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BUDZINSKI, Jason William

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

M.Sc. (Biology)

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In Vitro Interactions of Natural Health Products with P-Glycoprotein (P-gp) and Cytochrome P450 3A4 (CYP3A4): Relevance to HIV Therapies

John T. Arnason

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

T.W. Moon

B.J.R. Philogène

M. Smith

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

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In vitro interactions of natural health products with P-glycoprotein (P-gp) and cytochrome P450 3A4 (CYP3A4): Relevance to HIV therapies.

Jason William Budzinski

Thesis submitted to the
School of Graduate Studies and Research
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In partial fulfillment of the requirements for the
MSc degree in the
Ottawa-Carleton Institute of Biology

DEDICATION

For Paul Skory... gone but not forgotten.

Papa: Here is something for you to read while enjoying a Coke at the Kresge's in the sky.

ABSTRACT

This thesis examines the mechanisms by which natural health products (NHP)s may affect drug disposition pathways. Adverse reactions from concomitant NHP-drug use may result from competing interactions at the level of the metabolic enzyme cytochrome P450 3A4 (CYP3A4) or the xenobiotic efflux pump P-glycoprotein (P-gp). NHPs and drugs relevant to HIV were assessed for their ability to affect these drug disposition pathways.

NHP extracts were examined for their ability to inhibit CYP3A4 and stimulate P-gp ATPase. Goldenseal (GS) NHPs were the most inhibitory of CYP3A4; GS and milk thistle (MT) teas stimulated P-gp ATPase more than the positive control verapamil.

GS and MT extracts, related phytochemicals, and several HIV drugs were screened for their ability to modulate *CYP3A4* and P-gp (*ABCB1* gene product)expression in caco2 cell monolayers. *ABCB1* expression did not change from control levels; *CYP3A4* expression was more responsive, and thus a better marker for cellular response.

RÉSUMÉ

Cette thèse examine les mécanismes par lesquels les produits de santé naturels (PSN) peuvent altérer le métabolisme des médicaments. Les réactions adverses associées à l'usage concomitants des PSN peuvent résulter d'interactions compétitives au niveau de l'enzyme metabolique cytochrome P450 3A4 (CYP3A4), ou au niveau de la pompe xénobiotique P-glycoprotéine (P-gp). Une variété de PSN et de drogues relatives au VIH ont été testées pour leur capacité à affecter ces mécanismes d'élimination des médicaments.

Les extraits de PSN furent examinés pour leur capacité à inhiber le CYP3A4, et à stimuler l'activité de la P-gp ATPase. Les produits de l'hydraste du Canada (HC) étaient les plus inhibiteurs du métabolisme effectué par le CYP3A4; le thé de HC et de chardon-marie (CM) ont stimulé la P-gp ATPase à un plus fort degré que le contrôle positif de référence, le verapamil.

Les extraits de HC et de CM, produits phytochimiques reliés, et quelques médicaments pour le VIH furent évalués pour leur capacité à moduler les niveaux d'expression de *CYP3A4* et du gène de la protéine de transport P-gp (dénommé *ABCB1*) dans les cellules caco2. L'expression de *ABCB1* était peu affectée; *CYP3A4* réagissait plus aux différents traitements et était donc un meilleur marqueur de la réponse cellulaire.

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So this is it.... the culmination of the last 3 years of my research and life at the University of Ottawa (4 years if one considers my undergraduate 4th year project which provided a strong background and was the precursor for the work contained herein). I must confess that it has been both the best and worst time of my life. It has been a time of change and transition. There were days that I loved and days that I loathed, but I guess that is just as much a part of research as it is life. And hey... I learned a lot and am finally beginning a new phase in my academic career. But I would not be here now if it were not for the strength, support, compassion, friendship, and guidance of various individuals.

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I must honestly say that I did not have any great difficulty in writing this work... it was just sitting down and getting it started that was the hard part! (which reminds me of one more... thanks to Lise Maisonneuve).

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LIST OF ABBREVIATIONS

ABCB1 (MDR1)	adenosine triphosphate-binding cassette efflux transporter B1; also known as: multi-drug resistance protein 1
AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
ATP	adenosine triphosphate
AU	absorbance units
Caco2	human intestinal epithelial adenocarcinoma cell line
CAM	complimentary or alternative medicine
CC	cat's claw (*Uncaria tomentosa*)
cDNA	complimentary deoxyribonucleic acid
CYP3A4	cytochrome P450 3A4
ddH2O	deionized/distilled water
DEPC	diethyl pyrrocarbonate
DMEM	Dulbecco's modified Eagle media
EFV	efavirenz (Sustiva®)
EtOH	ethanol
FBS	fetal bovine serum
GITC	guanidinium thiocyanate
GRAS	generally regarded as safe
GS	goldenseal (*Hydrastis canadensis*)
HIV	human immunodeficiency virus

HPLC	high pressure liquid chromatography
IC_{50}	median inhibitory concentration
LCY-GS	Li Chung Yun goldenseal
MDD	maximal daily dose
MeOH	methanol
mRNA	messenger ribonucleic acid
MT	milk thistle (*Silybum marianum*)
NFV	nelfinavir (Viracept ®)
NHP	natural health product
NNRTIs	non-nucleoside reverse transcriptase inhibitor
NRP	natural research product
NVP	nevirapine (Viramune®)
P-gp	p-glycoprotein
PCR	polymerase chain reaction
Pnx gin	*Panax ginseng*
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SHR-MT	Swiss Herbal Remedies milk thistle
Sib gin	Siberian ginseng (*Eleutherococcus senticosus*)
SJW	St. John's wort (*Hypericum perforatum*)

Sol-GS	Solaray goldenseal
Sol-MT	Solaray milk thistle
SQV	saquinavir (Invirase®)
SSC	standard sodium citrate
TCM	traditional Chinese medicine

LIST OF TABLES AND FIGURES

CHAPTER 1: GENERAL INTRODUCTION

There has been a dramatic increase in the use of natural health products (NHP)s, often combined with conventional drug therapies (Brevoort, 1998). This is especially evident among individuals living in a diseased state, who tend to seek out additional complementary or alternative therapies. The use of many NHPs is generally regarded as safe (GRAS) and indeed many cultures have a well established reliance and tradition surrounding their use in improving human health. For example, traditional Chinese medicine (TCM) has been demonstrated to have unique efficacy in treating numerous diseases, including many different conditions (Xiaomei and Weibo, 1999). However, despite the acceptance and credibility of many "natural therapies" (mainly in their country or culture of origin), little is still known of the interactions of such treatments when combined with the allopathic medicines used in the treatment of various diseases (such as HIV/AIDS) (McKnight and Scott, 1996). The potential for adverse reactions occurring with concomitant administration of conventional drugs and NHPs is high. Numerous NHPs have biologically active constituents and reportedly have significant pharmacological activities capable of producing both potent therapeutic effects and adverse reactions (Foster *et al.*, 2001). Many NHPs and pharmaceuticals may affect human drug disposition and significantly increase the risk for serious clinical adverse reactions (Ameer and Weintraub, 1997; Budzinski *et al.*, 2000; Eagling *et al.*, 1999; Foster *et al.*, 1999; Foster *et al.*, 2001; Laroche *et al.*, 1998; Miller, 1998; Piscitelli *et al.*, 2002a; Piscitelli *et al.*, 2002b)

1.1 Complementary and Alternative Medicine (CAM) Use Among HIV Infected Individuals

"Natural therapies" are considered to be *complementary* when taken concomitantly with conventional medical therapies to assist in ameliorating an individual's health. By contrast, therapies are considered *alternative* when utilized exclusively instead of treatments offered by allopathic

medicine. CAM treatments typical fall into 2 categories: (1) substances that are ingested as a solid or liquid (these include vitamins and NHPs); and (2) holistic treatments that rely on an individual's interaction with the environment or its elements (these include massage, aromatherapy, acupuncture, and meditation) (McKnight and Scott, 1996). With respect to the first category, individuals living with HIV/AIDS may take a variety of NHPs to: help boost the immune system and aid it repair the damage caused by the virus; help prevent or combat AIDS-related infections; help alleviate HIV-related conditions such as dementia, depression, and wasting; increase their amount of systemic antioxidants; and improve their sense of general well-being (CATIE, 2000). CAM use among individuals with HIV/AIDS is difficult to determine and estimates are highly variable. This is mainly due to the fact that many patients are reluctant to inform their physician or health-care provider that they are using CAM, surveying methods themselves are highly variable, and CAM use varies dramatically among individuals, regions, countries, and cultures. For example, estimates of CAM use among Australian HIV+ patients are as high as 80% (McKnight and Scott, 1996). Similarly, a nationwide survey of US HIV+ outpatients (191 respondents) estimated that individuals had used CAM at some time to control HIV (67%) or were currently using CAM therapies (40%) (Duggan *et al.*, 2001). A survey of the metropolitan area of Minneapolis/St. Paul (US) conducted by Harnack *et al.* (2001) reported CAM use at 61.2% (n = 376 respondents). Despite this variability, it is essential to consider CAM use, and in particular the use of NHPs, with respect to drug disposition in order to address all potential confounding factors that may lead to the occurrence of an adverse event, or an improvement in health. Presently, there are many novel NHPs derived from ethnopharmacological research that have promising anti-HIV activities. These include plant extracts that interfere with viral formation, infectivity, replication, and protease activity (Vermani and Garg,

2002).

1.2 Drug Disposition Mechanisms

Drug disposition is the ingestion, absorption, distribution, metabolism, transport, and elimination of drugs and xenobiotics (describes all foreign compounds but is largely used in the context of this thesis to refer to drugs and the phytochemical components of NHPs) which enter the body (Benet and Zech, 1994).

Cytochrome P450 3A4 (CYP3A4)

Located in high amounts in the small intestinal epithelium and liver, CYP3A4 is a major contributor to the presystemic metabolism of drugs administered orally. Because CYP3A4 has an extremely wide substrate specificity and many *in vitro* and *in vivo* interactions involving bioactive compounds, it appears to mediate the oxidation of approximately 40 - 50% of the drugs administered to humans (Thummel and Wilkinson, 1998). With respect to the effect of NHPs and drug-drug interactions, the most widely studied natural product is grapefruit juice, which has been found to increase the bioavailabilty and/or prolong the metabolic elimination of many drugs, such as the dihydropyridine-type calcium channel blockers, the histamine-1 receptor antagonist terfenadine, quinidine, the benzodiazepine midazolam, 17-β-estradiol, and caffeine (Ameer and Weintraub, 1997; Bailey *et al.*, 1998), thereby significantly increasing their plasma concentrations. The specific mode of action of grapefruit juice components was found to be the disruption of first pass degradation detoxification reactions mediated by CYP3A4 (Ameer and Weintraub, 1997; Chan *et al.*, 1998; Fuhr, 1998; He *et al.*, 1998; Lown *et al.*, 1997). Consequently, many of the studies initially performed with grapefruit juice and/or its components sparked much of the interest into the area of NHP-drug interactions at the metabolic level.

Clearly, inhibition of CYP3A4 has important implications for possible drug-drug interactions, and it is logical to assume that natural products other than grapefruit juice may exhibit similar biological activities. Figure 1.1 highlights the typical mode of action of phase I enzymes.

The ABCB1 gene product P-glycoprotein

P-glycoprotein is an ATP-dependent membrane-bound glycoprotein of approximately 170 kDa molecular weight, constitutively found in normal tissues including the gastrointestinal epithelium, the canalicular membrane of the liver, the kidney, and capillary endothelial cells in the central nervous system (Wandel *et al.*, 1999). P-gp is a product of the *ABCB1* (ATP binding cassette) gene (formerly known as *MDR1* (multi-drug resistance); HUGO [Human Genome Organisation] nomenclature change) and a member of the ABC transporter superfamily. Overexpressed P-gp in malignant cells has been associated with the development of the multidrug resistance phenomenon often seen in cancer chemotherapy, and much of the early work studying and characterizing P-gp has related it to anticancer agents (Wandel *et al.*, 1999). Figure 1.2 highlights some of the structural features and transport activity of P-gp.

As with CYP3A4, grapefruit juice and several of its components have also been found to influence the activity of P-gp efflux of concomitantly administered substrates. For example, Eagling *et al.* (1999) found that both naringin and 6',7'-dihydroxybergamottin, 2 compounds naturally occurring in grapefruit juice, caused a minor but significant reduction in the efflux of SQV by P-gp in caco2-2 cell monolayers.

1.3 Project Overview and Rationale

Ultimately, the goal of using NHPs is to improve human health in a safe and efficacious manner. In order for this to occur: (1) NHP manufacturers should be regulated with respect to quality

control techniques and all consumer products should have well documented and implemented standardization techniques; (2) NHP manufacturers should be held accountable for label claims; and (3) NHPs must be evaluated as thoroughly as possible, and ideally all NHPs used, especially those in CAM therapy, should be scrutinized in laboratory studies and clinical trials in the allopathic medical tradition (McKnight and Scott, 1996). Unfortunately, this is an impossible task due to the sheer magnitude and difficulty associated with fully evaluating NHPs: (1) there are too many products to evaluate; (2) working with crude mixtures of NHPs increases the number of possible potential interactions that can occur and it is overwhelming and impossible to consider them all; (3) clinical trials are time consuming and expensive; and (4) one must not only fully consider the NHPs themselves, but also the interindividual genotypic variabilities that exist among different groups of people with respect to drug disposition mechanisms. For example, the phase I xenobiotic metabolic activity of various isozymes (CYP450 superfamily) can range from 10 to 1000 fold among individuals (Zülhsdorf, 1998). Thus, the main safety concern surrounding NHP use lies primarily with the possibility of unknown NHP-drug interactions.

The goal of *in vitro* studies such as the ones described in this thesis is one of risk assessment in determining the likelihood of an adverse event occurring due to the interaction of NHPs with drug disposition mechanisms. In the first study, various NHP extracts, related phytochemicals, and a few HIV drugs were screened for their ability to inhibit CYP3A4 mediated metabolism of a reference substrate and stimulate P-gp ATPase activity (which would promote substrate transport). I hypothesized that extracts from commercial goldenseal (GS) (*Hydrastis canadensis*) and milk thistle (MT) (*Silybum marianum*) NHPs would be highly active because these plants are known to have constituents that are both inhibitory of CYP3A4-mediated metabolism and are substrates for P-gp

transport (Budzinski *et al.*, 2000; Maitrejean *et al.*, 2000). These extracts were found to exhibit moderate levels of CYP3A4 inhibition and to cause significant stimulation of P-gp ATPase as compared to the positive control and other tested NHPs (Chapter 2). In the second study, various GS and MT extracts, related phytochemicals, and the HIV drug saquinavir were further evaluated in caco2 cell monolayers (a model for drug absorption) for their ability to modulate *CYP3A4* and *ABCB1* expression. It was hypothesized that the various extracts of GS and MT would increase transcripts for both genes.

