on the efflux activity of P-gp, using the fluorescent substrate Rh123, within Caco-2 cells was also measured. The aqueous extracts of each spice (5 mg/ml) tested influenced Rh123 levels in differing manners (Figure 23). The aqueous extract of turmeric powder slightly decreased Rh123 levels at 15 min and 60 min by 9.9% ± 3.7% and 18.4% ± 8.6%, respectively relative to the control, whereas no change was observed at 18 h. The aqueous extract of chilli powder influenced Rh123 levels in a biphasic manner, with a noticeable 46.2% ± 7.1% increase in Rh123 levels at 60 min, followed by a 44.1% ± 10.8% decrease following an 18 h incubation, relative to the control. The aqueous extract of black pepper moderately lowered Rh123 levels at 15 min, 60 min and 18 h, in relation to the control, resulting in a 19.0% ± 1.9%, 12.2% ± 2.7% and 10.2% ± 2.1% decrease, respectively. The methanolic extracts (250 μg/ml) of the spices tested, did not influence Rh123 levels to any considerable extent for the three spices tested (Figure 24). The Rh123 levels remained consistent at the three time points tested with the methanolic

Figure 23: Influence of aqueous extracts of *Curcuma longa* (turmeric powder), *Capsicum annuum* (chilli powder) and *Piper nigrum* (black pepper) on P-glycoprotein (P-gp) efflux activity within Caco-2 cells.

The efflux activity of P-gp within Caco-2 cells was determined using the fluorescent substrate rhodamine 123 (Rh123; 150 ng/ml) in the presence of freshly prepared aqueous extracts of turmeric powder, chilli powder and black pepper (NRP – 261, 262 and 263, respectively). Following 15 min, 60 min and 18 h incubations at 37°C and 5% CO_2, the amount of Rh123 accumulated within the Caco-2 cells was measured using a CytoFluor Series 4000 Multi-Well Plate Reader. (Control = 100%, % Rh123 increase ± SD, n = 6. Ritonavir is the positive control. The final concentration of extract in the reaction medium is equivalent to 5 mg/ml). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in P-gp efflux activity in Caco-2 cells treated with turmeric powder, chilli powder or black pepper compared to untreated controls.

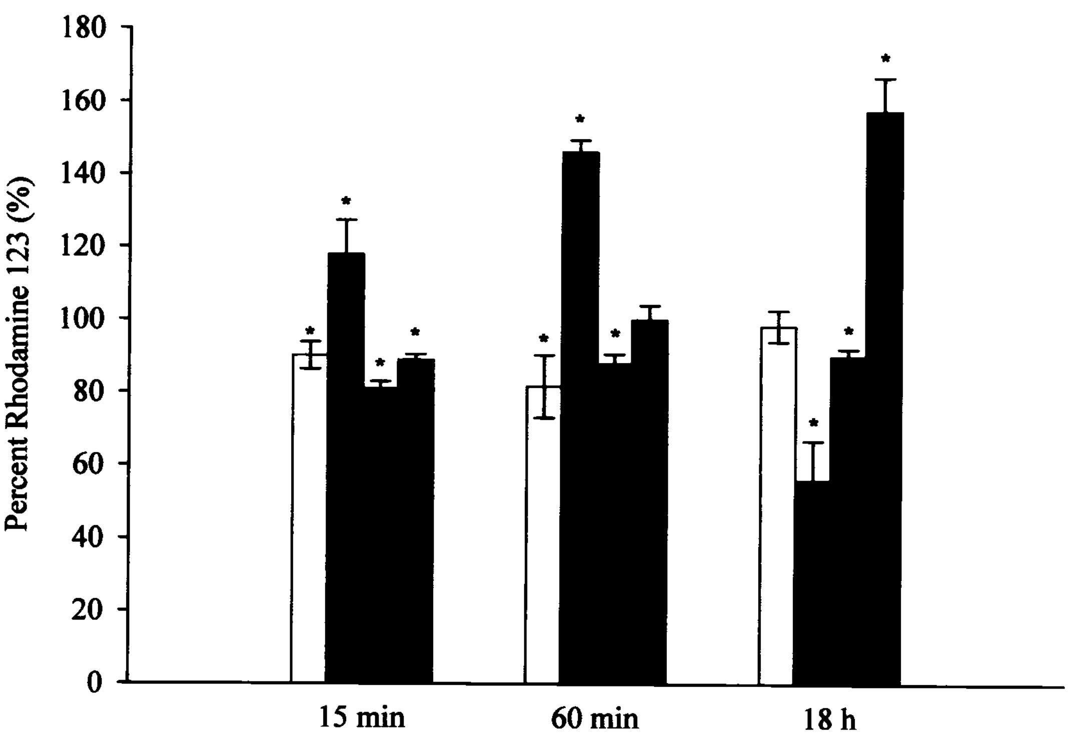
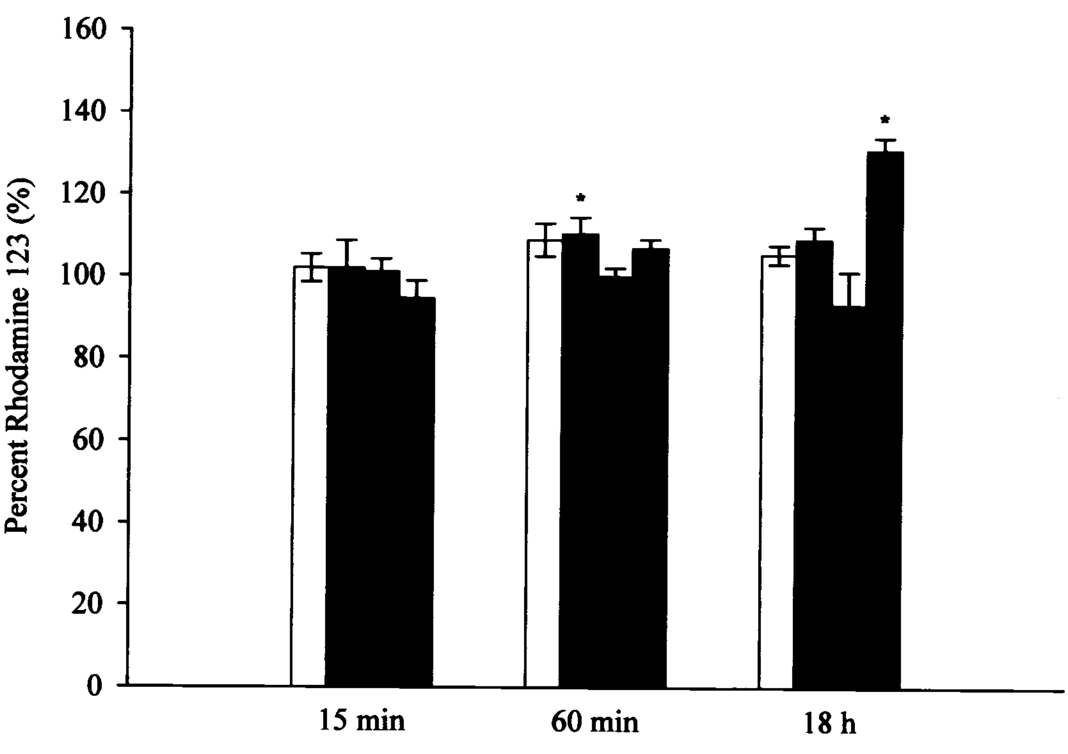

Percent Rhodamine 123 (%)
180
160
140
120
100
80
60
40
20
0
*
15 min
60 min
18 h
Turmeric Powder
Chilli Powder
Black Pepper
Ritonavir (27.5 µg/ml)

Figure 24: Influence of methanolic extracts of *Curcuma longa* (turmeric powder), *Capsicum annuum* (chilli powder) and *Piper nigrum* (black pepper) on P-glycoprotein (P-gp) efflux activity within Caco-2 cells.

The efflux activity of P-gp within Caco-2 cells was determined using the fluorescent substrate rhodamine 123 (Rh123; 150 ng/ml) in the presence of freshly prepared 55% methanolic extracts of turmeric powder, chilli powder and black pepper (NRP – 261, 262 and 263, respectively). Following 15 min, 60 min and 18 h incubations at 37°C and 5% CO_2, the amount of Rh123 accumulated within the Caco-2 cells was measured using a CytoFluor Series 4000 Multi-Well Plate Reader. (Control = 100%, % Rh123 increase ± SD, n = 6. Ritonavir is the positive control. The final concentration of extract in the reaction medium is equivalent to 250 μg/ml). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in P-gp efflux activity in Caco-2 cells treated with turmeric powder, chilli powder or black pepper compared to untreated controls.

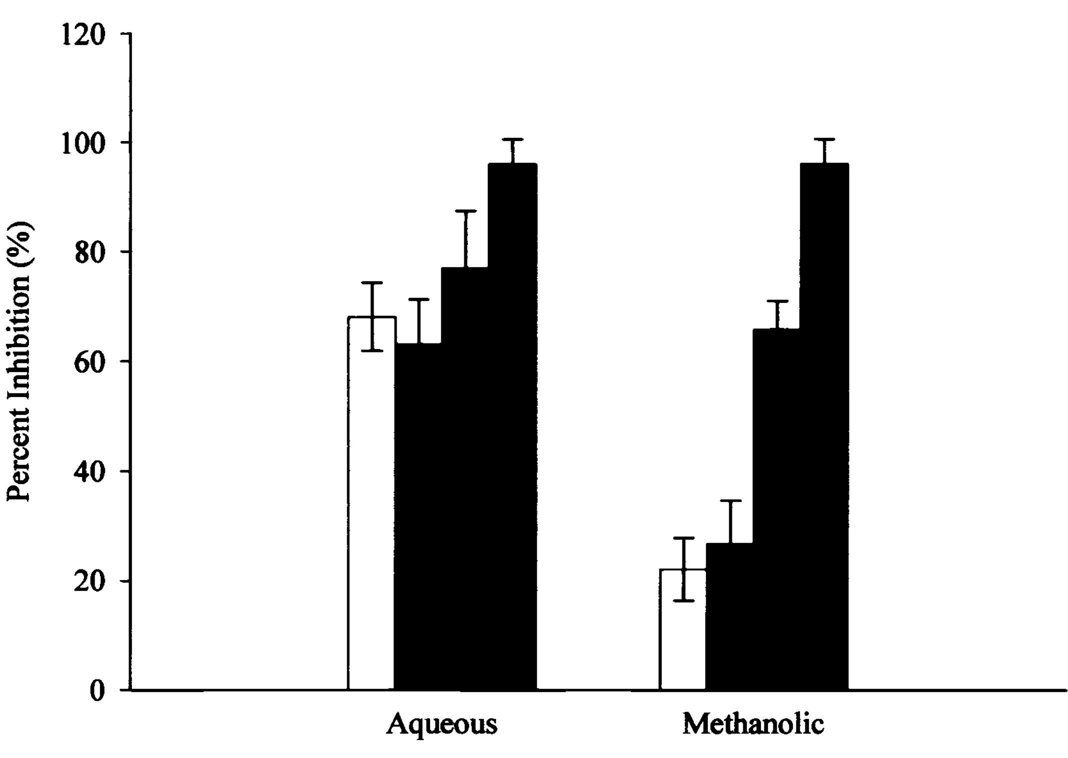

Percent Rhodamine 123 (%)
160
140
120
100
80
60
40
20
0
15 min
60 min
18 h
*
*
Turmeric Powder
Chilli Powder
Black Pepper
Ritonavir (27.5 μg/ml)

extracts of turmeric powder and black pepper with no observable changes, relative to the control. A slight 10.4% ± 3.9% increase in Rh123 levels was observed for the methanolic extract of chilli powder at 60 min, however the biphasic effect observed with the aqueous counterpart was not observed at 18 h

The influence of the extracts of the three bulk product spices on the isoenzyme activity of CYP 3A4, 3A5 and 3A7 was determined, via a measurement of fluorescein production from the metabolism of DBF by these isoenzymes. The aqueous extracts (5 mg/ml) of all three spices were able to markedly inhibit the activity of CYP 3A4, with the aqueous extract of turmeric powder producing the least inhibition at 68.1% ± 6.2% and the aqueous extract of black pepper providing the greatest inhibition at 76.9% ± 10.5% (Figure 25). The methanolic extracts (250 μg/ml) of turmeric powder and chilli powder were also able to moderately inhibit CYP 3A4 activity, with a 22.0% ± 5.7% and 26.7% ± 7.8% inhibition, respectively, whereas the methanolic extract of black pepper strongly inhibited isoenzyme activity at 65.8% ± 5.2%. CYP 3A5 activity was also influenced by both aqueous (5 mg/ml) and methanolic (250 μg/ml) extracts of the three spices tested (Figure 26). The inhibitory influence of the three aqueous extracts was 43.2% ± 9.6%, 93.0% ± 1.0% and 71.6% ± 2.6% inhibition for turmeric powder, chilli powder and black pepper, respectively. The effect of the methanolic extracts on CYP 3A5 activity resulted in consistently substantial inhibition among the three spices, with 100.3% ± 0.5%, 85.4% ± 2.0% and 92.4% ± 0.5% inhibition for turmeric powder, chilli powder and black pepper, respectively. A moderate inhibitory effect on CYP 3A7 activity was observed for the aqueous (5 mg/ml) extracts of the three spices tested, whereas a considerable high

Figure 25: Influence of aqueous and methanolic extracts of *Curcuma longa* (turmeric powder), *Capsicum annuum* (chilli powder) and *Piper nigrum* (black pepper) on CYP 3A4 isoenzyme activity *in vitro*.

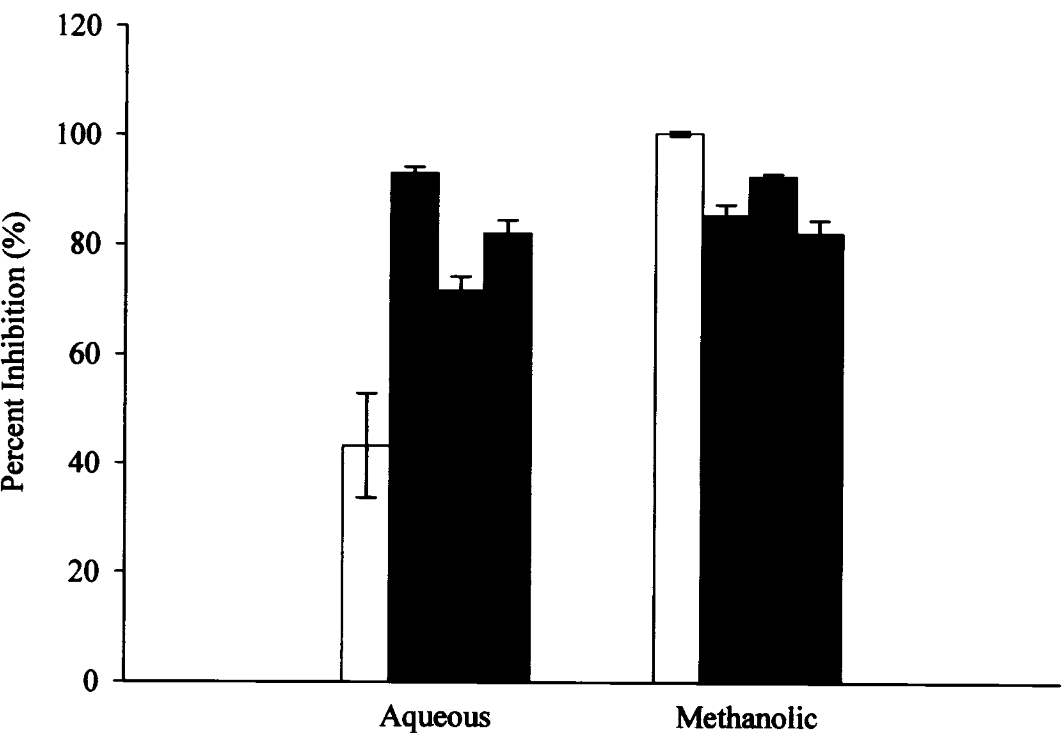
The enzymatic activity of CYP 3A4 in the metabolic conversion of 200 μM dibenzylfluorescein to fluorescein was assessed in the presence of freshly prepared aqueous and methanolic extracts of turmeric powder, chilli powder and black pepper (NRP – 261, 262 and 263, respectively). Following a 20 min incubation at 37°C, enzymatic activity was measured based on fluorescence produced using a CytoFluor Series 4000 Multi-Well Plate Reader. (% Inhibition ± SD, n = 6. Ketoconazole is the positive control. The final concentration of aqueous and methanolic extracts in the reaction medium is equivalent to 5 mg/ml and 250 μg/ml, respectively). All data presented have p-values ≤ 0.05 using the paired t-test, indicating a statistically significant difference in CYP 3A4 isoenzyme activity when treated with turmeric powder, chilli powder or black pepper compared to untreated controls.