Net Reaction

$$NADPH + H^+ + O_2 + \underline{R}H$$

$$\downarrow$$

$$NADP^+ + H_2O + \underline{R}OH$$

or

$$\underline{R}H + O_2 + 2e^- + H^+$$

$$\downarrow$$

$$\underline{R}OH + OH^-$$

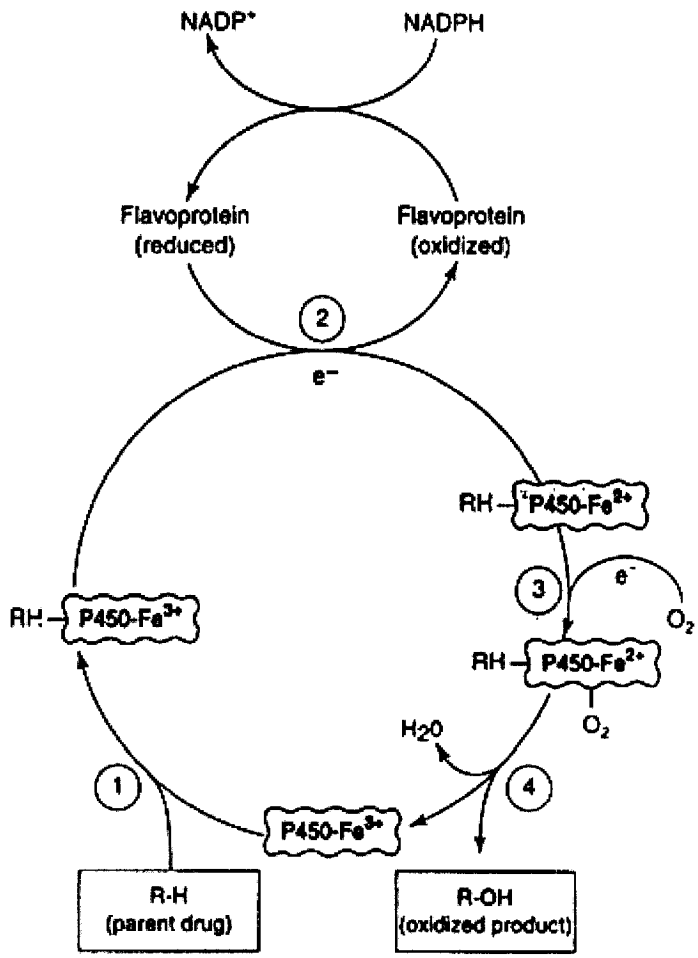
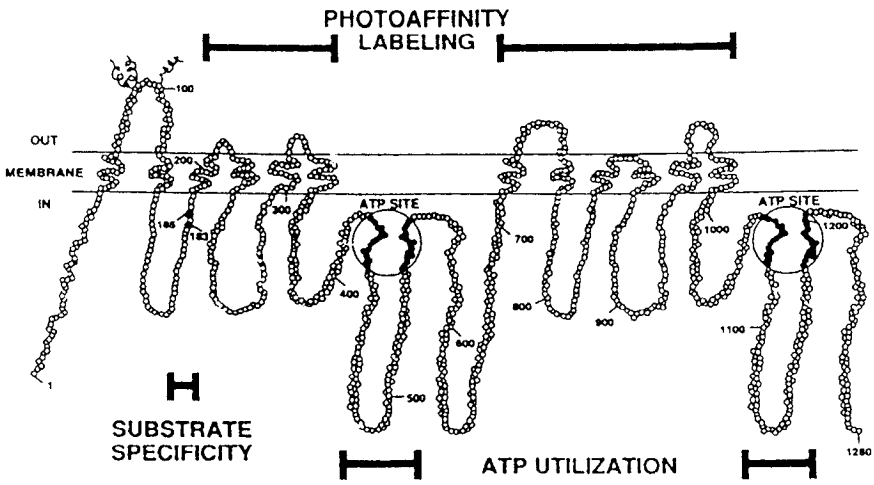


Figure 1.1: Cytochrome P450 cycle in xenobiotic drug oxidations. (RH, parent compound; ROH, oxidized metabolite; e-, electron). (Adapted and modified from Katzung, 1998).

A



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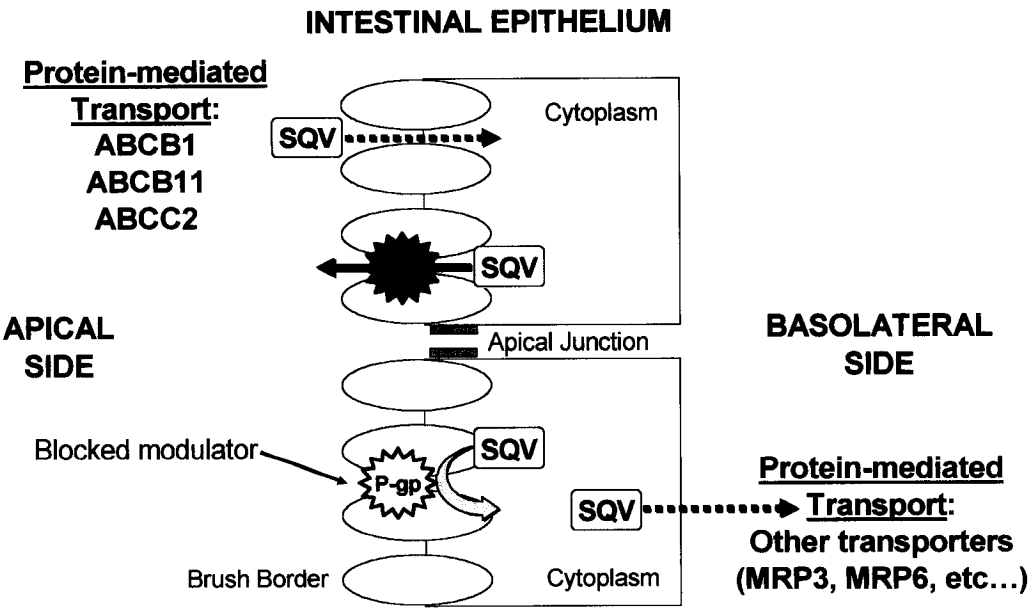


Adapted and modified from the original; courtesy of D. Back

Figure 1.2: (**A**) The 1280 amino acids that comprise human p-glycoprotein deduced from the cDNA sequence is shown, including glycosylation sites (wiggly lines), ATP-binding sites (circled), and the position of 2 mutations that affect transport specificity (residues 183 and 185). The 2 homologous halves each consist of 6 membrane-spanning domains that form 3 transmembrane loops. The functional domains indicated are the ATP sites, photoaffinity-labeling sites, and transport specificity sites (adapted and modified from van der Heyden *et al.*, 1995). (**B**) A depiction of the intestinal epithelium containing p-glycoprotein on the membrane surface. The transport of a P-gp substrate (the HIV protease inhibitor saquinavir [SQV]) is depicted under normal and blocked conditions.

CHAPTER 2 - The interaction of commercial source natural health products (NHP)s with human cytochrome P450 3A4 (CYP3A4) and P-glycoprotein (P-gp).

2.1 - INTRODUCTION

Among the HIV community, the use of NHPs (and other CAM) is increasing, and reasons commonly cited by these patients for their use of CAM include: expectation of a cure, reduction of symptoms from the disease itself (AIDS) or coinfections, a reduction of the side effects of various HIV drugs, or a desire for increased control over the disease process (Duggan *et al.*, 2001). Unfortunately, these patients often take complementary and alternative drugs despite limited data on efficacy and an increasing body of published research that suggests that numerous NHPs and the compounds therein can alter the metabolism of antiretroviral drugs (Piscitelli *et al.*, 2002b). This is due to the fact that NHPs can affect cytochrome P450-mediated metabolism (Budzinski *et al.*, 2000; Budzinski *et al.*, 2001; Foster *et al.*, 1999; Foster *et al.*, 2001), in particular CYP3A4, for which many HIV drugs (such as the protease inhibitors like SQV) are substrates (Washington *et al.*, 1998; Profit *et al.*, 1999). Hence, if an HIV drug and NHP are coadministered there is an increased risk for interaction. For example, if an NHP were to inhibit the CYP3A4-mediated metabolism of another substrate (e.g. SQV), the toxicity associated with that substrate could potentially increase due to a prolonged drug residence time within the body resulting from decreased metabolic breakdown. Similarly, due to overlapping substrate specificities for CYP3A4 and P-gp (Wacher *et al.*, 1995; Benet and Cummins, 2001), NHPs may act upon the same transport mechanisms as HIV drugs; the protease inhibitors are also substrates for P-gp-mediated efflux (Kim *et al.*, 1998).

Therefore, as a preliminary step towards assessing possible human exposure to adverse NHP-therapeutic interactions, the evaluation of the inhibitory potential of a variety of commercial herbal

product capsule formulations (of significance to the HIV community) against CYP3A4 is considered here using a rapid *in vitro* fluorometric microtiter plate assay. Similarly, a variety of commercial NHPs (of significance to the HIV community) were also assessed for their potential to interact with co-administered therapeutic products at the level of the membrane bound drug pump P-gp using a colorimetric microtiter assay.

2.2 - MATERIALS AND METHODS

2.2.1 - Commercial Herbal Products

Commercial herbal product preparations were purchased from local retail outlets (Ottawa, Canada) between September 2000 and May 2001. Table 2.1 summarizes the NRPs used in the subsequent studies.

2.2.2 - Pure Compounds

Phytochemical markers associated with several of the tested herbal extracts (Harborne and Baxter, 1993) and other pure compounds were tested for their P-gp ATPase activity, and/or used for expression studies (Chapter 3), and/or used for standards in the HPLC analysis of milk thistle or goldenseal products. Firstly, four anti-HIV drugs were kindly provided by Dr. Rolf van Heeswijk in the form of methanolic stock solutions. These included the protease inhibitors saquinavir (SQV) (0.165 mM) and nelfinavir (NFV) (0.441 mM), and the non-nucleoside reverse transcriptase inhibitors efavirenz (EFV) (0.798 nm) and nevirapine (0.962 mM). The phytochemical dillapiol was used as an inhibitor of CYP3A4 (Budzinski *et al.*, 2000) and was obtained from Drs. John Arnason and Tony Durst, formerly isolated from *Piper aduncum* as described in Bernard *et al.* (1995). The remaining phytochemicals were obtained from Sigma Aldrich, and included: silibinin and the silymarin group (a mixture of toxifolin, silichristin, silidianin, silybin A, silybin B, isosilybin A, and

isosilybin B) from milk thistle, hydrastine (hydrastine HCl) and berberine (berberine HCl) from goldenseal, and the P-gp modulator vinblastine (vinblastine sulfate) (Parasrampuria *et al.*, 2001). All pure compounds were crystalline or powdered solids with the exception of dillapiol which was an oily liquid.

2.2.3 - CYP3A4 Inhibition

Aqueous extracts of selected herbal products (Table 2.1) were prepared at an initial concentration of 100 mg/ml. Briefly, 1 ml of ddH_2O was added to 100 mg of each herbal product in a sterile 1.5 ml microcentrifuge tube, vortexed for 1 min, and sonicated for 1 min. The resultant suspension was centrifuged at 14 000 g (Eppendorf model 5414C) for 10 min. The supernatant was then collected, microfiltered through a Millex-LCR_4 0.5 μm filter unit (Millipore), and serially diluted to yield 7 concentrations (100 mg/ml - 1.56 mg/ml).

The various aqueous herbal product extracts were then evaluated for their ability to inhibit CYP3A4-mediated metabolism of a reference substrate using an *in vitro* fluorometric microtitre plate assay. The procedure used was adapted and modified from the one originally reported by Crespi *et al.* (1997), later described by GENTEST Corporation (GENTEST, 1998), and utilized by Budzinski *et al.* (2000). Briefly, assays were performed in clear-bottom, opaque-welled microtitre plates (96 well, Corning Costar, model # CSOO-3632). Wells were designated as either "Control", "Blank", "Test" or "Test-Blank". Control wells consisted of ddH_2O and NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form) (Sigma) solution; blank wells consisted of ddH_2O and buffer solution; test wells consisted of the herbal product extract at a particular concentration and NADPH solution; and test-blank wells consisted of the corresponding herbal product extract and buffer solution. Enzyme solution was added to all wells. Solutions were added to the appropriate

designated wells in the following order and quantity: 90 µl of buffer or NADPH solution, 10 µl of ddH_2O or test solution, and 100 µl of enzyme solution. Buffer solution (pH 7.4) consisted of an approximate 1:4.7 (v/v) mixture of 0.5 M potassium phosphate dissolved in ddH_2O. NADPH was mixed with buffer to yield a 15 mg/ml solution and stored at - 4°C for up to 2 weeks before use. Prior to addition, NADPH solution was thawed and further diluted with buffer to 1.07 mg/ml. An enzyme stock solution (760 µl/ml buffer, 200 µl/ml distilled water, 10 µl/ml substrate solution, and 30 µl/ml enzyme) was made by pre-warming the water/buffer mixture to 37°C for 10 min in a sand bath, adding the substrate solution of 1.5 mg/ml 7-benzyloxyresorufin (Sigma) in acetonitrile, vortexing the mixture for 5 seconds, and adding rapidly thawed CYP3A4 (300 pmoles, CYP3A4+OR+b_5SUPERSOMES) (GENTEST). Enzymes were stored at -80°C until used and were not subjected to more than two freeze-thaw cycles.

Once all of the solutions were sequentially added (buffer or NADPH solution, ddH_2O or herbal test mixture, enzyme solution) to the designated wells, plates were incubated for 1 h at 37C. After incubation, 100 µl of stop solution consisting of a 1:4 (v/v) acetonitrile to 0.5M Tris base (Tris-[hydroxymethyl]aminomethane) (Sigma) in distilled water (pH 9) was added to each well. A Millipore Cytofluor 2350 Fluorescence Measurement System set to 530 nm excitation filter (30 nm bandwidth) and a 590 nm emission filter (35 nm bandwidth) was used to analyse each plate. Percent inhibition calculations were based on differences in fluorescence between the control/blank wells and test/test blank wells. All assays were performed under gold fluorescent lighting (Industrial Lighting).

Statistical Analysis

Percent inhibition values for each assay were plotted against the log-transformed

concentration and analysed via simple linear regression using SYSTAT (version 9, SPSS Inc.). Median inhibitory concentration (IC_{50}) values were obtained for dilutions that exhibited clear dose-dependency (a one-tailed p-value < 0.05) by the formula: $y = mx + b$; where (y) is the percent inhibition, (x) is the relative concentration, (m) is the slope, and (b) is the constant, given by the linear regression analysis.

2.2.4 - P-gp ATPase

P-gp-expressing membranes were purchased from GENTEST and the P-gp ATPase activity in the presence of herbal product extracts or pure compounds (phytochemicals or HIV drugs) was determined via a colorimetric microtiter plate assay that measures the amount of inorganic phosphate released from ATP. All products were compared to the ATPase activity of the positive control verapamil (20 μM). Briefly, membranes (25 μg) were incubated at 37C for 5 min in 30 μl Tris-MES (2-[N-morpholino] ethanesulfonic acid) (Sigma) buffer (pH 6.8) (50 nM Tris-MES, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA) (Polli *et al.*, 2001) and the test compound or herbal product in the presence or absence of 200 μM sodium orthovanadate. Stock solutions of pure compounds were maintained at 60 μM and represented ⅓ of the reaction volume when added to the wells (20 μM). Similarly, herbal product extracts (capsules and teas) were also added to the wells at ⅓ of the total reaction volume. Each product was analyzed in triplicate on the same 96 well plate (Corning Costar, model # CSOO-3632). The reaction was started via the addition of 30 μl of 10 mM ATP (magnesium salt) (Sigma) and allowed to proceed for 20 min at 37C. The reaction was stopped with the addition of 10% SDS containing antifoam A (Sigma). In order to visualize the amount of inorganic phosphate liberated, 200 μl of a detection reagent was added to each well and the plate was incubated at 37C for an additional 20 min. The detection reagent consisted of 1:4 (v/v) 35 mM

ammonium molybdate (Sigma) in 15 mM zinc acetate [pH 5] (Sigma), and 10% ascorbic acid [pH 5] (Sigma) (Polli *et al.*, 2001). The absorbance at 650 nm was measured using a THERMOmax microplate reader. The amount of stimulatory ATPase activity of a herbal product extract or pure compound was delimited as the difference in the amount of inorganic phosphate released from ATP in the presence and absence of sodium orthovanadate as compared to a phosphate standard curve.

Herbal Extracts - Teas

Herbal teas (Table 2.1) were screened for their potential ability to interact with P-gp using the ATPase assay. After addition of three tea bags to 600 ml of boiling ddH_2O at 100C, teas were steeped at room temperature conditions for 15 min. Resultant tea concentrations in the final reaction were as follows: goldenseal, 59 mg/ml; milk thistle, 100 mg/ml; cat's claw, 60 mg/ml; *Echinacea*, 80 mg/ml; Panax ginseng, 70 mg/ml; Siberian ginseng, 80 mg/ml; and St. John's wort, 68 mg/ml.

Herbal Extracts - Capsules

Herbal product capsule formulations (Table 2.1) were also screened for their ability to stimulate P-gp ATPase. Briefly, 300 mg of each product was suspended in 1 ml of ddH_2O or 1 ml of 70% EtOH in a sterile 1.5 ml microcentrifuge tube, vortexed for 1 min, and sonicated for 5 min. The resultant solution was centrifuged at 13 000 g (Eppendorf model 5414C) for 10 min. The supernatant was then collected and diluted 10X in Tris-MES buffer. This stock solution (30 mg/ml) was subsequently microfiltered through a Millex-LCR_4 0.5 μm filter unit (Millipore) prior to testing.

Statistical Analysis

The orthovanadate-sensitive release of phosphate (ATPase activity) of each product was compared to the P-gp inhibitor verapamil (20 μM) using a one-way ANOVA, followed by Dunnett's

2-sided pairwise comparison (significance set to $p < 0.05$). As well, to control for the variability observed among all of the assays for the ATPase activity of 20 μM verapamil, values from individual assays were expressed as a ratio to the positive control (relative ATPase activity). The relative ATPase activities associated with each herbal product or pure compound tested were compared to one another using a one-way ANOVA, followed by Tukey's pairwise comparison (significance set to $p < 0.05$). All analyses were carried out using SYSTAT (version 9, SPSS Inc.).

2.2.5 - HPLC Analysis

Goldenseal and milk thistle products (Table 2.1) were analysed for levels of their constituent marker compounds: hydrastine and berberine for goldenseal; silibinin and the silymarin group for milk thistle. Briefly, 0.5 g of herbal product was extracted in a 50 ml centrifuge tube with 8 ml of 70% EtOH, MeOH, or ddH_2O, 3X sequentially. Each time solvent was added to the tube, the mixture was sonicated for 5 min, vortexed for 1 min, and centrifuged at 1000 g for 5 min. Supernatants were pooled in a separate tube and the final concentration was adjusted to 25 ml. Prior to analysis, sample solutions were microfiltered through a Millex-LCR_4 0.5 μm filter unit (Millipore). All solvents used were of analytical grade.

Goldenseal - Instrumentation and Chromatographic Conditions

The protocol used was adapted and modified from the one developed by Abourashed and Khan (2001). Briefly, all goldenseal samples were analysed on a Varian ProStar 230 component HPLC system equipped with a photodiode array detector, column temperature controller, and autosampler. Samples were eluted by gradient from a Waters Symmetry C-18 column (150 mm x 4.6 mm; 5 μm particle size; serial no. T63451L). The mobile phase consisted of 50 mM sodium phosphate (NaH_2PO_4) buffer (pH 2.1) (solvent A) and acetonitrile (solvent B), with solvent A

varying from 75% to 25% over the first 15 min, and from 25% to 10% from 15 - 18 min. The solvent flow rate was 1.0 ml/min. The injection volume was 5 µl for all the samples tested. The signal was monitored at 295 nm for hydrastine and 350 nm for berberine. Data collection and integration were performed using the accompanying Varian software.

Goldenseal - Instrumentation and Chromatographic Conditions

The protocol used was adapted from the one developed by John Livesey (Dr. John Arnason's lab) and based upon the method published online by the Institute for Nutraceutical Advancement (2002). Briefly, all milk thistle samples were analysed on an Agilent 1100 Series HPLC system equipped with a photodiode array detector, in-line degasser, column temperature controller, and autosampler. Samples were eluted by gradient from a Waters YMC column (50 mm x 2.0 mm; 3 µm particle size; serial no. 1973101521D04). The mobile phase consisted of ddH_2O (solvent A), acetonitrile (solvent B) and MeOH (solvent C). Solvent C was held constant at 10%, with solvent B varying from 5% to 50% over the first 10 min, and from 50% to 90% from 10- 12 min. The solvent flow rate was 0.5 ml/min. The injection volume was 1 µl for all the samples tested. Several MT samples were diluted 10 fold prior to analysis (methanolic and ethanolic extracts of both NRP126 and NRP18). The signal was monitored at 285 nm for silibinin and the silymarin group (considered to be all peaks from 3.5 min to 11 min). Data collection and integration were performed using the accompanying Hewlett Packard software.

2.3 - RESULTS

2.3.1 - NHP Analysis:

A first step was to characterize the various NHPs used phytochemically. In particular, goldenseal and milk thistle capsules used in subsequent expression studies were analyzed in greater

detail than other herbal products. For three of the four products, a discrepancy was found between the mean capsule content weight of each product (n = 10) and the capsule weight indicated on individual product labels (Table 2.2). Capsules of LCY-GS (NRP-121) were the exception since capsule content weights fell within the range of 450 mg (0.444 ± 0.016 g). The amount of excipient occurring in each capsule was not indicated on any product label (Table 2.1).

Alkaloid constituents of the goldenseal products (hydrastine and berberine) were determined by the HPLC analysis of three different solvent preparations: ddH_2O, 70% EtOH, and MeOH (Table 2.3). The water extracts represent an infusion type of dosage form, while the two alcoholic extracts are representative of commercial herbal product extraction methods. For all GS products, aqueous extracts had far lower concentrations of these two alkaloids than alcoholic extracts did. Extracts from Alvita GS (tea) had much lower levels of hydrastine and berberine than did the capsule preparations. Of all the products evaluated, only Sol-GS (NRP17) had specific label claims as to total alkaloid content (10%) (Table 2.1), and when evaluated, all three prepared extracts of this product supported this company's statement. Extracts of Sol-GS were also found to have greater levels of hydrastine and berberine than the other two GS products.

Flavanolignan constituents of the milk thistle products (silibinin and the "silymarin group") were also determined by the HPLC analysis of the three different solvent preparations: ddH_2O, 70% EtOH, and MeOH (Table 2.4). For all MT products, aqueous extracts had extremely low levels of silibinin and total silymarins. As seen with the GS tea extracts, Alvita MT (tea) had much lower levels of silibinin and total silymarins than did the other two MT capsule preparations. According to label claims, both SHR-MT (NRP126) and Sol-MT (NRP18) were made (in part) with milk thistle extracts standardized to 80% silymarins. Extracts tested by HPLC were based on the weight of the

total capsule, and the highest observed levels of total silymarins were found to be roughly 19%.

2.3.2 - CYP3A4 Inhibition

Serial dilutions of aqueous extracts from herbal products were assessed for their ability to inhibit CYP3A4-mediated metabolism of a reference substrate 7-benzyloxyresorufin (Table 2.1). Figure 2.3 depicts representative regression lines used to determine the median inhibitory concentration (IC_{50}) for the tested GS and MT extracts, while the relevant statistical values for the regression curves of all of the tested products are summarized in Table 2.5. IC_{50} values ranged from 3.03 mg/ml for the Solaray goldenseal herbal extract to 4. 68 x 10^1 mg/ml for cat's claw. Both Solaray and Li Chung Yun goldenseal were approximately one order of magnitude more inhibitory than both milk thistle products tested in this assay. Herbal products were also ranked according to their IC_{50} value, with 1 being assigned to the most inhibitory extract: Solaray goldenseal(Table 2.5).

2.3.3 - P-gp ATPase Activity

Among the Alvita herbal teas (Fig. 2.4), only milk thistle and goldenseal extracts (NRP #s 113 and 115 respectively in Table 2.1) had significantly higher vanadate-sensitive ATPase activity than the reference P-gp inhibitor and verapamil (20 μM). All other herbal teas evaluated had some degree of ATPase activity, but were all significantly less active than the positive control (verapamil).

Similarly, both aqueous and 70% EtOH extracts of herbal preparations (Table 2.1) were assessed for their ability to stimulate P-gp ATPase activity. When compared directly to the positive control (20 μM verapamil), most products had significantly lower P-pg ATPase activity. Similarly, aqueous extracts had equal or greater P-gp ATPase than their corresponding ethanolic extract (Fig. 2.5 and Fig. 2.6). Among the herbal products tested, only aqueous Li Chung Yun goldenseal (Fig. 2.5) and ethanolic *Echinacea* (NRP127) (Fig. 2.6) extracts were as active as the positive control. The

only herbal product extract tested with higher P-gp-ATPase activity than the positive control was the aqueous extract of Solaray goldenseal (Fig. 2.5). In all cases, the tested goldenseal products had markedly higher ATPase activity than the analyzed MT products. When the ATPase activity of all herbal product extracts was expressed relative to the activity of the positive control verapamil (20 μM) (Fig. 2.8), the majority of products were found to have relative levels of ATPase stimulation below 1.0 and are therefore less active than the positive control (Polli *et al.*, 2001). Only 2 product extracts were considered to stimulate ATPase activity at a level greater than the positive control: 70% EtOH extracts of Li Chung Yun goldenseal (ratio > 2.0) and aqueous extracts of Solaray goldenseal (ratio > 1.0).

A variety of pure compounds were also assessed for their ability to stimulate P-gp ATPase activity. When compared directly to the positive control (20 μm verapamil), most phytochemicals and anti-HIV drugs had significantly lower P-pg ATPase activity (Fig. 2.7). Two of the pure compounds were equally as active as verapamil (20 μM): the vinca alkaloid vinblastine (20 μM) and the protease inhibitor saquinavir (20 μM). The remainder of the of the tested pure compounds, however, were found to be less active than verapamil (20 μM), since all had relative values < 1.0 (Fig. 2.9).

Table 2.1: Summary of the commercial Natural Research Products (NRPs) used in the various studies, including individual product label information.

Herbal Product Name	Company (Source)	Product Type	NRP #	Stated Unit Weight (mg); Source; Amount of Product	Suggested Dosage	Other Ingredients	Experiments Used In	Expiration Date	Retail Cost ($C)
Cat's Claw (*Uncaria tomentosa*)	Organika (Richmond, BC)	Capsules	114	500 mg; 6X extract powder from wildcrafted bark; 90 capsules	1 - 2 capsules 2X daily	none listed	CYP, ATP	Jan. 2002	13.49
	Alvita (Twin Labs) (American Fork, UT)	Tea	122	36 g (net weight); none listed; 24 tea bags	1 tea bag in 6 - 8 oz. of water by decoction	none listed	ATP	none listed	11.45
Echinacea angustifolia *Echinacea purpurea*	Natural Source HSC (Winnipeg, MB)	Capsules	127	400 mg; *E. purpurea* herb & *E. angustifolia* herb & root; 90 capsules	1 capsule 3X daily with meals	Mg stearate	CYP, ATP	Feb. 2002	13.79
	Alvita (Twin Labs) (American Fork, UT)	Tea	112	48 g (net weight); none listed; 24 tea bags	1 tea bag in ≤ 6 oz. of water by infusion	none listed	ATP	none listed	13.19
Goldenseal (*Hydrastis canadensis*)	Li Chung Yun (Burnaby, BC)	Capsules	121	450 mg; root powder; 90 capsules	1 - 3 capsules daily with meals	none listed	ES, CYP, ATP	Nov. 2003	19.71
	Solaray (Park City, UT)	Capsules	17	250 mg of root extract standardized to 10% alkaloids); 65 mg of root; 60 capsules	1 -2 capsules daily with meals	gelatin (capsule), Mg stearate	ES, CYP, ATP	Apr. 2004	34.99
	Alvita (Twin Labs) (American Fork, UT)	Tea	115	35.44 g (net weight); herb; 30 tea bags	1 tea bag in ≤ 6 oz. of water by infusion	none listed	ATP	none listed	13.09
Milk Thistle (*Silybum marianum*)	Swiss Herbal Remedies (Richmond Hill, ON)	Capsules	126	250 mg, extract standardized to 80% silymarins; 60 capsules	1 - 2 capsules 3X daily with meals	rice flour, di calcium phosphate, silica, Mg stearate	ES, CYP, ATP	Jan. 2005	16.09
	Solaray (Park City, UT)	Capsules	18	175 mg of seed extract standardized to 140 mg [80%] silymarin; 120 capsules	1 capsule 1 - 2X daily with meals	cellulose, gelatin (capsule), silica, Mg stearate	ES, CYP, ATP	Feb. 2004	45.99
	Alvita (Twin Labs) (American Fork, UT)	Tea	113	60 g (net weight); none listed; 24 tea bags	1 tea bag in ≤ 6 oz. of water by infusion	none listed	ATP	none listed	7.01

Note: **ES** = expression studies (includes Caco2 cell treatments, mRNA detection by dot blots, and protein determination by immuno-blotting); **CYP** = CYP3A4 inhibition studies (median inhibitory concentration determination of extracts); and **ATP** = P-glycoprotein ATPase studies (comparison of extracts' ATPase activity to 20 μm verapamil).

Table 2.1: continued...

Herbal Product Name	Company (Source)	Product Type	NRP #	Stated Unit Weight (mg); Source; Amount of Product	Suggested Dosage	Other Ingredients	Experiments Used In	Expiration Date	Retail Cost ($C)
Asian Ginseng (*Panax ginseng*)	Li Chung Yun (Seoul, Korea)	Tablet	123	500 mg; 6 year old root powder; 50 tablets	1 tablet 3X daily	none listed	CYP	Jul. 2003	9.82
	Alvita (Twin Labs) (American Fork, UT)	Tea	117	42 g (net weight); none listed; 24 tea bags	1 tea bag in ≤ 6 oz. of water by infusion	none listed	ATP	none listed	14.01
St. John's Wort (*Hypericum perforatum*)	Quest (Vancouver, BC)	Caplet	128	1500 mg (provided by 300 mg P.E. 1:5 standardized to 0.3% hypericin); 90 caplets	up to 3 caplets daily with meals	herbal base: kelp, peppermint leaf, scullcap, siberian ginseng, wood betony, calcium phosphate, microcrystalline cellulose, vegetable stearin, croscarmaellose sodium, silicon dioxide, magnesium stearate, vegetable cellulose complex (CellCote coating)	CYP, ATP	Aug. 2003	14.93
	Alvita (Twin Labs) (American Fork, UT)	Tea	105b	40.8 g (net weight); none listed; 24 tea bags	1 tea bag in ≤ 6 oz. of water by infusion	none listed	ATP	none listed	6.29
Siberian Ginseng (*Eleutherococcus senticosus*)	Quest (Vancouver, BC)	Capsules	125	500 mg; none listed; 90 capsules	1 - 3 capsules daily	none listed	ATP	Apr. 2005	12.9
	Alvita (Twin Labs) (American Fork, UT)	Tea	116	48 g (net weight); none listed; 24 tea bags	1 tea bag in ≤ 6 oz. of water by infusion	none listed	CYP, ATP	none listed	6.53

Note: **ES** = expression studies (includes Caco2 cell treatments, mRNA detection by dot blots, and protein determination by immuno-blotting); **CYP** = CYP3A4 inhibition studies (median inhibitory concentration determination of extracts); and **ATP** = P-glycoprotein ATPase studies (comparison of extracts' ATPase activity to 20 μM verapamil).