120
100
80
60
40
20
0
Percent Inhibition (%)
Aqueous
Methanolic
Turmeric Powder
Chilli Powder
Black Pepper
Ketoconazole (0.5 μg/ml)

Figure 26: Influence of aqueous and methanolic extracts of *Curcuma longa* (turmeric powder), *Capsicum annuum* (chilli powder) and *Piper nigrum* (black pepper) on CYP 3A5 isoenzyme activity *in vitro*.

The enzymatic activity of CYP 3A5 in the metabolic conversion of 200 μM dibenzylfluorescein to fluorescein was assessed in the presence of freshly prepared aqueous and methanolic extracts of turmeric powder, chilli powder and black pepper (NRP – 261, 262 and 263, respectively). Following a 20 min incubation at 37°C, enzymatic activity was measured based on fluorescence produced using a CytoFluor Series 4000 Multi-Well Plate Reader. (% Inhibition ± SD, n = 6. Ketoconazole is the positive control. The final concentration of aqueous and methanolic extracts in the reaction medium is equivalent to 5 mg/ml and 250 μg/ml, respectively). All data presented have p-values ≤ 0.05 using the paired t-test, indicating a statistically significant difference in CYP 3A5 isoenzyme activity when treated with turmeric powder, chilli powder or black pepper compared to untreated controls.

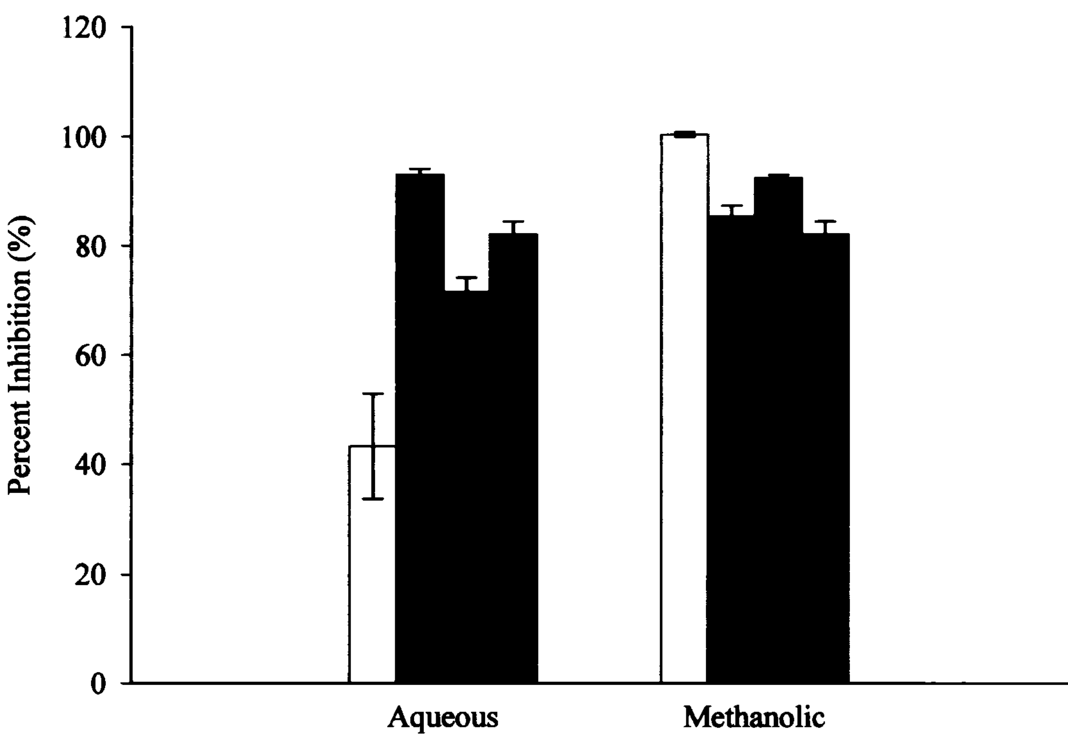


Percent Inhibition (%)
120
100
80
60
40
20
0
Aqueous
Methanolic
Turmeric Powder
Chilli Powder
Black Pepper
Ketoconazole (0.5 μg/ml)

inhibitory effect resulted in the presence of the methanolic (250 μg/ml) extracts (Figure 27). The aqueous and methanolic extracts of turmeric powder resulted in 75.7% ± 5.1% and 98.5% ± 1.3% inhibition. A similarly high inhibitory influence was observed for the aqueous and methanolic extracts of chilli powder resulting in 101.3% ± 0.6% and 92.4% ± 0.4% inhibition. CYP 3A7 activity was also inhibited at 89.2% ± 3.2% by the aqueous extract of black pepper, and the methanolic extract counterpart was able to influence isoenzyme activity resulting in a 94.3% ± 2.9% inhibition.

Figure 27: Influence of aqueous and methanolic extracts of *Curcuma longa* (turmeric powder), *Capsicum annuum* (chilli powder) and *Piper nigrum* (black pepper) on CYP 3A7 isoenzyme activity *in vitro*.

The enzymatic activity of CYP 3A7 in the metabolic conversion of 200 μM dibenzylfluorescein to fluorescein was assessed in the presence of freshly prepared aqueous and methanolic extracts of turmeric powder, chilli powder and black pepper (NRP – 261, 262 and 263, respectively). Following a 40 min incubation at 37°C, enzymatic activity was measured based on fluorescence produced using a CytoFluor Series 4000 Multi-Well Plate Reader. (% Inhibition ± SD, n = 6. Ketoconazole is the positive control. The final concentration of aqueous and methanolic extracts in the reaction medium is equivalent to 5 mg/ml and 250 μg/ml, respectively). All data presented have p-values ≤ 0.05 using the paired t-test, indicating a statistically significant difference in CYP 3A7 isoenzyme activity when treated with turmeric powder, chilli powder or black pepper compared to untreated controls.



120
100
80
60
40
20
0
Percent Inhibition (%)
Aqueous
Methanolic
Turmeric Powder
Chilli Powder
Black Pepper
Ketoconazole (0.5 μg/ml)

4 Discussion

4.1 C3435T, G2677T/A, C1236T Haplotype and P-gp Activity/Expression

4.1.1 Identification of the C3435T, G2677T/A, C1236T Haplotype

To determine the C3435T, G2677T/A, C1236T haplotype relationship, initially 80 healthy male Caucasian individuals were screened for the C3435T polymorphism. The 19 individuals that were identified as either CC3435 or TT3435, were also screened for G2677T/A and C1236T. Eight out of nine individuals that were screened for TT3435 also contained polymorphisms within the 2677 and 1236 regions, and all nine TT3435 patients had at least one additional polymorphism. Polymorphisms within the 2677 and 1236 regions can also occur independently from a TT3435 mutation, as was seen with one CC3435 individual that had heterozygous mutations at the other two loci of interest. These observations agree with what has been suggested previously [41]. One study suggested a 62% linkage disequilibrium between the TT3435, TT2677 and TT1236 polymorphisms in a European American population, while another study found a 93.8% association between the TT3435 and TT2677 polymorphisms within a Japanese population [94,99]. The results mentioned in the Kim and Tanabe studies are based on population sizes between 60 to100 individuals, whereas the results from this study were obtained from 19 participating individuals. Initially, 80 healthy male Caucasians were enrolled within this study, with the expectation that half would screen for either the wild-type CC3435 or the polymorphic TT3435 for further analysis of the C3435T, G2677T/A, C1236T haplotype [100]. Unfortunately the number of individuals successfully identified with either the CC3435 or TT3435 genotype and willing to participate for the duration of the study decreased to 19 individuals.

4.1.2 C3435T, G2677T/A, C1236T Haplotype and P-gp Efflux Activity

An analysis of whether the C3435T, G2677T/A, C1236T haplotype influences P-gp efflux activity was addressed. Data obtained from the P-gp efflux studies between TT3435 and CC3435 individuals found a substantial range in the percent of Rh123 accumulated within the isolated macrophage cells in both populations. It was expected that TT3435 individuals, believed to have lower P-gp efflux activity, would have a high percent of Rh123 levels within the cells relative to wild-type controls. Alternatively, CC3435 individuals should have a lower percent of Rh123 levels relative to polymorphic controls, based on the theory that a higher P-gp efflux activity exists within these individuals. Observations from this study indicate a large range in the percent Rh123 within the cells for both populations. Additionally, based on the scatter plot diagram, no statistically significant difference in Rh123 levels between the TT3435 and CC3435 populations was observed. None of the C3435T, G2677T/A, C1236T haplotype variations identified, markedly influenced the percent Rh123 within the cells in comparison with each other.

4.1.3 C3435T, G2677T/A, C1236T Haplotype and P-gp mRNA Expression

The influence of the C3435T, G2677T/A, C1236T haplotype, on the expression of the *ABCB1* gene was then examined. As observed within the P-gp efflux activity study, a broad range in the levels of P-gp mRNA within the identified haplotype populations was observed. The broad range observed was mainly due to outliers within two, TT3435, GT2677, CT1236 and CC3435, GG2677, CC1236, of the haplotype variations. Taking into account such outliers leads to the observation that the P-gp mRNA expression levels

between the haplotype variations studied does not differ. The scatter plot representation of P-gp mRNA levels for the two populations also indicate no statistically significant difference in mRNA levels between the five C3435T, G2677T/A, C1236T haplotype variations studied.

The observations suggesting no difference in P-gp efflux activity and mRNA expression between the TT3435 and CC3435 population, with consideration of a C3435T haplotype relationship with G2677T/A and C1236T, is supported by other published studies. One study looking at the influence of the C3435T and G2677A/T polymorphisms on P-gp activity within LLC-PK1 cells, found no significant differences in the transport of four P-gp compounds – verapamil, digoxin, vinblastine, and cyclosporine A – between the polymorphic and wild type cells studied [101]. P-gp efflux of Rh123 within peripheral blood lymphocytes isolated from 46 healthy male volunteers was also studied, and results suggest no significant differences in activity in the presence of the C3435T and G2677A/T polymorphisms [100]. An additional study using healthy male Caucasian volunteers investigated the influence of the C3435T polymorphism, and found no association between this polymorphism and the ability of P-gp to efflux the substrate loperamide [102]. However other publications support our initial hypothesis and the opposite to what was observed in our studies and the research referenced above. A study performed by Hitzl, discovered that P-gp efflux activity and expression within CD56+ natural killer cells isolated from healthy male Caucasians, is influenced based on the presence of the C3435T polymorphism [42]. Another study using 21 healthy Caucasian volunteers also observed a significant difference in P-gp efflux activity and expression in

the duodenum, between TT3435 and CC3435 populations [38]. The clinical studies mentioned above used healthy male Caucasian volunteers similar to our study, therefore pathological or ethnic differences are not likely reasons for the contradictory results. The studies that did observe P-gp activity and expression differences focused solely on the C3435T polymorphism, and did not consider any possible haplotype relationships that may also exist. Therefore the results, from the above mentioned studies, that were observed with the C3435T polymorphism may have been linked to another polymorphism that is responsible for influencing P-gp efflux activity and expression. Further studies which consider haplotype relationships between C3435T and other polymorphic loci within the *ABCB1* gene should be considered to explain this discrepancy. A study of Japanese patients suggests the possible role of the T-129C polymorphism within the promoter region of the *ABCB1* gene in influencing P-gp expression within human placenta [99]. Whether the T-129C polymorphism does have a haplotype relationship with the more commonly studied C3435T and G2677A/T still needs to be determined.

4.2 Influence of Trans-β-carotene (β-carotene) on P-gp Expression

The influence of the NHP β-carotene on P-gp expression was examined through a clinical study involving HIV infected patients with a carotene deficiency. Initially, the study was designed to involve 14 patients, however the study did not accrue to the expected number and a total of 5 HIV infected patients participated in the study. Following a 28-day β-carotene supplementation regimen, P-gp expression levels within isolated PBMCs were measured and compared to expression levels prior to β-carotene supplementation.

Results obtained from the β-carotene study with these HIV infected patients do not indicate a statistically significant difference in P-gp mRNA expression following a 28 day supplementation regimen. However, a trend towards an increase in P-gp mRNA expression was observed from the five patients that have been tested thus far. Metabolites of β-carotene are believed to be involved in the activation of transcription factors such as the 9-*cis* retinoic acid receptor (RXR), which has the ability to heterodimerize with PXR [61,103]. The activation of PXR would result in an increase in *ABCB1* gene expression. Therefore the trend increase observed with the five patients studied, although not statistically significant, may suggest a possible activation of *ABCB1* gene expression. Future analysis on the influence of β-carotene supplementation within a larger population would provide further insight in determining whether an increase in P-gp mRNA expression exists. Due to the fact that the study was performed using PBMCs isolated from HIV infected individuals, it may be possible that HIV infected cells do not effectively respond to stimuli, such as the activation of PXR by metabolites of β-carotene. Therefore a non-substantive difference in P-gp mRNA expression within infected PBMCs is observed instead of the increase in P-gp mRNA expression expected, following β-carotene supplementation.

4.3 Influence of NHPs on P-gp and CYP Activity

The effect of natural health products (NHPs) on drug metabolism varies between each product. Several NHPs, such as St John's Wort, grapefruit juice and garlic, have been shown to influence the activity of drug metabolizing proteins such as P-gp and members within the CYP3A subfamily [2,48,52,54]. The potential for NHPs to influence drug

metabolism in a biphasic manner, either through a single dose or multiple doses, represents a difficulty in ensuring that the bioavailability of conventional medication is present at therapeutic levels and adverse drug reactions do not develop.

4.3.1 Trans-β-carotene (β-carotene)

The influence of aqueous and methanolic extracts of β-carotene on P-gp efflux activity within Caco-2 cells was assessed. Both aqueous and methanolic extracts of β-carotene did not influence the efflux activity of P-gp at 15 min and 18 h. A slight, yet statistically significant, decrease in Rh123 levels which corresponds to an increase in P-gp efflux activity was observed at 60 min for the methanolic extract of β-carotene. This slight increase in P-gp activity at 60 min may represent an increase in activity due to the presence of β-carotene, however if this were the case a further increase in P-gp efflux activity would have been expected at 18 h, as seen with other NHPs discussed below. A recent study using L1210 mouse lymphoma cells and human breast cancer cells, also observed no influence on P-gp efflux activity for Rh123 in the presence of β-carotene [104]. Another study suggested that β-carotene is involved in enhancing P-gp efflux activity, via the activation of PXR which in turn increases the expression of the *ABCB1* gene, however our results at 18 h do not agree with this suggestion [61].