Table 2.2: Mass of 10 product units from selected herbal products. Values represent the mean ± SD.

Herbal Product	NRP #	Intact Capsules: Mean Weight (g)	Empty Capsules: Mean Weight (g)	Contents: Mean Weight (g)	Weight of Product Indicated On Label (g)
Goldenseal	17	0.414 ± 0.004	0.073 ± 0.001	0.341 ± 0.004	0.315
	121	0.543 ± 0.015	0.099 ± 0.003	0.444 ± 0.016	0.45
Milk Thistle	18	0.548 ± 0.016	0.093 ± 0.002	0.454 ± 0.016	0.175
	126	0.600 ± 0.019	0.096 ± 0.002	0.504 ± 0.019	0.25

Table 2.3: Constituent analysis for hydrastine and berberine from selected goldenseal products as determined by HPLC. Berberine content was determined at 350 nm. and hydrastine content was determined at 295 nm. Extracts were prepared in triplicate and values represent the mean ± SD.

Goldenseal Product (NRP #)	Extract Solvent	Hydrastine Content (%)	Berberine Content (%)
Alvita (NRP 115)	Water	0.20 ± 0.00	1.00 ± 0.00
	70% Ethanol	0.43 ± 0.06	4.53 ± 0.06
	Methanol	0.33 ± 0.06	2.93 ± 0.12
Li Chung Yun (NRP 121)	Water	2.03 ± 0.15	0.97 ± 0.12
	70% Ethanol	3.00 ± 0.17	3.73 ± 0.06
	Methanol	2.67 ± 0.15	3.47 ± 0.15
Solaray (NRP 17)	Water	8.73 ± 2.32	15.47 ± 0.40
	70% Ethanol	13.87 ± 0.68	17.37 ± 0.81
	Methanol	16.03 ± 0.06	19.70 ± 0.10

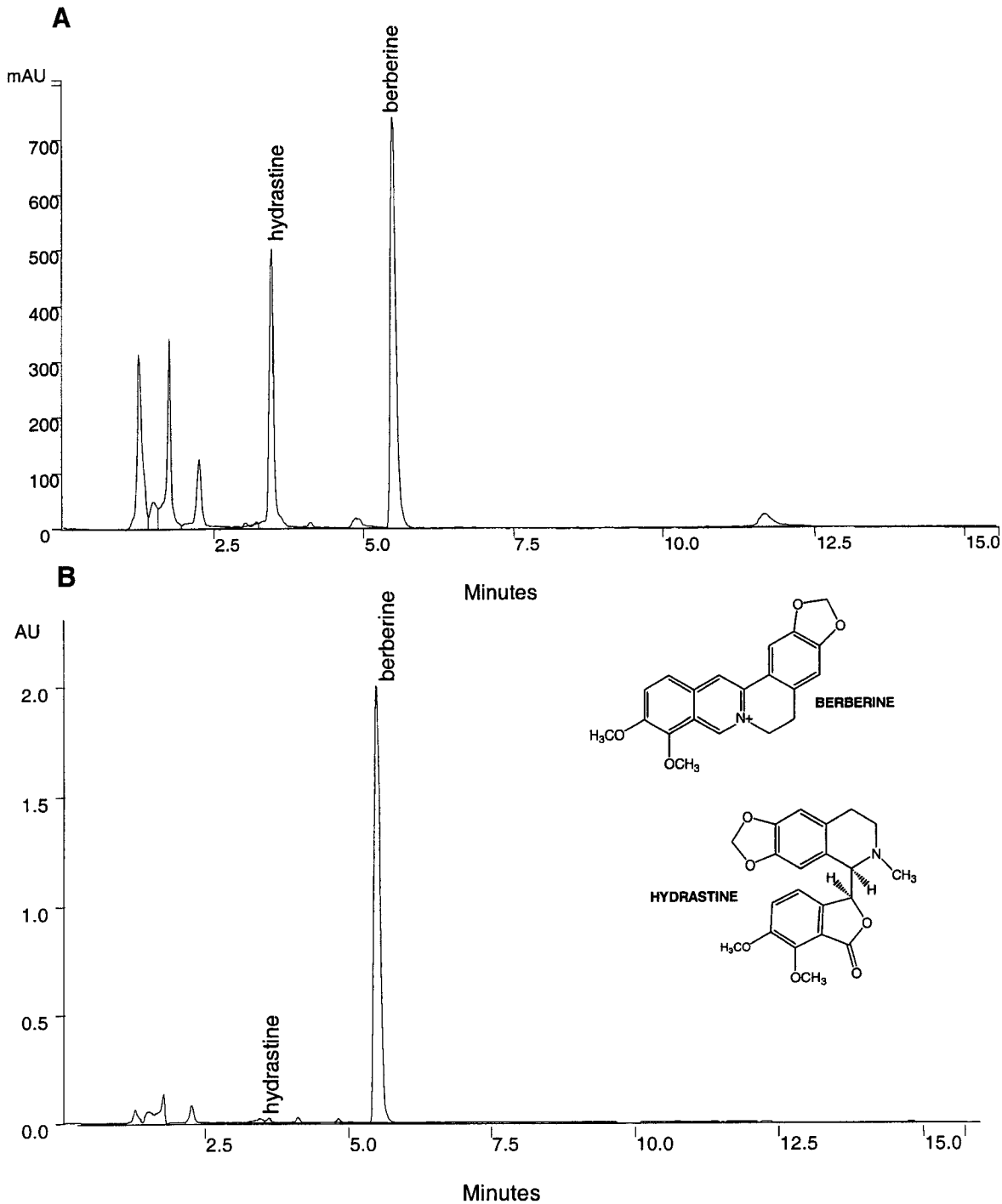


Figure 2.1: Representative chromatograms of methanolic extracts from goldenseal products: (A) Solaray goldenseal (NRP17) - 295 nm; and (B) Solaray goldenseal (NRP17) - 350 nm. Note the structures of berberine and hydrastine (inset).

Table 2.4: Constituent analysis for silibinin and the silymarin group (includes toxifolin, silichristin, silidianin, silybin A, silybin B, isosilybinin A, and isosilybin B) from selected milk thistle products as determined by HPLC. Both were determined at 285 nm. Extracts were prepared in triplicate and values represent the mean ± SD.

Milk Thistle Product (NRP #)	Extract Solvent	Silibinin Content (%)	Silymarin Group Content (%)
Alvita (NRP 113)	Water	0.02 ± 0.02	0.12 ± 0.12
	70% Ethanol	0.86 ± 0.12	1.07 ± 0.12
	Methanol	0.91 ± 0.05	1.08 ± 0.06
Swiss Herbal Remedies (NRP 126)	Water	0.10 ± 0.01	0.74 ± 0.06
	70% Ethanol	8.86 ± 1.18	17.94 ± 1.31
	Methanol	15.22 ± 0.79	18.23 ± 2.25
Solaray (NRP 18)	Water	0.12 ± 0.03	0.69 ± 0.17
	70% Ethanol	10.10 ± 0.33	18.71 ± 0.33
	Methanol	12.83 ± 1.17	16.3 ± 1.21

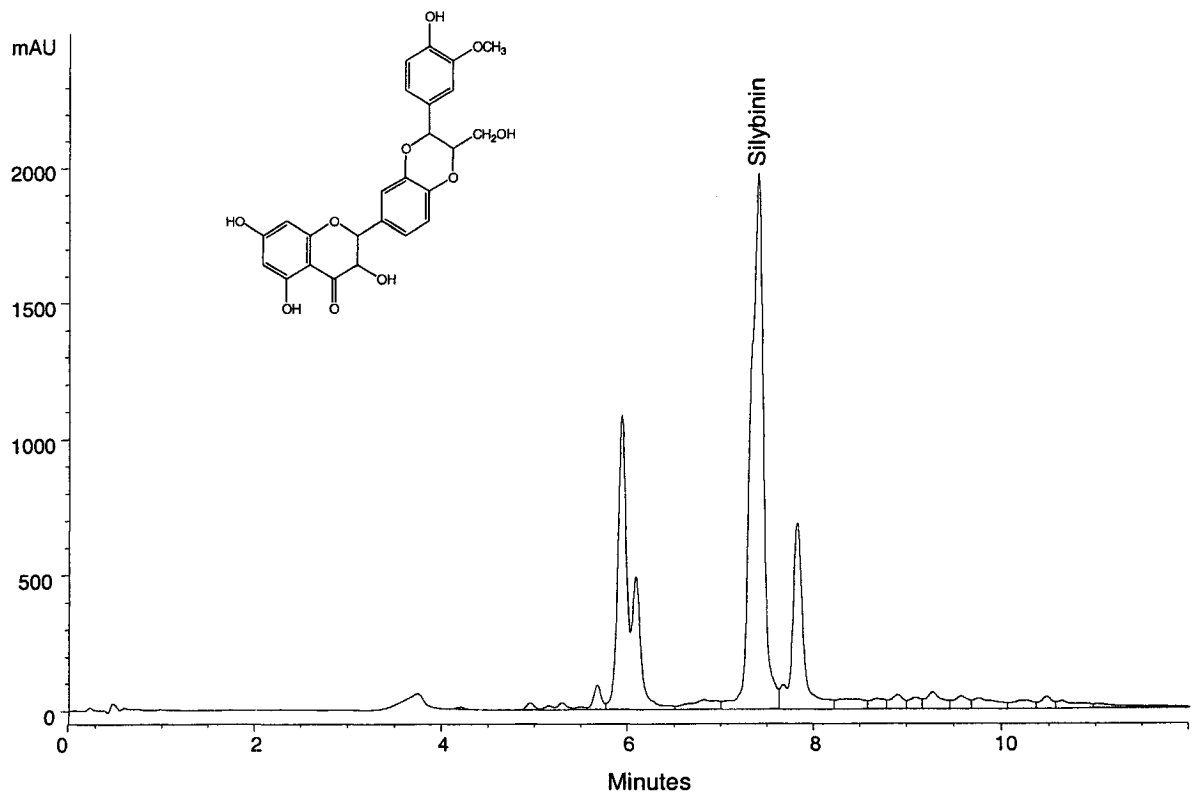


Figure 2.2: Representative chromatogram of methanolic extracts from milk thistle products. Shown is Solaray milk thistle (NRP18) - 285 nm. Note that the "Silymarin Group" included numerous compounds and considered all peaks occurring from 3.5 minutes to 11 minutes. Note the structure of silibinin (inset).

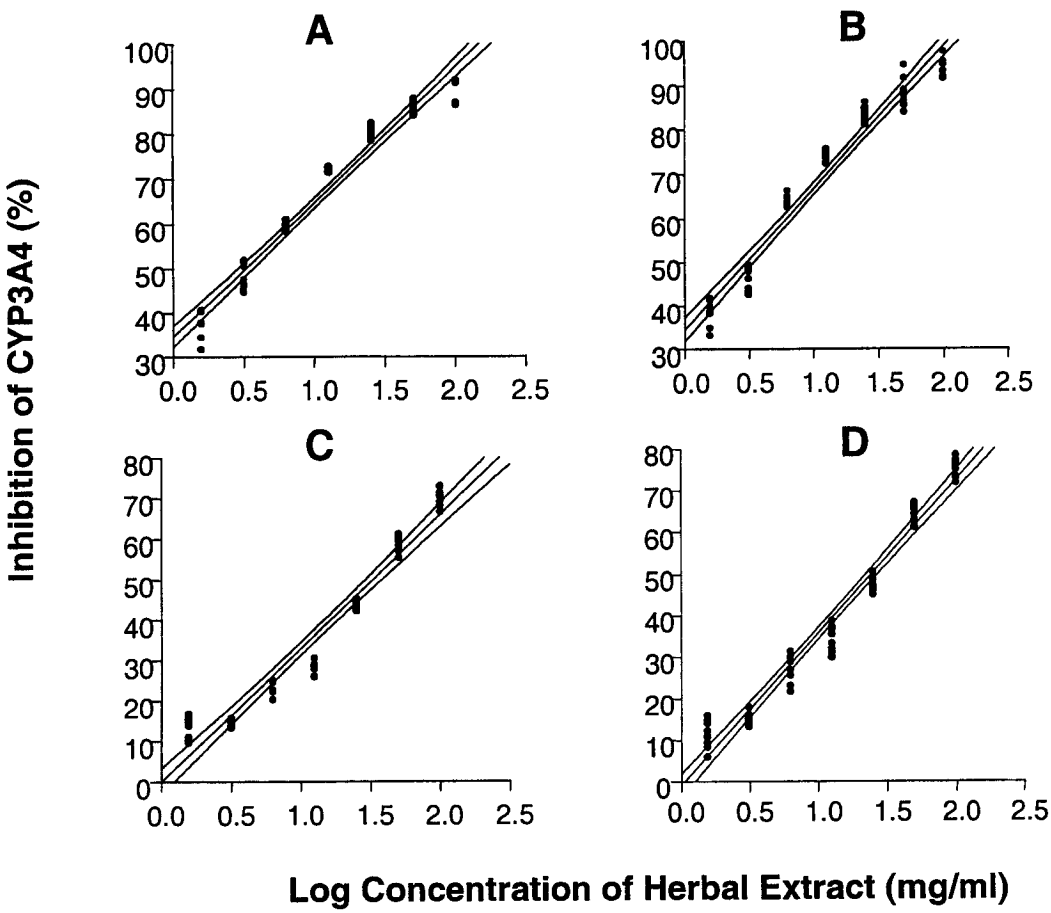


Figure 2.3: Representative regression lines with the 95% confidence intervals used in determining the median inhibitory concentration (IC_{50} value) of aqueous herbal extracts against cytochrome P450 3A4 (CYP3A4). Extracts include: (**A**) Li Chung Yun goldenseal; (**B**) Solaray goldenseal; (**C**) Swiss Herbal Remedies milk thistle; and (**D**) Solaray Milk Thistle.

Table 2.5: The median inhibitory concentration (IC_{50}) values for aqueous extracts of various commercial herbal product capsules to effect the cytochrome P450 3A4-mediated metabolism of a reference substrate (n = 9). Herbal products were ranked according to their inhibitory capability.

Herbal Product (Company)	IC_{50} Concentration[1] (mg/ml)	Regression Line:					Rank
		Slope	Constant	*N*	R^2	*p*-value (1 tail)	
Goldenseal (Li Chung Yun)	**3.23** (4.03, 2.65)	30.09 (28.54, 31.65)	34.68 (32.731, 36.618)	63	0.961	0	2
Milk Thistle (Swiss Herbal Remedies)	**3.30 x 10^1** (5.10, 2.24) x 10^1	32.98 (30.86, 35.11)	-0.07 (-2.721, 2.589)	63	0.941	0	7
Goldenseal (Solaray)	**3.03** (3.84, 2.46)	31.09 (29.05, 33.14)	36.38 (33.817, 38.938)	63	0.938	0	1
Milk Thistle (Solaray)	**2.45 x 10^1** (3.37, 1.83) x 10^1	37.23 (35.12, 39.34)	-1.56 (-4.201, 1.087)	63	0.953	0	6
St. John's Wort (Quest)	**5.74** (8.59, 4.14)	100.25 (89.68, 110.82)	-26.07 (-33.763, -18.376)	36	0.916	0	3
Panax ginseng (Li Chung Yun)	**2.02 x 10^1** (4.66, 1.05) x 10^1	44.25 (38.74, 49.75	-7.74 (-14.623, -4.422)	63	0.809	0	5
Echinacea (Natural Source HSC Vita Health)	**1.55 x 10^1** (2.26, 1.13) x 10^1	48.85 (45.64, 51.53)	-8.11 (-11.797, 8.277)	63	0.947	0	4
Cat's Claw (Organika)	**4.68 x 10^1** (9.71, 2.57) x 10^1	26.95 (24.32, 29.59)	4.98 (1.68, 8.28)	63	0.873	0	8

[1] Numbers in brackets correspond to the lower and upper 95% confidence limits of the particular value respectively.

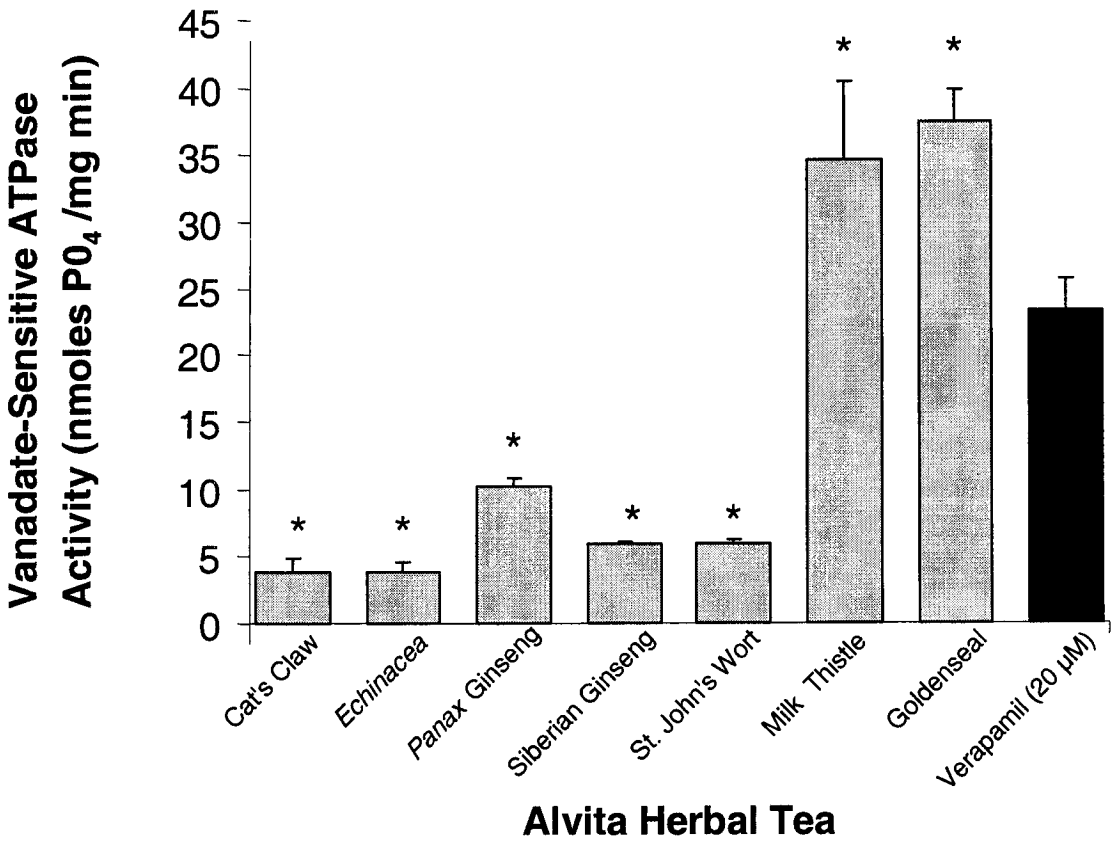


Figure 2.4: Vanadate-sensitive ATPase activity of a variety of aqueous extracts from Alvita herbal teas. Goldenseal and milk thistle teas had significantly higher ATPase activity than the reference P-gp inhibitor verapamil (20 μM). All other teas were significantly less active than the positive control (verapamil). Means were analysed via a one-way ANOVA (n = 9) followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

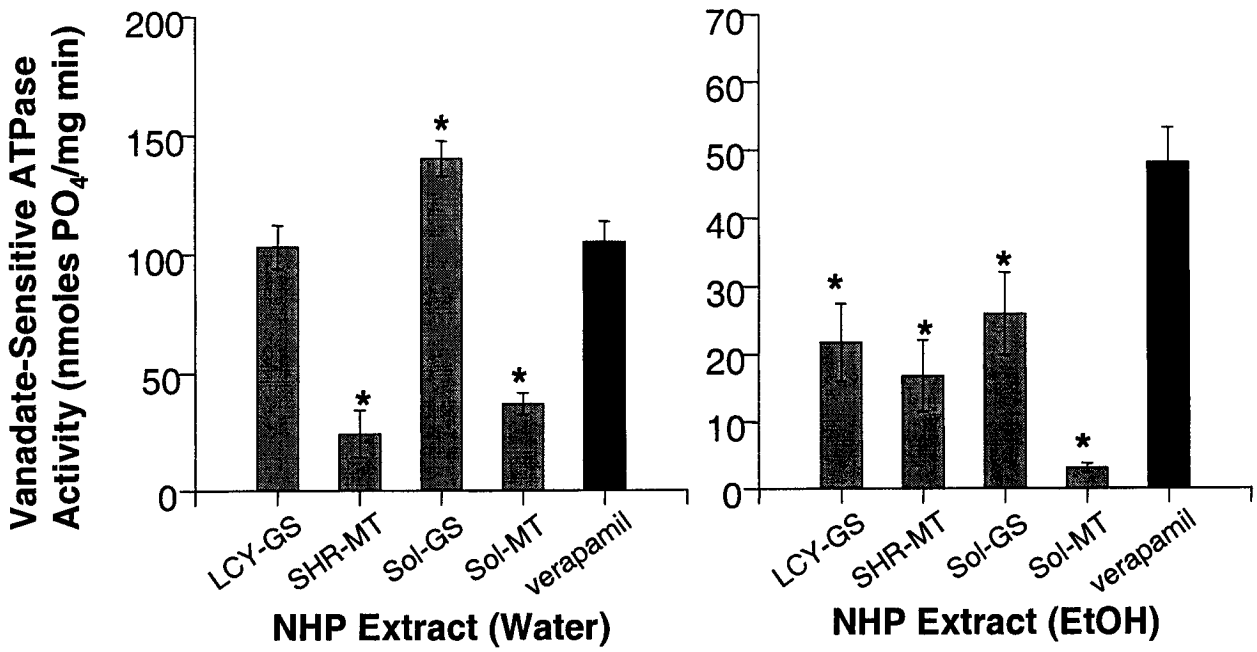


Figure 2.5: Vanadate-sensitive ATPase activity of a variety of aqueous and 70% ethanolic (EtOH) extracts of Solaray goldenseal (Sol-GS), Li Chung Yun goldenseal (LCY-GS), Swiss Herbal Remedies milk thistle (SWH-MT), and Solaray milk thistle (Sol-MT) capsules. Aqueous LCY-GS extracts had an ATPase activity equal to that of verapamil (20 μM). All other extracts were significantly less active than the positive control (verapamil). Means were analysed via a one-way ANOVA (n = 9) followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

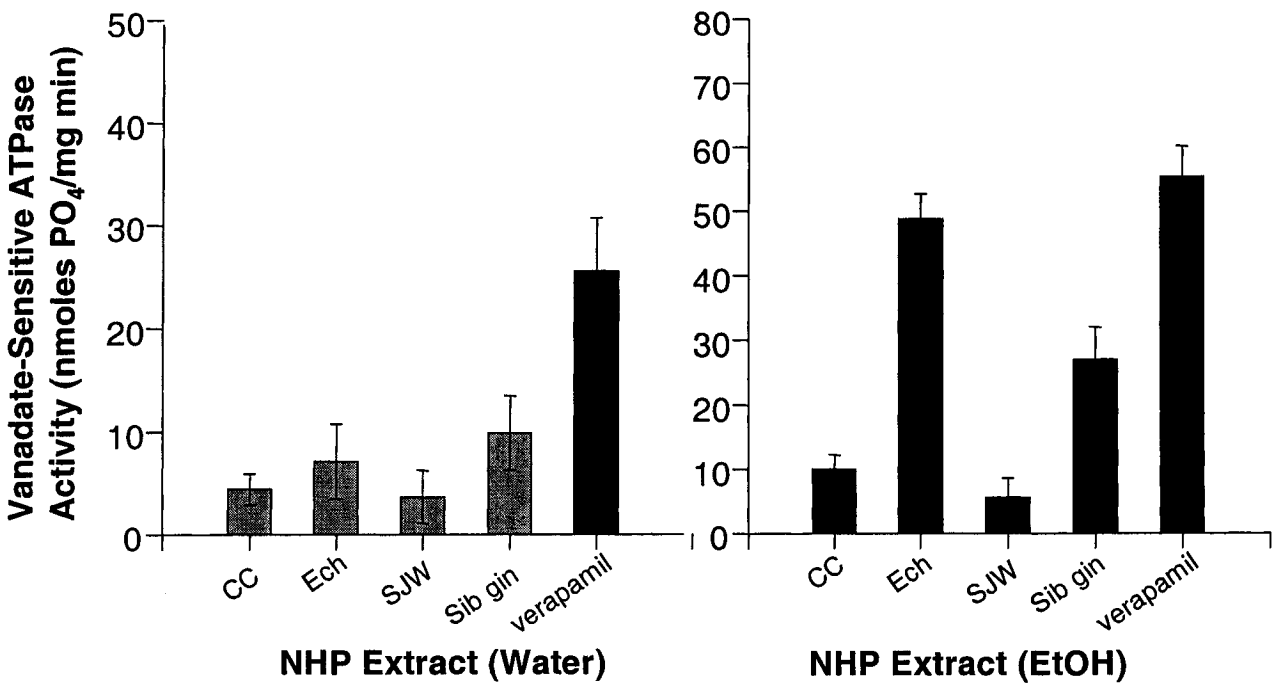


Figure 2.6: Vanadate-sensitive ATPase activity of a variety of aqueous and 70% ethanolic (EtOH) extracts of capsules of other natural health products (NHPs). Ethanolic Echinacea (Ech) extracts were as active as verapamil (20 μM) for ATPase activity. All other extracts (cat's claw [CC], St. John's wort [SJW], and Siberian ginseng [Sib gin]) were significantly less active than the positive control (verapamil). Means were analysed via a one-way ANOVA ($n = 9$) followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

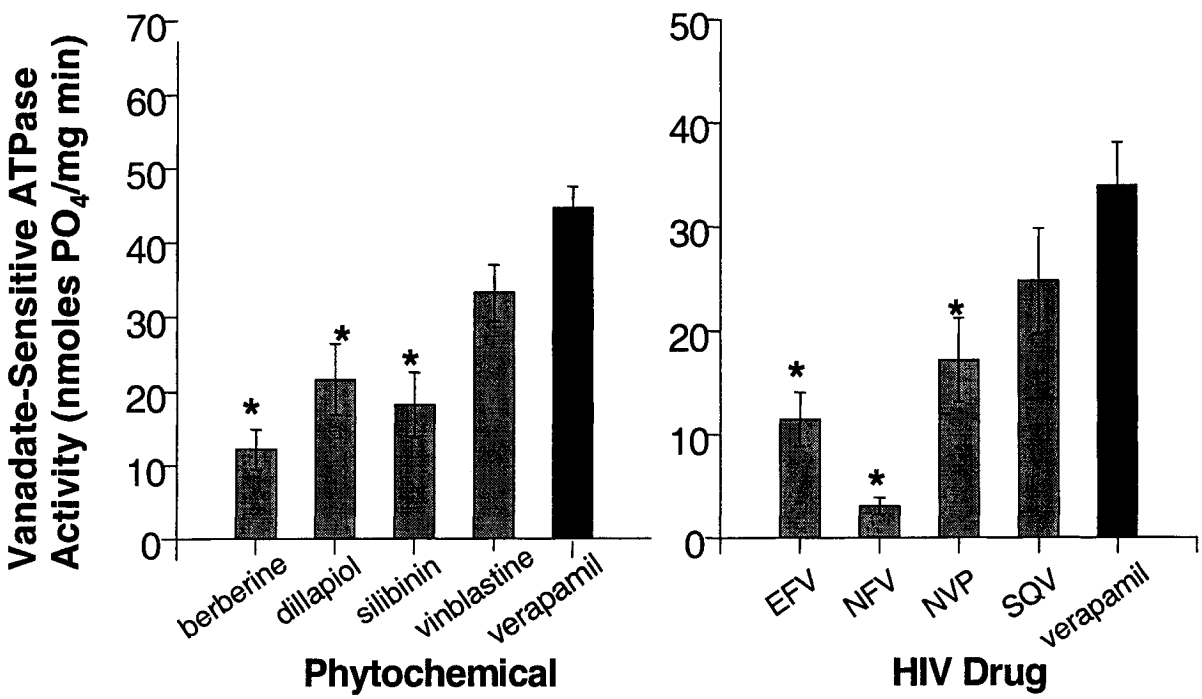


Figure 2.7: Vanadate-sensitive ATPase activity of a variety of phytochemicals and HIV drugs: EFV (efavirenz); NFV (nelfinavir); NVP (nevirapine), and SQV (saquinavir). All compounds were tested at 20 μM and compared to verapamil for ATPase activity. Vinblastine and SQV were as equally active as the positive control (verapamil). Means were analysed via a one-way ANOVA (n = 9) followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