The influence of aqueous and methanolic extracts of β-carotene on CYP 3A4, 3A5 and 3A7 isoenzyme activity produced quite interesting observations, with the aqueous extracts resulting in significant increase in DBF metabolism by the CYP 3A4 isoenzyme, however not influencing the activity of CYP 3A5 and 3A7. The methanolic extracts of β-

carotene also produced statistically significant results, where an enhancement of DBF metabolism was observed for CYP 3A4 and 3A5, however an inhibition in activity was found for CYP 3A7. At the three concentrations tested (3.3 mg/ml, 1.7 mg/ml and 1.1 mg/ml), the increase in CYP 3A4 isoenzyme activity does not seem to be concentration dependent. A possible reason for this may be due to the fact that at 1.7 mg/ml the influence on isoenzyme activity is saturated. A preliminary study was been performed at lower β-carotene concentrations (0.5 mg/ml and 0.25 mg/ml), which suggest a concentration dependent increase in CYP 3A4 activity. The results to this study have not been presented, since additional information is required regarding the influence of lower β-carotene concentrations on P-gp efflux activity within the Caco-2 cell line, as well as on CYP 3A5 and 3A7 isoenzyme activity.

No change in CYP 3A5 isoenzyme activity was observed in the presence of aqueous extracts of β-carotene. The inability of the aqueous extracts of β-carotene to influence CYP 3A5 isoenzyme activity may be due to a decrease in the substrate selectivity of the constituents that are soluble within the aqueous extract. Results from CYP 3A5 isoenzyme activity studies with the methanolic extracts of β-carotene, suggest an enhancement of DBF metabolism at the three concentrations studied. The decrease in DBF metabolism associated with an increased concentration of methanolic extract of β-carotene may be due to the multiple binding of substrates to CYP 3A5. Studies have suggested that CYP 3A4 may have more than one substrate binding pocket at the active site of the isoenzyme, as well as an allosteric "effector" site which is not involved in substrate metabolism, but influences the binding and metabolism of substrates at the

active site [105,106]. If a similar characterization of substrate binding is also present within CYP 3A5, this may explain the observed decrease in DBF metabolism associated with an increase in the concentration of methanolic extracts of β-carotene. At high concentrations, such as 3.3 mg/ml, it may be possible that binding of β-carotene to this allosteric site occurs more frequently, whereas at lower concentrations, such as 1.1 mg/ml, binding to the allosteric site is limited. Therefore at lower β-carotene concentrations, the reduced binding at the CYP 3A5 allosteric site would not influence the binding and metabolism of DBF at the active site of the isoenzyme. This phenomenon may not be observed with CYP 3A4 or CYP 3A7, since they share a greater sequence homology with each other within the substrate binding section, than CYP 3A5 [105]. Future studies should consider lower concentrations of β-carotene extracts in order to determine their influence on CYP 3A5 activity as well as kinetic assays which determine whether allosteric modulation of isoenzyme activity by β-carotene exists within CYP 3A5.

The isoenzyme activity of CYP 3A7 in the presence of aqueous extracts of β-carotene ranged at the three concentrations tested. At the highest and lowest concentrations tested, no influence on CYP 3A7 isoenzyme activity was observed, however at 1.7 mg/ml a small, yet statistically significant, increase in isoenzyme activity was observed. This observation may be due to the presence of some trace amounts of organic constituents that may have remained within the aqueous extract during sample preparation. The influence of the methanolic extracts of β-carotene on CYP 3A7 activity suggests a concentration dependent inhibition, with a possible plateau in inhibition beginning at the

3.3 mg/ml concentration. Further tests studying the influence of lower concentrations of β-carotene extracts on CYP 3A7 activity, as performed with CYP 3A4, can verify whether a concentration dependent inhibition does exist.

It has been suggested that β-carotene can increase the expression of *CYP3A4*, *CYP3A5* and *CYP3A7* genes via the activation of PXR [61]. Results from this study suggest that β-carotene has the ability to increase metabolism of DBF by CYP 3A4 and 3A5 *in vitro*. An increase in CYP activity by β-carotene could result in the increased metabolism of conventional medication taken concomitantly, with the potential to reduce the bioavailability of the drug, resulting in an inability to invoke a therapeutic effect. Additionally, an increase in CYP activity due to β-carotene may lead to increased metabolism of carcinogenic compounds, which have the potential to increase oxidative stress [57,107]. Whether the antioxidant effect of β-carotene would outweigh the indirect influence it may have in the development of oxidative stress needs to be addressed.

4.3.2 *Arctostaphylos uva-ursi* (Kinnikinnick)

This study was initiated as limited information was available on the effect of Kinnikinnick extracts on P-gp efflux activity and CYP-mediated metabolism. Five Kinnikinnick products, three bulk and two capsulated, were tested to determine their influence on P-gp efflux activity within human Caco-2 cells and CYP 3A4, 3A5 and 3A7 activity *in vitro*.

The studies performed, which considered the influence of aqueous extracts of

Kinnikinnick on P-gp efflux activity within Caco-2 cells, differed between the five extracts tested, with NRP 247A producing the greatest range at the three time points tested. The initial increase in Rh123 levels at 15 min and the further increase at 60 min demonstrate a statistically significant inhibition in P-gp efflux activity. A statistically significant decrease in levels of Rh123 was observed at 18 h, indicating an intracellular response to the initial inhibition resulting in an increase in P-gp efflux activity. The increase in P-gp efflux activity was observed for all five aqueous extracts of Kinnikinnick at 18 h. This increase in P-gp activity may be due to an increase in P-gp mRNA expression, possibly via the activation of PXR which was previously mentioned to be involved in modulating the expression of the *ABCB1* gene. Further studies need to be performed to determine if the increase in P-gp efflux activity is a result of increased *ABCB1* gene expression or increased release of P-gp from intracellular stores. This biphasic effect was also present for the studies performed with methanolic extracts of NRP 247A within Caco-2 cells, although to a lesser degree due to the fact that the extract concentration was 1 mg/ml versus 5 mg/ml for the aqueous counterpart. However, at 18 h all five methanolic extracts of Kinnikinnick resulted in a statistically significant increase in the efflux activity of P-gp.

Preliminary studies were performed within human THP-1 cells using aqueous and methanolic extracts of Kinnikinnick. The results obtained with both the aqueous and methanolic extracts of Kinnikinnick indicate a biphasic influence on P-gp efflux activity. Although both aqueous and methanolic extracts produced the biphasic effect, the methanolic extracts seemed to be more effective in inducing this effect, as can be seen

with an inhibition in P-gp efflux activity initially at 15 min, opposed to 60 min as seen with the aqueous extracts, and secondly even at a lower concentration (1 mg/ml), a strong influence on P-gp efflux activity was observed. In comparison with studies performed within the Caco-2 cells, the results suggest that P-gp within THP-1 cells were more sensitive to the influence of the extracts tested. The observations from this Kinnikinnick study demonstrates how NHPs can influence drug disposition in a biphasic manner, as well as recognizes that the extent of this influence may change depending on the type of cell or tissue exposed.

The influence of aqueous extracts of Kinnikinnick on CYP 3A4, 3A5 and 3A7 mediated metabolism of DBF was also studied. All three isoenzymes studied within the CYP3A subfamily were significantly inhibited in the presence of the aqueous extracts of Kinnikinnick, with inhibition from most to least in the order of CYP 3A5 $\geq$3A7>3A4. Although CYP 3A4 was inhibited to a lesser degree than CYP 3A5 and CYP 3A7, due to its high content within the liver, up to 60% of all hepatic CYPs, and ability to metabolize approximately 50% of all conventional medication, such an inhibitory effect would likely have a greater influence in the elimination of xenobiotics [12,108].