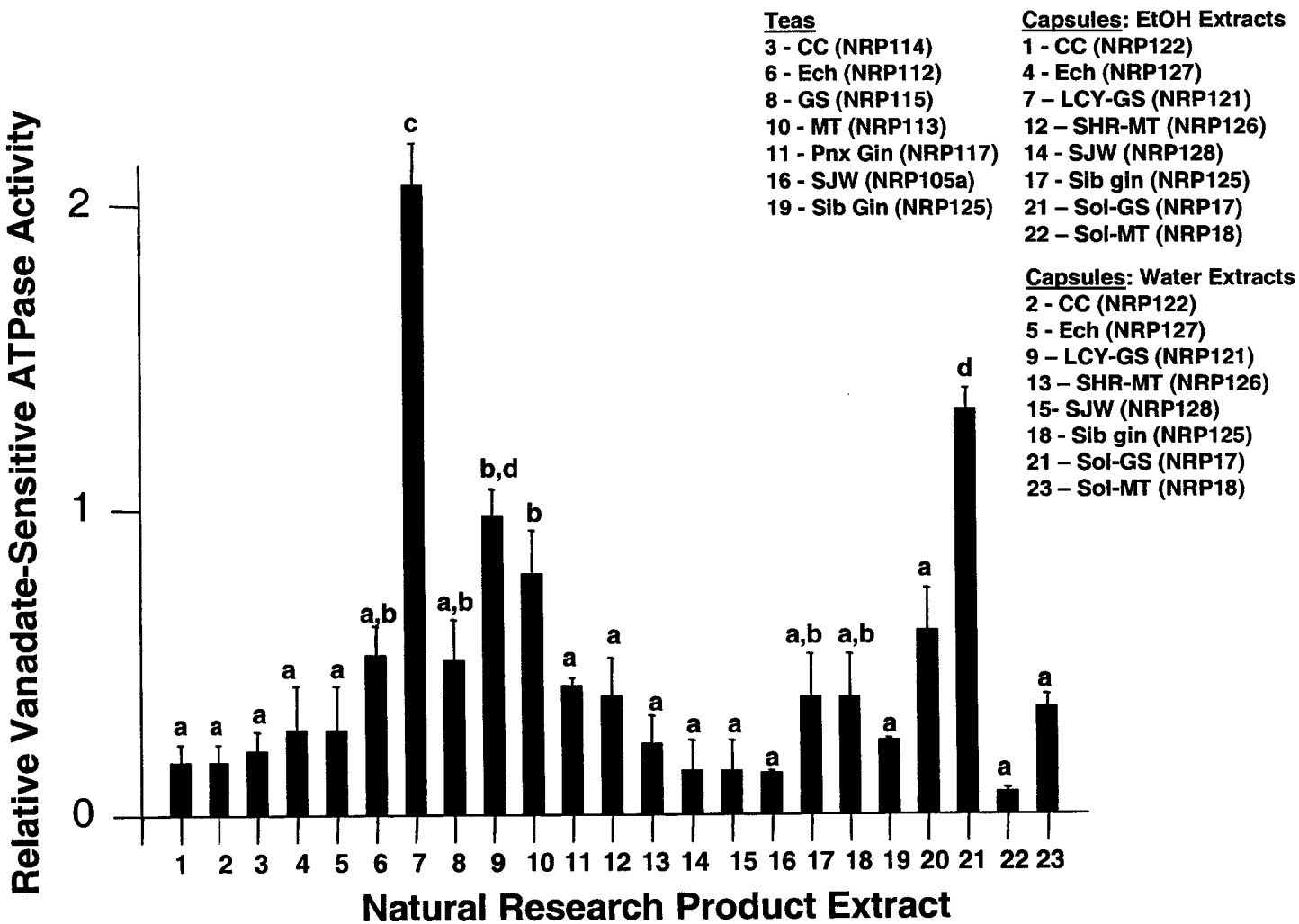
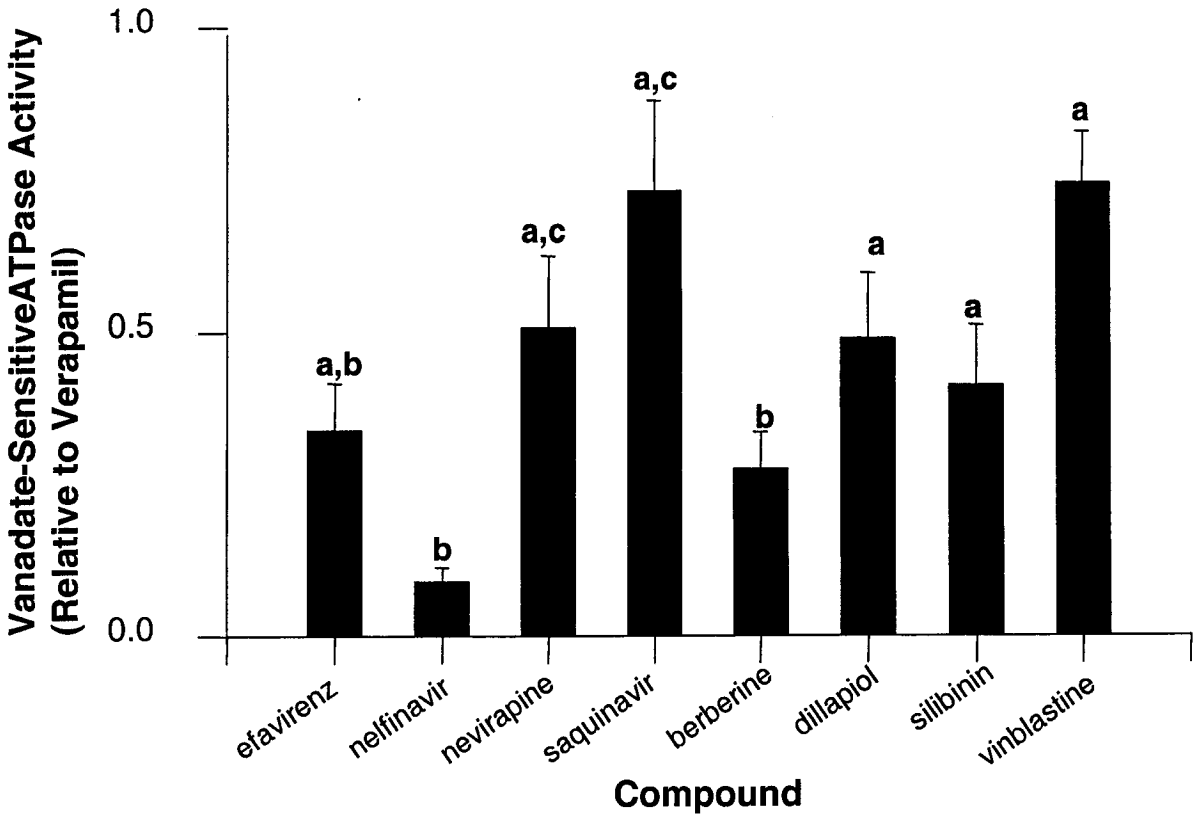


Figure 2.8: A comparison of the relative vanadate-sensitive ATPase activity of all herbal product preparations tested. Values represent the ratio of each product's ATPase activity versus that of the positive control verapamil (20 μM) and control for differences among the separate experiments. Letters indicate similar means, as determined by a one-way ANOVA ($n = 9$) followed by Tukey's pairwise comparison (p



< 0.05).

Figure 2.9: Relative vanadate-sensitive ATPase activity of a variety of phytochemicals and HIV drugs: efavirenz, nelfinavir, nevirapine, and saquinavir. All compounds were tested at 20 μM and compared to verapamil for ATPase activity. Vinblastine and SQV were as equally active as the positive control (verapamil). Means were analysed via a one-way ANOVA (n = 9) followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

2.4 - DISCUSSION

When it comes to manufacturing and marketing a safe and efficacious NHP, clearly the keystones of product assurance are product identity and quality. According to Canada's Food and Drug Act at this time, NHPs can be marketed as food if they do not make label claims that represent the product's use in: (1) the diagnosis, treatment, mitigation, or prevention of a disease, disorder, abnormal physical state, or the symptoms thereof in man or animal; or (2) restoring, correcting, or modifying organic functions in man or animal. Consequently, NHPs sold as food are not required to be subjected to as stringent or rigorous a review process as drugs (which normally include information on drug formulation, efficacy, and safety (Kozyrskyj, 1997). Thus the onus of assuring product identity and quality for most NHPs lies mainly with the manufacturer. As a result, NHPs may be highly variable in their potencies or quality among different companies, and even among lots of the exact same marketed product. Also, herbal product crops can often vary in their levels of biomarkers and other secondary metabolites because of genetic and environmental factors (Büter *et al.*, 1998) and seasonal variations (Southwell & Bourke, 2001).

In this study, goldenseal and milk thistle products were analyzed by HPLC for their content of marker compounds. Clearly, all of the tested products passed the species identity issue. Based on the chromatogram profile under current experimental conditions they appear to contain marker phytochemicals for the species on the label. Despite the fact that no extraneous peaks were visible in the HPLC chromatograms (Fig. 2.1 and Fig. 2.2), it is not possible to know if the tested products have been adulterated with other NHPs or drugs.

Among all of the goldenseal products (Table 2.1), only Solaray goldenseal (NRP17) had specific label claims with respect to total alkaloid content (250 mg of a standardized extract at 10%

alkaloids; capsules also contain 65 mg of root). Mean content weight for this product was found to be 341 ± 4 mg (Table 2.2) despite a label claim of 315 mg. Nevertheless, alkaloid content was very high in these capsules (Table 2.3) and actually exceeded label indications. However it is not specified exactly which alkaloids they are referring to as "total alkaloids" and this company was unwilling to reveal any of their standardization procedures when contacted (personal communication, 2001).

There is further confusion when one considers the milk thistle capsules. The analyses conducted here were performed on total capsule content weights (note the discrepancies for NRP18 and NRP126 when comparing the indicated label weight of each product with that of the actual content weight) which obviously included the excipients. The highest determined content for total silymarins (which includes silibinin) was determined to be 18.7% ± 0.3% (Table 2.4: Sol-MT; NRP18; 70% EtOH fraction). If one examines the labels of each product closely, both claim to have a content of at least 80% silymarins. It is manufacturing practices such as these that may mislead consumers, since the 80% silymarins merely refers to the silymarin content of an extract that represents a portion of the capsule weight.

With respect to the excipient content of NHPs, companies will often add other NHPs to the final manufactured product, which further confounds studying them. Consider the St. John's wort product (NRP128) evaluated for CYP3A4 inhibition (Table 2.5: $IC_{50} = 5.74$ [8.59, 4.14]). One cannot with any great deal of confidence attribute this IC_{50} solely to the activity of St. John's wort, since there are numerous NHPs indicated on the label as additives, some of which (e.g. scullcap: *Scutellaria lateriflora*) are also established CYP3A4 inhibitors (Awad *et al.*, 2002).

With respect to the assays for CYP3A4 inhibition and P-gp ATPase determination, these

techniques allow for the assessment of numerous products to interact with important drug disposition pathways in a relatively short time thereby identifying possible candidates for NHP-drug interactions that can be advanced to more detailed *in vitro* or *in vivo* studies (Budzinski *et al.*, 2000). In fact, it was the initial results of preliminary P-gp ATPase testing with teas (Fig. 2.4) that helped in deciding to pursue further investigations with milk thistle and goldenseal products. Chapter 3 highlights these experiments.

CHAPTER 3 - The modulation of human cytochrome P450 3A4 (CYP3A4) and P-glycoprotein (P-gp) in caco2 cell monolayers by a variety of commercial-source milk thistle and goldenseal products.

3.1 - INTRODUCTION

Two popular herbs utilized among individuals with HIV/AIDS include goldenseal (GS) and milk thistle (MT) (CATIE, 2000). Traditionally, GS has been used to treat infections of the eyes, ears, and upper respiratory tract, and may also be useful in treating liver disorder and cirrhosis (CATIE, 2000). It's biological activity is largely attributed to the phototoxic alkaloid berberine (Philogène *et al.*, 1984), which has demonstrated anti-fungal properties and is primarily responsible for the plant's medicinal effects (CATIE, 2000). Recent studies have investigated the use of berberine in patients with congestive heart failure as a therapy overcoming premature ventricular beats (Zeng and Zeng, 1999). Also, berberine is a known inhibitor of CYP3A4 (Budzinski *et al.*, 2000) and can inhibit multidrug resistance efflux pumps, and act as a synergist with P-gp substrates (Stermitz *et al.*, 2000).

Similarly, milk thistle has been used traditionally to treat jaundice and liver damage, and may also help lower liver enzyme levels and remedy co-infections of hepatitis (CATIE, 2000). It's biological activity is largely attributed to the flavanolignan silymarin, which is also responsible for the plant's medicinal effects. Silymarin is the parent compound, which readily dissociates into three compounds known as silibinin, silidianin, and silicristin, with silibinin being predominant. Both milk thistle and its constituents have been studied extensively (Brown, 1993). Milk thistle has been shown to decrease the activity of numerous liver enzymes including CYP3A4 and uridine diphosphoglucuronosyl transferase (Venkataramanan *et al.*, 2000), aid in the treatment of liver diseases such as cirrhosis or hepatitis (Ferenci *et al.*, 1989; Flora *et al.*, 1998), and can promote

neuronal differentiation and survival *in vitro* (Kittur *et al.*, 2002). Silibinin is the most prevalent component of silymarin and has also been studied extensively. Silibinin has been linked to an increase of phase II enzyme activity in mice (Zhao and Agarwal, 1999), is a known modulator of P-gp (Maitrejean *et al.*, 2000), can decrease nephrotoxicity in kidney cells (Sonnenbichler *et al.*, 1999), and inhibits the insulin-like growth factor I receptor-mediated signaling pathway (Zi *et al.*, 2000).

In Chapter 2, various herbal extracts of these 2 NHPS were found to significantly stimulate P-gp ATPase. Thus, the goal of this study was to examine responses of living cells to these biologically active products. Briefly, caco2 cell monolayers were treated with varying concentrations of the numerous GS and MT extracts, along with several related pure compounds including phytochemicals and an HIV protease inhibitor (SQV). Treatments consisted of a short term exposure (48 hours) and monitored levels of *ABCB1* and *CYP3A4* expression in response to the treatments.

3.2 - MATERIALS AND METHODS

3.2.1 - Herbal Product Extracts

Commercial herbal product preparations were purchased from local retail outlets (Ottawa, Canada) between September 2000 and May 2001, as summarized in Table 2.1. In order to arrive at a biologically relevant test concentration, aqueous extracts of four selected herbal products were made based upon the calculated maximal daily dosage indicated on each product's label. It was decided that a volume of 200 ml (i.e.: a fairly typical volume for a glass of water) was an appropriate volume for concentration calculations. Aqueous extracts of each product were made as stock solutions that were 10X more concentrated than the maximal daily dosage/200 ml ddH_2O. Briefly, the appropriate amount of herbal product was weighed out from the contents of 3 - 5 capsules (combined in a small weigh-boat) and added to 20 ml of ddH_2O in a 50 ml Falcon centrifuge tube

(note that the 10X stock solutions were scaled down from 200 ml to a volume of 20 ml). This suspension was then vortexed for 1 min, sonicated for 1 min, vortexed again for 1 min, sonicated again for 1 min, and finally centrifuged at 500 g for 10 min. The resultant supernatant was transferred to a fresh 50 ml tube and used to prepare the decade series of dilutions, resulting in four test concentrations for each herb. These herbal extracts were ultimately tested in the caco2 cell monolayers at 10% of the media volume, resulting in the following final test concentrations: X, X/10, X/100, and X/1000. For Sol-GS (NRP17), X = 3.15 mg/ml; for Sol-MT (NRP18), X = 1.75 mg/ml); for LCY-GS (NRP121), X = 6.75 mg/ml; and SHR-MT (NRP126), X = 7.50 mg/ml.

Pure compounds (silibinin, berberine, vinblastine, saquinavir, and dillapiol) were obtained as outlined in Chapter 2, and maintained as 10 mM stock solutions in 70% EtOH. For specific caco2 cell treatments, the appropriate amount of pure compound was diluted directly into the media from the stock solution to yield the final test concentration.

3.2.2 - Caco2 Cell Culture

Caco2 cells (C2BBe1: human adenocarcinoma colon cells; clone of Caco2) were routinely subcultured, grown, and maintained in 50 ml cell culture flasks (Nunclon) in a Fisher Scientific 605 incubator set to 37C and 5% CO_2. Culture feeding media consisted of DMEM (Dulbecco's modified Eagle media) (Gibco) supplemented with 20% FBS (fetal bovine serum) (Gibco), 50 units of penicillin-streptomycin (Gibco) per ml of media, and 0.25% human transferrin (lyophilized; 4 mg/ml) (Gibco). Cell culture health was routinely assessed by: (1) direct observation of cultured cells via microscopy for contamination; (2) cell density count comparisons; (3) pH measurements; and (4) determination of glucose utilization via analysis with the Glucose LiquiColor enzymatic-colorimetric test kit (Stanbio).

For the individual experiments, caco2 cells were subcultured and plated at an initial density of 1 x 10^6 cells in 8 ml of 20% FBS DMEM and allowed to grow for 120 hours at 37C. Subsequently, the formed caco2 cell monolayers were then treated for 48 h with media containing either a 10% herbal extract (10X stock solution and corresponding dilutions: 1X, 0.1X, and 0.01X), 10% ddH_2O with a pure compound dilution, or 10% ddH_2O (control). Cells were then washed, trypsinized, and harvested. Briefly, the media from each flask was discarded and the cell monolayer surface rinsed with 5 ml of trypsin EDTA (Gibco). Cells were then trypsinized for 10 min with another 5 ml volume of trypsin EDTA and then collected in a 15 ml Falcon centrifuge tube. A 10 ml volume of fresh media was added to the tube and the cells were then resuspended. A small aliquot (approximately 0.2 ml) was obtained from each cell suspension for the purposes of counting, while the remaining cells were centrifuged at 1100 rpm for 10 min. The supernatant was then discarded and the remaining cell pellet was flash frozen in liquid N_2 and stored at - 80C until needed.

3.2.3 - RNA Extraction

Total RNA was isolated from frozen pellets of treated or untreated caco2 cells using the acid guanidinium thiocyanate (GITC)-phenol-chloroform method (Chomczynski and Sacchi, 1987; Chomczynski and Mackey, 1995). Briefly, frozen cell pellets were homogenized for 1 min in 1.0 ml of denaturing solution [solution D; per 100 ml: 73 ml of diethyl pyrrocarbonate (DEPC) (Bio Basic Inc.) treated water, 62.5 g of solid GITC (Bio Basic Inc.), 4.4 ml of 0.75 M sodium citrate (pH 7.0) (Bio Basic Inc.), 6.6 ml of 10% sarkosyl (10 g of sodium lauryl citrate (Bio Basic Inc.) per 100 ml DEPC-treated water), and 0.3 ml of ß-mercaptoethanol (Sigma)]. Afterwards, 0.5 ml of phenol (BDH) saturated with DEPC-treated water was added to the suspension and the solution was vortexed. Subsequently, 0.2 ml of chloroform was added and the resultant solution was vortexed

again. The solution was then transferred to an RNase-free 2.0 ml microcentrifuge tube and centrifuged at 12 000 g for 20 min in an Eppendorf microfuge. The supernatant (aqueous phase) was subsequently transferred to a new 2.0 ml microcentrifuge tube and the remaining solution was frozen in liquid N_2 and stored at -80C for future protein purification from the organic phase. To the supernatant, 0.6 ml of isopropanol was added and the resultant mixture was vortexed and centrifuged again at 14 000 g for 15 min. This new supernatant was then discarded and the RNA pellet at the bottom of the tube was washed with 1.0 ml of ice-cold 80% EtOH and centrifuged again for 10 min at 14 000 g. This washing procedure was repeated twice and following the second wash, the RNA pellet was air dried for 10 min and afterwards suspended in 100 µl of DEPC-treated water. The RNA concentration of individual samples was determined by drawing a 2 µl aliquot from each, suspending it in 98 µl of filtered 0.1X TE (Tris-EDTA (Bio Basic Inc.)), and quantified using a Pharmacia Gene Quant DNA/RNA calculator spectrophotometer. RNA purity was determined from the ratio of absorbancies at 260 nm and 280 nm (A_{260}/A_{280}). The remaining stock solution of RNA was frozen in liquid N_2 and stored at -80C.

3.2.4 - RT-PCR and cDNA Synthesis

First-strand cDNA was synthesized from 1 - 5 µg of total RNA (isolated from untreated caco2 cells) using Oligo (dT) primer and Superscript II reverse transcriptase (Gibco BRL). Specific oligoprimers for ABCB1 and ß-actin were designed to amplify and isolate specific fragments of each gene via PCR. Oligoprimers for ABCB1 were modeled after those used by Pu *et al.* (1996) and were designed to amplify ABCB1 cDNA with a product size of 249 nucleotides from exon 13 to exon 15 of the *ABCB1* gene. The sense strand primer sequence was (5' - GGA AGC CAA TGC CTA TGA CT - 3') and the anti-sense stand primer was (5' - CGA TGA GCT ATC ACA ATG GT - 3'). The ß-

actin gene was used to control for variations in RNA loading within the experimental conditions; oligoprimers for ß-actin were also modeled after those used by Pu *et al.* (1996). The sense strand primer sequence was (5' - CAT CCT CAC CCT GAA GTA CC - 3') and the anti-sense stand primer was (5' - GGT GAG GAT CTT CAT GAG GT - 3') with a PCR product size of 394 nucleotides. Individual PCR reactions were carried out in 0.5 ml microcentrifuge tubes in a total reaction volume of 50 µl [per reaction: 36.25 µl of DEPC-treated water, 5 µl of 10X PCR buffer (Gibco BRL), 1.5 µl of 50 mM $MgCl_2$ (Gibco BRL), 1 µl of 10 mM dNTP mix (Gibco BRL), 2 µl of each oligoprimer (maintained as a 25 µM stock solution), 2 µl of cDNA template, and 0.25 µl of *Taq* polymerase (Gibco BRL)]. PCR reaction was performed for 25 cycles (*ABCB1* gene) or 20 cycles (*ß-actin* gene) under the following schema: predenaturation at 95C for 5 min, denaturation at 94C for 40 s, annealing at 54C (*ABCB1*) or 57C (*ß-actin*) for 40 s and extension at 72C for 1 min. Amplified products were then size-fractionated by electrophoresis in a 50 ml 1.2% agarose gel (Agarose A: Bio Basics Inc.) and visualized. Bands corresponding to the expected size were excised and purified using the QIAquick gel extraction kit (Qiagen). Following PCR reactions, purified cDNA was ligated into the pCRII-TOPO cloning vector (Invitrogen) and used to transform One Shot competent cells (Invitrogen). Plasmids were subsequently isolated and purified from transformed bacterial cultures using the Wizard Miniprep kit (Promega) and sent to the Canadian Molecular Research Services (Ottawa, Canada) for sequencing. Nucleotide sequences were subsequently verified via the internet using GenBank.

First strand cDNA for CYP3A4 was also synthesized using the RT-PCR technique described above. Instead of using total RNA isolated from untreated caco2 cells as a template however, a CYP3A4 expression vector (*p*UV1) obtained from Dr. Frank Gonzalez at the National Institutes of

Health (NIH, USA) was used. The sense strand primer sequence was (5' - CAA GAC CCC TTT GTG GAA AA- 3') and the anti-sense stand primer was (5' - TCT GAG CGT TTC ATT CAC CA - 3') with a PCR product size of 498 nucleotides. The PCR reaction was performed as previously described for 30 cycles under the following schema: predenaturation at 95C for 5 min, denaturation at 94C for 40 s, annealing at 55C for 40 s and extension at 72C for 1 min. Amplified products were isolated, purified, ligated into pCRII-TOPO, used to transform competent cells, and ultimately sent for sequencing as previously described.

3.2.5 - Dot Blotting and Hybridization

Total RNA (7 μg) isolated from various caco2 cell treatments was denatured in 8 μl of denaturation buffer per μg of RNA at 65C for 10 min. RNA denaturation buffer consisted of: 50% deionized formamide (Sigma), 2.2 M formaldehyde (BDH), 20 mM MOPS (morpholino-propanesulfonic acid) (pH 7.0) (Bio Basics Inc.), 5 mM sodium acetate (Bio Basics Inc.), and 1 mM disodium EDTA (Bio Basics Inc.). RNA was then chilled on ice and diluted 1:2 (v/v) with 20X SSC (standard sodium citrate). The final volume was then adjusted to 0.5 ml with 10X SSC prior to loading. RNA was loaded onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech) pre-soaked with 10X SSC using a Schleicher and Schuell Minifold I dot blot apparatus. The RNA was applied to each well from the resultant 0.5 ml solution and then subsequently rinsed twice with 0.5 ml of 10X SSC. Membranes were allowed to dry under vacuum for approximately 10 min and then subjected to intense UV light for 5 min in order to covalently bind the RNA to the membrane.

For hybridization, membranes were washed twice in a solution of 0.1X SSC and 0.1% SDS (sodium dodecyl sulfate) for 20 min each at 65C. Hybridization solution (10 % dextran sulfate (Bio Basics Inc.), 1% SDS, 6X SSC, 100 μg/ml of salmon sperm DNA, 5X Denhardt's solution) was

added (10 ml per 100 cm^2 of membrane) and the membrane was pre-hybridized for 3 hours at 65C. During the pre-hybridization, radiolabelled probes were prepared by: (1) diluting 25 ng of the appropriate template cDNA in 45 µl of 1X TE (10 mM Tris, 1.0 mM EDTA); (2) denaturing the cDNA by placing the mixture at 100C for 5 min; (3) radiolabelling the cDNA using Redivue stabilized [α^{32}P]dCTP and the Rediprime II random prime DNA labeling system (Amersham Pharmacia Biotech); and (4) purifying the radiolabelled probe using a Probe Quant G-50 Micro Column (Amersham Pharmacia Biotech) in order to remove unbound radioactive nucleotides. After the pre-hybridization was complete, the hybridization solution was replaced with an equal volume of fresh solution and supplemented with the radiolabelled probe. The mixture was left to hybridize overnight for roughly 18 hours at 65C. Membranes were subsequently washed at 65C as follows: (1) rinsed vigorously with 1X SSC and 0.1% SDS; (2) 10 min with 1X SSC and 0.1% SDS; and (3) 10 min with 0.1X SSC and 0.1% SDS. Membranes were then placed on Whatman filter paper, wrapped in Saran wrap, and exposed to a Kodak K phosphor screen for 48 h. Phosphor screens were scanned with a Bio-Rad phosphor imager and dot intensities analyzed using the Bio-Rad Quantity One quantitation software (release 4).

Hybridizations for all membranes occurred in the following order: ABCB1, CYP3A4, and ß-actin. Between hybridizations, membranes were stripped of all radioactivity by washing them with 1% SDS for 20 min at 85C. A lack of radioactivity was verified by observing membranes after a short-term exposure to Kodak K screens (6 h) and visualizing them as previously described.

Statistical Analysis

For a given membrane, the densitometric data for the relative expression of *ABCB1* and *CYP3A4* (corrected for ß-actin to control for variation in loading) was calculated as the (counts x

mm^2 for each blot)/(mean counts x mm^2 for all blots). This allowed for differences among membranes and thus ratios from multiple membranes were then pooled and analyzed via a one-way ANOVA, followed by Dunnett's 2-sided pairwise comparison (significance set to $p < 0.05$) carried out using SYSTAT statistical software (version 9, SPSS Inc.).

Note: The following protocols were carried out in collaboration with our laboratory's post-doctoral researcher Dr. Mitra Panahi. I provided her with the organic fractions collected during the various total RNA isolations and she performed the subsequent protein purification, immuno-blotting techniques, and densitometrical analysis. I use this information with her permission.

3.2.6 - Protein Isolation and Western Blot Analysis

The total protein was recovered from the organic phase leftover during the RNA isolation previously described. Briefly, 0.8 volume of 100% EtOH was added to the organic phase and after centrifugation at 2000 g for 5 min at 4C, 2 volumes of isopropanol was added to the supernatant. After centrifugation at 13000 g for 10 min at 4C, the pellet was washed by 90% EtOH 3 times. The pellet was subsequently air dried and dissolved in 1% SDS. The protein concentration was then determined using Bio-Rad's protein assay with bovine serum albumin (BSA) serving as a standard.

Samples were subjected to electrophoresis on a 7.5% poly accrylamide gel and transferred to nitrocellulose using standard techniques. The blot was blocked in 3% non-fat milk and incubated with primary antibody: C494 (ID Labs Inc. - 1:500 dilution) for ABCB1 and CYP3A4Mabr (Research Diagnostics Inc. - 1:500 dilution) for CYP3A4. The blot was then washed with TTBS (Tris-buffered saline containing 0.05% Tween-20) and incubated with secondary alkaline phosphatase-conjugated antibody: goat anti-mouse for ABCB1 (Sigma) or goat anti-rabbit for CYP3A4 (Bio-Rad) with a dilution of 1:3000. The signal was observed using tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) and was quantified using a Kodak image

scan program. Each Western blot was repeated two times per sample set and due to the low amount of protein recovered, organic fractions were pooled from multiple experiments ($N = 1$).

3.3 - RESULTS

3.3.1 - Expression of *ABCB1* and *CYP3A4*

Membranes dotted with total RNA isolated from caco2 cells treated with NHP extracts or pure compounds were hybridized with a radiolabelled cDNA probe for the specific mRNAs. Figure 3.1 highlights some typical representative dot blot membranes. It was consistently observed that the levels of relative gene expression among all of the membranes were as follows: *ABCB1* < *CYP3A4* < ß-actin. Densitometric data was compared among multiple membranes, grouped, and summarized by experimental treatment (Fig. 3.2 to Fig. 3.10).

Briefly, after 48 hours of exposure, extracts of Sol-GS (X = 3.15 mg/ml) had no significant effect on *ABCB1* or *CYP3A4* expression for any of the tested concentrations (Fig. 3.2). Sol-MT extracts (X = 1.75 mg/ml) did not have any significant effect on *ABCB1* expression, but two extract dilutions (X/10 and X/100) significantly down-regulated expression of *CYP3A4* (Fig. 3.3). Extracts of LCY-GS (X = 6.75 mg/ml) did not significantly effect *ABCB1* expression, but there was an observed bimodal modulation for these extracts on levels of CYP3A4 mRNA (Fig. 3.4); full strength LCY-GS (X) extracts upregulated *CYP3A4* expression while LCY-GS (X/100) extracts downregulated *CYP3A4* expression. It is interesting to note that although levels of ABCB1 mRNA in the caco2 cells were not significantly altered by LCY-GS treatments, a similar but non-significant trend of bimodal modulation was observed as with *CYP3A4* expression. A similar bimodal effect on *CYP3A4* expression was observed with SHR-MT (X = 7.50 mg/ml) extract treatments (Fig. 3.5), with an upregulation occurring for SHR-MT (X) and a downregulation occurring for SHR-MT

(X/100 and X/1000). SHR-MT extracts also decreased levels of ABCB1 mRNA for the 2 lowest tested concentrations (X/100 and X/1000).

Similarly, pure compounds including phytochemicals and HIV drugs were also examined for their ability to alter gene expression in treated caco2 cell monolayers. Various concentrations of the goldenseal alkaloid berberine (Fig. 3.6) had no significant effect on *ABCB1* expression, although *CYP3A4* was downregulated by the highest exposure concentration (10 μM). The milk thistle flavanolignan silibinin (Fig. 3.7) also had no appreciable effect on *ABCB1* expression but a clear dose-dependent downregulation of *CYP3A4* was observed. The vinca alkaloid and known P-gp inhibitor vinblastine (Parasrampuria *et al.*, 2001) did not affect levels of *ABCB1* mRNA, but did upregulate *CYP3A4* expression for treatments at 1 μM (Fig. 3.8). The HIV protease inhibitor saquinavir had no effect on *ABCB1* nor *CYP3A4* expression, except an apparent increase of CYP3A4 mRNA at the lowest tested concentration (100 nM) (Fig. 3.9). Dillapiol, a compound occurring in dill and *Piper* sp., is a known CYP3A4 inhibitor (Budzinski *et al.*, 2000) and was found to significantly upregulate CYP3A4 expression in a dose-dependent manner (Fig. 3.10). Dillapiol had no appreciable effect on ABCB1 mRNA levels at the concentrations tested.