When studying the influence of the methanolic extracts of Kinnikinnick on the metabolism of marker substrates, a statistically significant inhibition of all three CYPs studied was observed. The inhibitory effect of the methanolic extracts of Kinnikinnick were similar to that observed with the aqueous extracts in some cases, such as the influence of NRP 191 on CYP 3A4 activity. However, other methanolic extracts of

Kinnikinnick were able to have an even greater inhibitory effect on isoenzyme activity compared to the aqueous counterpart, considering the fact that a lower concentration of methanolic extract (250 μg/ml) was within the reaction medium compared to aqueous extracts (1.25 mg/ml). This can be seen with the influence of the NRP 247A extracts on CYP 3A5 activity, where similar levels of inhibition on isoenzyme activity exist in the presence of either extract.

The studies performed suggest a potential for Kinnikinnick to influence drug disposition by acting on P-gp and CYP3A isoenzymes. However the specific constituents within the Kinnikinnick extracts that produce the observed effects are still unclear. Future studies need to be performed in order to determine the specific active constituent(s) involved in the inhibition of P-gp efflux activity and CYP3A isoenzyme activity, and which form of extract (aqueous or methanolic), increases the solubility of the active constituents. This could potentially lead to the development of safer Kinnikinnick products that would provide all of the reported benefits to consumers, without seriously influencing the therapeutic effect of conventional medication taken concomitantly. One of the major implications of the present studies involves the neonatal isoenzyme CYP 3A7 which was found to be highly sensitive to inhibition by Kinnikinnick. Many label warnings along with the Natural Medicines Database suggest that Kinnikinnick could be unsafe to take during pregnancy or lactation [109]. These results substantiate warnings and suggest a strong possibility of Kinnikinnick being hazardous to neonates.

4.3.3 *Acorus americanus and Acorus calamus* (Acorus)

Studies were performed to assess the influence of Acorus on P-gp efflux activity within Caco-2 cells. The influence of aqueous and methanolic extracts of Acorus on P-gp efflux activity within Caco-2 cells did not produce the biphasic effect observed with some of the Kinnikinnick extracts. The aqueous extracts of Acorus did not influence P-gp efflux activity at 15 min and 60 min, and a slight, yet statistically significant, decrease in Rh123 levels, which represents a slight increase in P-gp efflux activity, was observed at 18 h. The increase in P-gp efflux activity at 18 h may suggest that the aqueous extracts of Acorus does slightly activate P-gp expression via the activation of PXR, however further studies measuring P-gp mRNA expression levels at the three time points would need to be performed to verify this. The methanolic extracts of Acorus were not able to influence P-gp efflux activity at all three time points, suggesting that any influence that Acorus may have of P-gp activity is minimal at most. Another possible explanation can be due to the lower concentrations (250 μg/ml) used for the methanolic extracts, which may have prevented any significant effect on P-gp efflux activity from being observed.

The influence of aqueous and methanolic extracts of Acorus on CYP 3A4, 3A5 and 3A7 activity was also studied. The isoenzyme activity of CYP 3A4 was influenced in a different manner by the two Acorus samples tested. The aqueous NRP 205 extract was not able to inhibit CYP 3A4 activity, whereas a weak inhibition in the presence of the aqueous NRP 240 extract was observed. When considering the influence of the methanolic extracts of Acorus on CYP 3A4 activity, greater evidence exists that shows the difference between NRP 205 and NRP 240. The methanolic extract of NRP 205

increased DBF metabolism by CYP 3A4, whereas the NRP 240 extract caused a moderate, yet statistically significant, inhibition on CYP 3A4 activity producing a greater inhibitory effect relative to the aqueous NRP 240 extract. This data suggests that although the two products are considered to be of an Acorus variety, their influence on isoenzyme activity substantially differs. A similar trend can be observed when studying the influence of the aqueous and methanolic extracts of Acorus on CYP 3A5 activity. The aqueous extract of NRP 205 also did not influence isoenzyme activity, however the aqueous NRP 240 extract produced an even greater inhibitory effect on CYP 3A5 than that observed with CYP 3A4. Similarly, the methanolic extracts of NRP 205 increased DBF metabolism by CYP 3A5, however to a lesser degree than seen with CYP 3A4, whereas the methanolic extracts of NRP 240 significantly inhibited isoenzyme activity to levels equivalent to the ketoconazole positive control. The methanolic extracts (250 μg/ml) of NRP 240 were observed to be more effective in the inhibition of CYP 3A4 and CYP 3A5 than the aqueous extracts (5 mg/ml), suggesting that the constituents involved in inhibition are lipophilic. For both CYP 3A4 and CYP 3A5 a stronger influence on activity was observed for the NRP 240 extract, which is of the *Acorus calamus* variety.

The presence of lipophilic β-asarones, may explain the distinction observed between the two products. The NRP 205 extract, which is of the *Acorus americanus* variety, does not contain β-asarones, and therefore would not influence isoenzyme activity in the same manner. Further studies analysing the chemical content of the two Acorus products tested should be performed, in order to confirm which specific chemical components are responsible for producing the opposing effects observed. The influence of the aqueous

extract of NRP 205 on CYP 3A7 activity differed from what was observed with CYP 3A4 and CYP 3A5. Both the aqueous extracts of NRP 205 and NRP 240 were able to cause a statistically significant inhibition of CYP 3A7 activity and a similar trend was observed with the methanolic extracts. Since both extracts have the ability to inhibit CYP 3A7 activity, constituents in addition to β-asarones are likely involved in CYP 3A7 inhibition. The degree of inhibition for both the aqueous and methanolic extracts was greater for NRP 240 in relation to NRP 205, which may be due to the presence β-asarones within NRP 240. Currently, there are no available publications that consider the influence of Acorus on the activity of CYP 3A7.

4.3.4 Spices (*Curcuma longa*, *Capsicum annuum*, *Piper nigrum*)

The influence of extracts of the three bulk product spices – turmeric powder, chilli powder and black pepper, on the efflux activity of P-gp within Caco-2 cells was also measured. The aqueous extracts of turmeric powder and black pepper produced statistically significant results, where a slight decrease in Rh123 levels within Caco-2 cells at 15 min and 60 min were observed, which suggests a minimal increase in P-gp efflux activity. The aqueous black pepper extract also produced a similar effect at 18 h. The decreased Rh123 levels observed may be due to the activation of PXR, resulting in an increased expression of P-gp, however the process of gene up-regulation would not occur within 15 min. An alternative explanation may be due to the quenching ability of the turmeric powder and black pepper extracts, which would decrease the fluorescent intensity measured. Since some spices exhibit natural fluorescence or quenching, a future study which considers the quenching ability of both aqueous and methanolic extracts of

the three spices tested on the fluorescent Rh123 should be considered [110]. The aqueous extract of chilli powder did produce a biphasic effect, at a concentration of 5 mg/ml, as previously observed with extracts of Kinnikinnick. A possible explanation for the initial inhibition at 15 min and 60 min, followed by induction of P-gp efflux activity at 18 h, may be due a cellular response to the inhibition, resulting in an increased translocation of intracellular P-gp to the cell membrane or increased expression of the *ABCB1* gene via the activation of a nuclear response element such as PXR. Further studies involving P-gp mRNA analysis within Caco-2 cells exposed to aqueous extracts of chilli powder would provide insight regarding the mechanism of action. One of the active compounds within chilli powder, capsaicin, is an alkaloid and as previously mentioned is believed to be involved in the inhibition of P-gp activity [83]. However, capsaicin is a lipophilic compound, and since our study observed a moderate effect of the aqueous chilli powder extract on P-gp efflux activity, some other chemical component within the bulk chilli powder sample may be responsible for the biphasic effect observed. A chemical analysis of the bulk chilli powder product should focus on the components that are within the aqueous samples of the chilli powder extract, in order to develop a more comprehensive knowledge of the actual components involved in inhibiting P-gp efflux activity. The methanolic extracts of the bulk spices did not result in any significant changes in P-gp efflux activity at the three time points tested, with the exception of the chilli powder extract that produced a slight increase in Rh123 levels at 60 min. A possible explanation for the unsubstantial result observed using the methanolic extracts of spice may be related to the lower concentrations (250 μg/ml) used due to potential cell sensitivity. Similar to the methanolic extracts of Acorus, a slightly higher final

concentration (between 500 μg/ml and 1 mg/ml) should be considered to determine any influence that these extracts would have on P-gp efflux activity.

A measurement of fluorescein production from the metabolism of DBF by CYP 3A4, 3A5 and 3A7, in the presence of extracts of the three bulk product spices was determined. A statistically significant inhibitory influence was observed for the three spice products extracts on CYP 3A4, 3A5 and 3A7 isoenzyme activity. The aqueous turmeric powder and chilli powder extracts (5 mg/ml) were able to inhibit CYP 3A4 to a similar degree compared to the methanolic turmeric powder and chilli powder extracts (250 μg/ml), however the methanolic black pepper extract seems to be more effective in inhibiting CYP 3A4 isoenzyme activity compared to the aqueous counterpart. Interestingly, the methanolic extracts of turmeric powder, chilli powder and black pepper were able to inhibit CYP 3A5 and 3A7 more effectively than the aqueous counterpart, when the lower concentrations of the methanolic extracts are considered. This data suggests that the chemical components within the methanolic extracts likely have a more potent inhibitory influence on CYP 3A4, 3A5 and 3A7 activity. When considering that the commonly studied constituents in turmeric powder (curcuminoids), chilli powder (capsaicin) and black pepper (piperine) are lipophilic, this suggests that these compounds may be involved in the inhibitory action on the CYP3A isoenzymes studied. Future examination to determine the chemical composition of the bulk products tested, would provide further insight of specific compounds involved in influencing isoenzyme activity within the CYP3A subfamily.

Studies which consider the influence of the tested spices on drug metabolism, tend to focus on the main active constituents instead of the more commonly consumed form. A study which considered the influence of curcumin and capsaicin on P-gp function within carcinoma KB-C2 cells found that both constituents were able to cause an inhibitory effect on P-gp activity [83]. Another study observed an inhibitory influence by piperine on P-gp transport within Caco-2 cells and CYP 3A4 isoenzyme activity *in vitro* [90]. Our studies considered the influence of the commonly consumed spice product, which contained between 0.01% to 7.0% active constituents. What was also observed in some instances was the aqueous extracts of the spices tested had a similar influence on P-gp efflux activity and CYP isoenzyme activity as seen with the methanolic extracts. This suggests that the commonly studied active constituents, which are lipophilic, may not be the only chemical compounds involved in influencing the activities of P-gp and the CYPs tested. Therefore future tests should consider employing pure chemical compounds in comparison to the commonly consumed extracts. Additionally, chemical analysis of the aqueous and methanolic extracts would assist in clarifying the role of the constituents found within these extracts.

4.4 Ritonavir

The HIV protease inhibitor ritonavir has been used as a positive control in our P-gp efflux studies within Caco-2 cells due its known influence as a P-gp inhibitor [111]. Throughout these studies, it has been consistently shown that ritonavir produces an inhibitory effect on P-gp efflux activity in a concentration dependent manner, resulting in statistically significant increased levels of Rh123 within Caco-2 cells (Figure 28,

Appendix A). An interesting observation with ritonavir exposure to Caco-2 cells, is that a biphasic influence observed with some of the NHPs tested does not exist. A possible reason for this may be due to the fact that a longer exposure time may be required to produce an induction of P-gp mRNA expression [30]. A study using primary cultures of human hepatocytes from six patients found that exposure to ritonavir for 42 h was able to activate PXR, yet a decrease in microsomal CYP 3A4 activity was observed [112]. This study explained the observed discrepancy by suggesting that since ritonavir is such a potent inhibitor of CYP 3A4, the increase in expression and levels of CYP 3A4 caused by PXR activation does not produce any significant changes in CYP 3A4 isoenzyme activity observed. Since P-gp is also inhibited by ritonavir, a similar explanation may apply to the observation of no change in P-gp efflux activity following a 24 h incubation period within the studies presented.

4.5 Conclusion

Our studies have considered whether a correlation exists between the C3435T, G2677A/T, C1236T haplotype and P-gp efflux activity or *ABCB1* gene expression. Our data indicates that there is no difference between CC3435 and TT3435 individuals, as well as the C3435T, G2677A/T, C1236T haplotype relationship, in relation to the efflux activity or expression of P-gp within isolated PBMCs. These observations agree with other published studies [101,102]. However an analysis of other polymorphisms within the ABCB1 gene, such as T-129C, should be considered in order to determine what influence they may have on P-gp efflux activity or expression. Additionally a larger study population, as initially planned, would have provided a better indication of whether

the C3435T, G2677A/T, C1236T haplotype does influence P-gp efflux activity or *ABCB1* gene expression.

The influence of a 28 day β-carotene supplementation on P-gp expression levels within HIV infected patients with a carotene deficiency, was also studied. Our results indicate no statistically significant change in P-gp mRNA expression following the supplementation period, however a general trend towards an increase in P-gp mRNA levels was observed. An increase in *ABCB1* gene expression, via the activation of PXR has been previously reported [61]. The potential for β-carotene to increase P-gp expression can have serious implications for an HIV infected individual taking this NHP concomitantly with anti-retroviral medication, since an increased activity in P-gp may ultimately decrease the bioavailability of the therapeutic drug. Further analysis using a larger patient population may provide a more conclusive result in determining whether any influence β-carotene supplementation may have on P-gp expression.

Studies involving the *in vitro* influence of NHPs on P-gp efflux activity within Caco-2 cells have shown that some NHPs, such as Kinnikinnick and the commonly consumed chilli powder, are able to cause a biphasic effect on P-gp efflux activity dependent on the duration of exposure with the NHP. Additionally, preliminary studies with THP-1 cells suggest that the influence of NHPs, such as Kinnikinnick, on P-gp efflux activity differs between cell types. Clinically, these observations suggests that certain types of NHPs, depending on whether a single dose or multiple doses of the NHP is taken, have the potential to influence drug disposition of conventional medication taken concomitantly,

and the extent of the influence by the NHP may change depending on the type of tissue/organ targeted.

The main focus of the current literature in the area of NHPs and CYP drug metabolism considers possible drug interactions involved with the CYP 3A4 isoenzyme. However, observations from this study suggest that other CYP3As (CYP 3A5 and CYP 3A7) are also significantly influenced by NHPs. A general observation from our *in vitro* studies suggests that CYP 3A4, 3A5 and 3A7 react to these NHPs in a different manner where in some cases an increase in metabolism is observed and in other cases substrate metabolism is inhibited. An interesting observation from this study was that both aqueous and methanolic extracts of the commonly consumed preparations of turmeric powder, chilli powder and black pepper were able to significantly inhibit CYP3A enzyme activity. Considering that a large proportion of individuals consume these spices on a consistent basis, it may be possible that drug bioavailability within these individuals is compromised compared to individuals that do not consume spices within their diets. The common trend seen between the NHPs and isoenzymes studied is that the neonatal CYP 3A7 is strongly inhibited, which raises the issue as to whether pregnant women should be consuming certain NHPs in their diets. With the increased use of NHPs within the population, further studies should be performed to determine the influence that these products have on the bioavailability of drugs consumed for therapeutic purposes.

Appendix A: Supplementary Data

Table 2: Natural Health Products Tested

Test product information on weight, suggested dose, sources and physical appearance of the product. All product extracts were prepared fresh on the day of study and under reduced lighting conditions. (NRP represents Nutraceutical Research Programme accession number).

NRP	Product Name (Brand) [Expiration Date, mm,yy]	Stated Weight/Units (Average Weight) Per Capsule	Suggested Dose	Sources & Appearance of Product
191 192 193	Kinnikinnick, *Arctostaphylos uva-ursi*	Bulk Product	-----	Powdered leaves, green/brown
247A	Kinnikinnick (Herbal Select) [07,04] *Arctostaphylos uva-ursi*	500 mg (491.6 mg)	Two capsules, 1-3 times daily	Powdered leaves, green/brown
247B	Kinnikinnick (Herbal Select) [09,05] *Arctostaphylos uva-ursi*	500 mg (484.8 mg)	Two capsules, 1-3 times daily	Powdered leaves, green/brown
157	B-Carotene (Exact) [04,06]	25000 IU (254.5 mg)	One capsule daily	Viscous liquid, dark organge/red
205	Sweet Flag, *Acorus Americanus*	Bulk Product	-----	Powdered root, light brown
240	Sweet Flag, *Acorus Calamus*	Bulk Product	-----	Powdered root, light brown
261	Turmeric Powder, *Curcuma longa*	Bulk Product	-----	Powdered root, dark yellow
262	Chilli Powder, *Capsicum annuum*	Bulk Product	-----	Powdered chilli, dark red
263	Black Pepper, *Piper nigrum*	Bulk Product	-----	Powdered peppercorns, brown/black

Table 3: Comparison of P-glycoprotein (P-gp) mRNA expression between TT3435 and CC3435 populations.

P-gp mRNA levels were measured from isolated peripheral blood mononuclear cells (PBMC) of healthy male Caucasian individuals containing a TT3435 (Table 3A) or CC3435 (Table 3B) genotype and one of five haplotype variations, with G2677T/A and C1236T, within the *ABCB1* gene. P-gp mRNA expression levels were determined using reverse-transcriptase followed by cDNA amplification using PCR methodology. Samples were analysed using a polyacrylamide gel electrophoresis system and band intensity measured using a Kodak 440-CF band analysis system. (Average mRNA band intensity ± SD, adjusted based on total RNA concentration within sample, n = 15 patients. Each individual value represents triplicate readings).

Table 3A

Individual Identification Number	Genotype			Average P-gp mRNA Band Intensity	Standard Deviation (%)	P-gp mRNA Value (adjusted based on total RNA concentration)
	C3435T	G2677T/A	C1236T			
9	TT	GT	TT	16230	11.6	4.6
33	TT	GT	CT	6200	10.4	5.2
52	TT	GT	CT	19900	3.0	34.3
75	TT	GT	CT	13580	1.0	9.2
140	TT	GT	CT	4720	8.8	4.1
128	TT	TT	TT	9900	5.3	3.5

Table 3B

Individual Identification Number	Genotype			Average P-gp mRNA Band Intensity	Standard Deviation (%)	P-gp mRNA Value (adjusted based on total RNA concentration)
	C3435T	G2677T/A	C1236T			
26	CC	GG	CC	15630	6.8	7.6
37	CC	GG	CC	28000	6.8	35.6
51	CC	GG	CC	7050	6.3	3.5
53	CC	GG	CC	6850	18.5	6.7
78	CC	GG	CC	10160	9.7	8.3
113	CC	GG	CC	3565	16.6	3.0
151	CC	GG	CC	10710	10.0	4.2
136	CC	GA	CC	4404	8.8	2.9
150	CC	GA	CC	10190	9.4	8.5

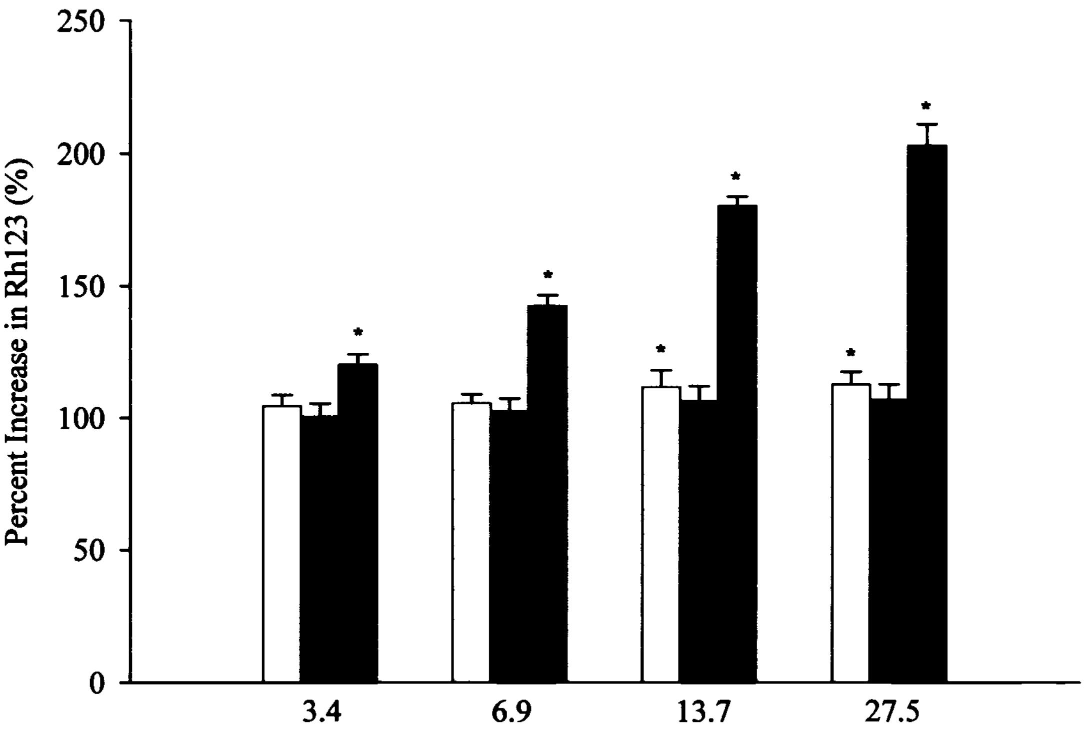
Table 4: Effect of β-carotene supplementation on P-glycoprotein (P-gp) mRNA expression.

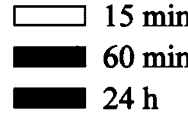
P-gp mRNA levels were measured from isolated peripheral blood mononuclear cells (PBMC) pre- and post- β-carotene (NRP 157) supplementation of HIV infected patients. P-gp mRNA expression levels were determined using reverse-transcriptase followed by cDNA amplification using PCR methodology. Samples were analysed using a polyacrylamide gel electrophoresis system and band intensity measured using a Kodak 440-CF band analysis system. (Average mRNA band intensity, adjusted based on total RNA concentration within sample, n = 5 patients. Each patient value represents triplicate readings).

Patient	Average Band Intensity Pre β-carotene	Average Band Intensity Post β-carotene	Increase in P-gp Expression (%)	Standard Deviation (%)
1	2258.09	5253.51	132.8	6.2
2	8642.32	6309.64	-27.1	7.7
3	11283.57	16675.04	46.6	17.1
4	10176.47	9963.25	-2.6	18.9
5	9487.02	14180.10	49.1	15.0

Figure 28: Influence of ritonavir on P-gp efflux activity within Caco-2 cells.

The efflux activity of P-gp within Caco-2 cells was determined using the fluorescent substrate Rh123 (150 ng/ml) in the presence of 3.4 µg/ml, 6.9 µg/ml, 13.7 µg/ml and 27.5 µg/ml ritonavir. Following 15 min, 60 min and 18 h incubations at 37°C and 5% CO_2, the amount of Rh123 remaining within the Caco-2 cells was measured using a CytoFluor Series 4000 Multi-Well Plate Reader. (Control = 100%, % Rh123 Increase ± SD; n = 6). Asterisk (*) represents data with a p-value ≤ 0.05 using the paired t-test, indicating a statistically significant difference in P-gp efflux activity in Caco-2 cells treated with ritonavir compared to untreated controls.


250
200
150
100
50
0
Percent Increase in Rh123 (%)
*
*
*
*
*
*
3.4
6.9
13.7
27.5
Ritonavir Concentration (μg/ml)


15 min
60 min
24 h

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