3.3.2 - Protein Levels of P-glycoprotein and CYP3A4

Protein fractions for all of the experiments were collected from the organic phase of the RNA isolation protocol, purified, pooled, and subjected to immuno-blot analysis (Fig. 3.11) by Dr. Mitra Panahi. Since the data reflect pooled protein (N = 1), it is difficult to draw any significant conclusions. Protein level from each treatment were expressed as a ratio to the corresponding control value and summarized in Table 3.1. Relative to the control, levels of CYP3A4 ranged from 0.10 (Sol-MT X/10) to 2.25 (Silibinin 1 μM). Similarly, levels of P-gp were apparently affected to a larger

degree ranging from 0.18 (Sol-GS X) to 10.4 (Silibinin 3 μM). The overall change in levels of mRNA for ABCB1 and CYP3A4 are compared with the observed changes in protein in a qualitative manner summarized in Table 3.2.

3.4 DISCUSSION

The caco2 cell line is considered an industry standard for modeling human intestinal absorption of new compounds (Cummins *et al.*, 2001), and in the case of this study, NHPs, related phytochemicals, and established HIV drugs. In general, rates of drug permeation across caco2 cell monolayers correlates well with the amount of passive drug absorption and actively transported compounds, since caco2 cells endogenously express numerous types of efflux transporters (P-gp being the predominant one). According to Cummins *et al.* (2001), there are currently two caco2 cell models that express CYP3A4 at high enough levels to allow for the concurrent study of transport and metabolism. These include: (1) caco2 cells in which levels of CYP3A4 have been artificially upregulated via the addition of 1α,25-dihydroxy vitamin-D_3 to the medium; and (2) caco2 cells transfected with the CYP3A4 gene (such as those produced by GENTEST). The caco2 cell model used in these experiments were neither of the two described, since the goal was to determine whether or not *CYP3A4* (and *ABCB1*) could be modulated from an endogenous state. As well, no transport studies were performed since the total RNA was isolated from stripped and trypsinized monolayers. In fact, *CYP3A4* was found to be highly responsive in our system as evidenced by a strong dose-dependent modulation (e.g. an observed upregulation by dillapiol and downregulation by silibinin). Perhaps this is one reason for the observed apparent poor responsiveness of *ABCB1* in this system. Since P-gp and CYP3A4 have numerous overlapping substrate specificities (Cummins *et al.*, 2002), it is conceivable that the majority of P-gp substrates (especially in the milk thistle and goldenseal

extracts) were extensively metabolized by the CYP3A4 and thus had less influence on *ABCB1* and its gene product.

More studies need to be completed in order to re-evaluate and confirm these results. Currently, due to the fact that the protein fractions were pooled for Western blot analysis ($N = 1$), it is difficult to draw any conclusions comparing the observed changes in ABCB1 or CYP3A4 mRNA with the corresponding protein levels (although the trends are described Table 3.1 and Table 3.2). As well, the results of a longer term exposure and/or repeated exposure study would be very useful in complementing the existing studies.

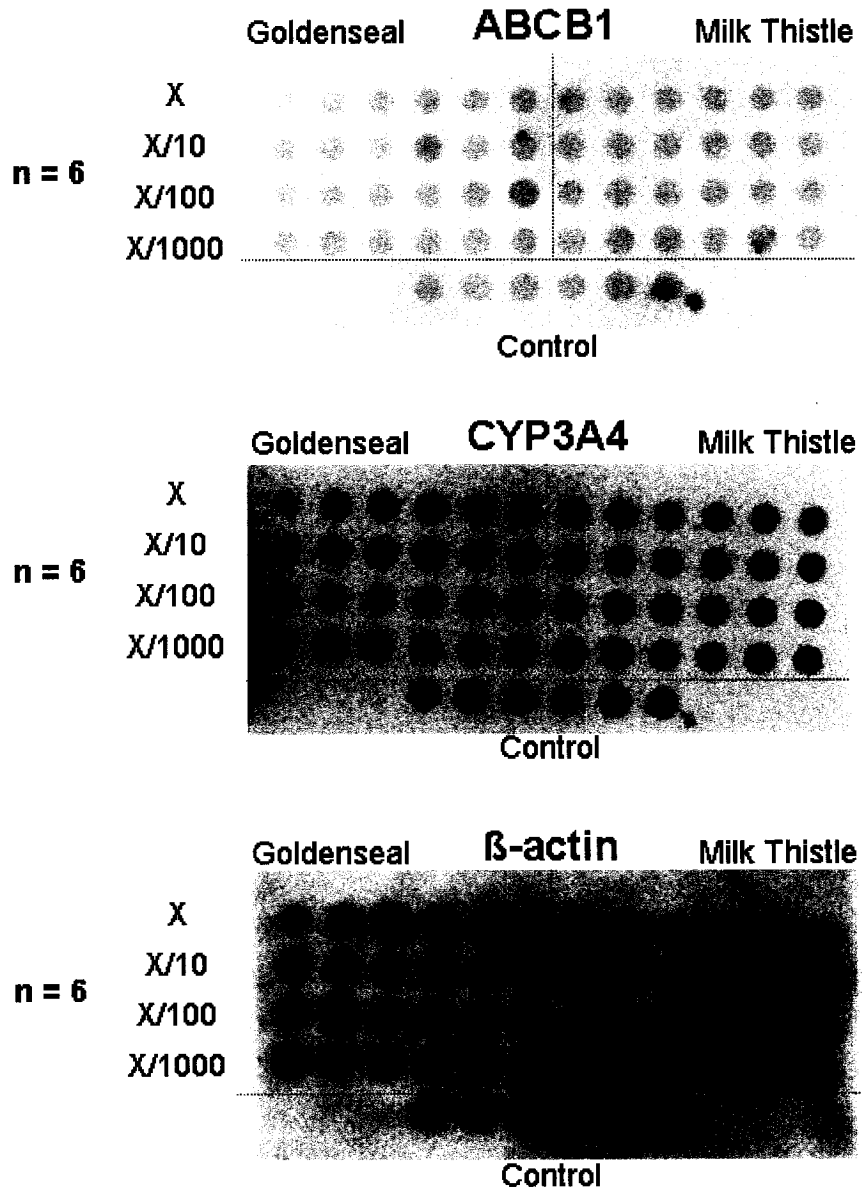


Figure 3.1: A representative dot blot membrane probed for ABCB1 mRNA, CYP3A4 mRNA, and ß-actin mRNA. This particular membrane was blotted with 7 µg of total RNA isolated from caco2 cell monolayers treated with aqueous extracts of Solaray goldenseal (NRP17; X = 3.15 mg/ml) or Solaray milk thistle (NRP18; X = 1.75 mg/ml). Images represent 48 hours of exposure of hybridized membranes to the Kodak K-Screen and imaged with the Bio-Rad phosphorimager.

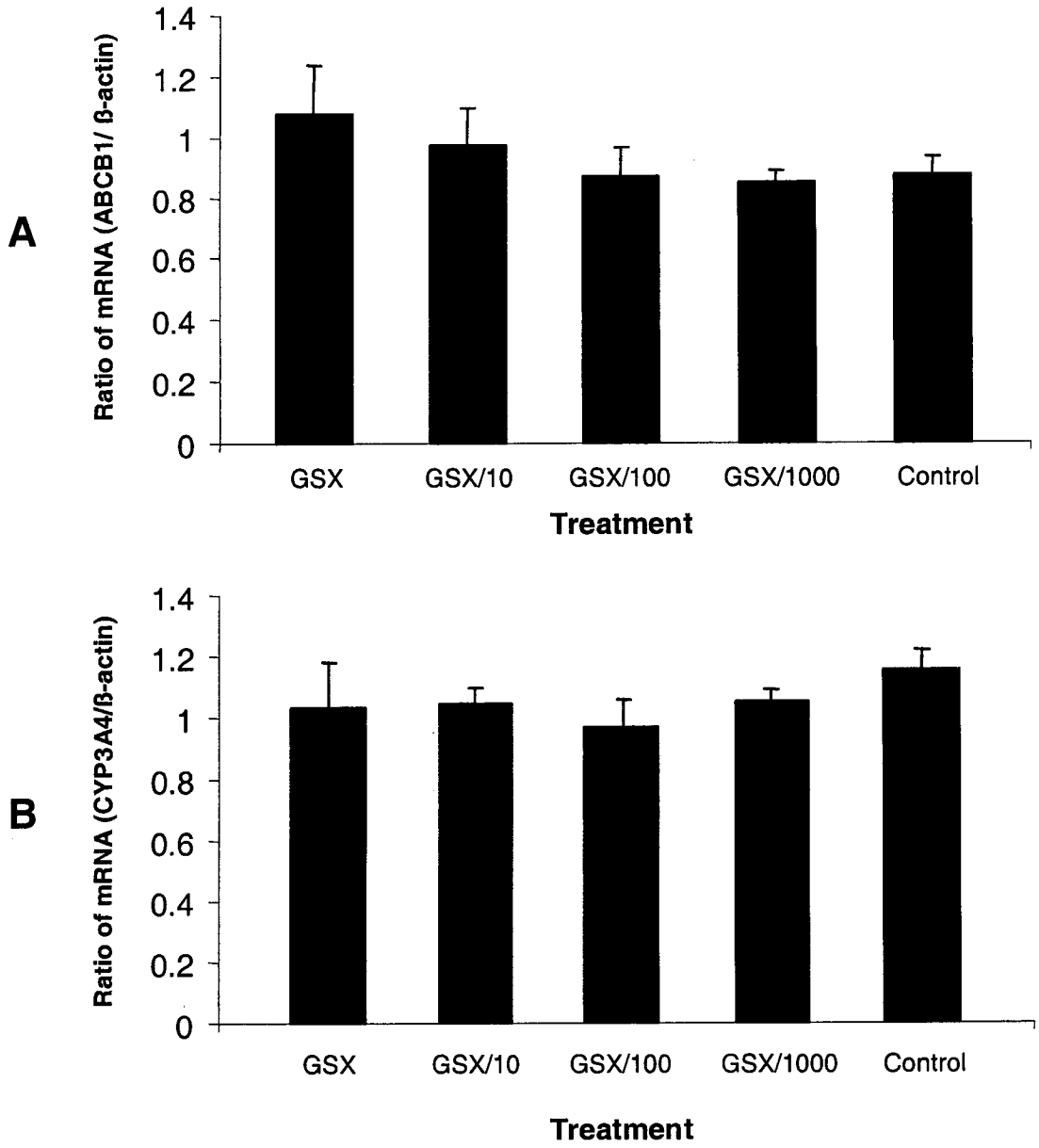


Figure 3.2: (**A**) ABCB1 and (**B**) CYP3A4 mRNA levels for caco2 cells treated with varying concentrations of aqueous extracts of Solaray goldenseal capsules (NRP17; X = 3.15 mg/ml). Data represent the mean ratio of *ABCB1* or *CYP3A4* expression relative to ß-actin ± SEM ($N = 18$). Note that all means are equal for the expression of both genes, as determined by a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

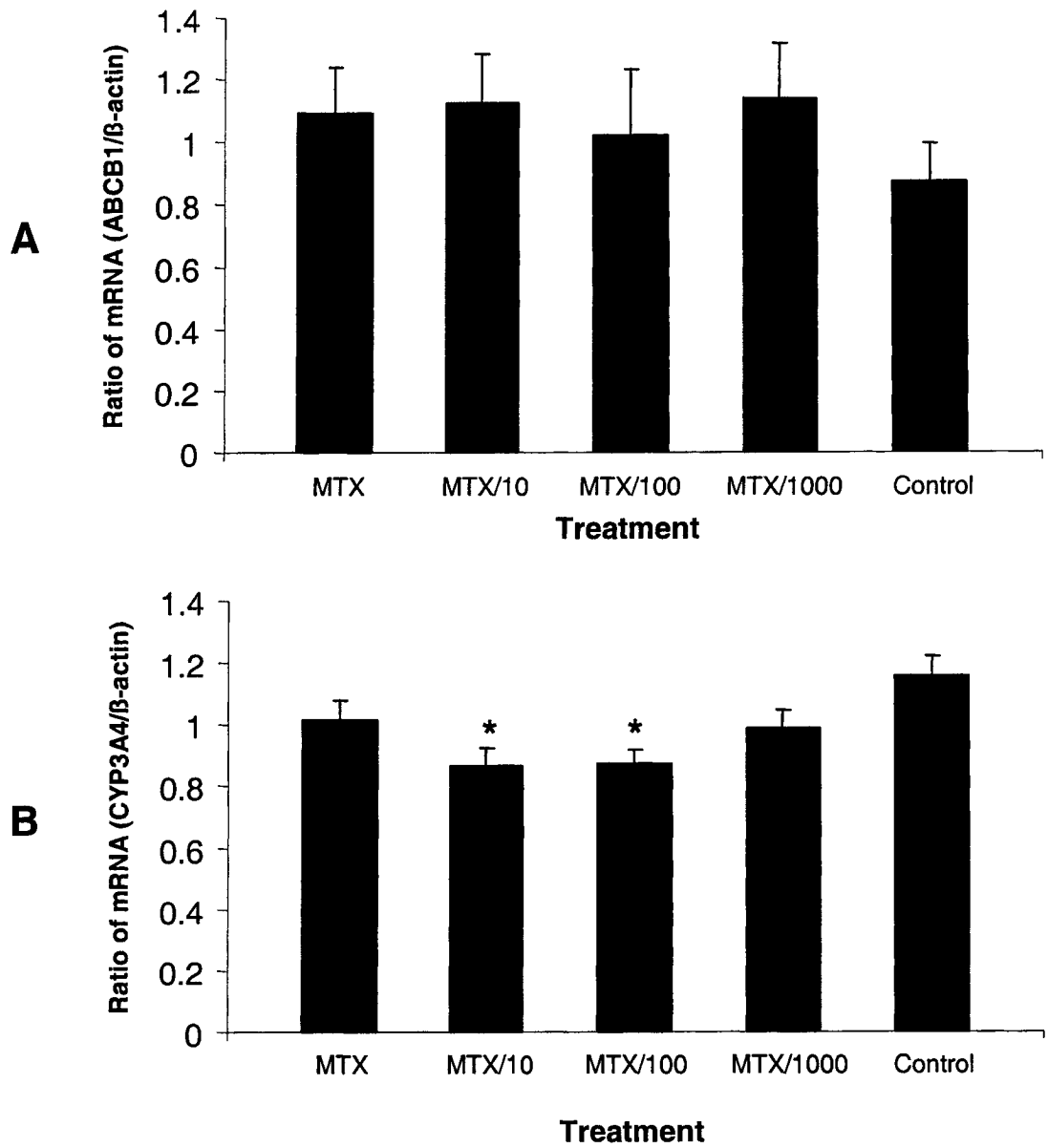


Figure 3.3: (**A**) ABCB1 and (**B**) CYP3A4 mRNA levels for caco2 cells treated with varying concentrations of aqueous extracts of Solaray milk thistle capsules (NRP18; X = 1.75 mg/ml). Data represent the mean ratio of *ABCB1* or *CYP3A4* expression relative to ß-actin ± SEM ($N = 18$). Note that the means for MT X/10 and MT X/100 were significantly lower than control levels of *CYP3A4* expression, as determined by a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

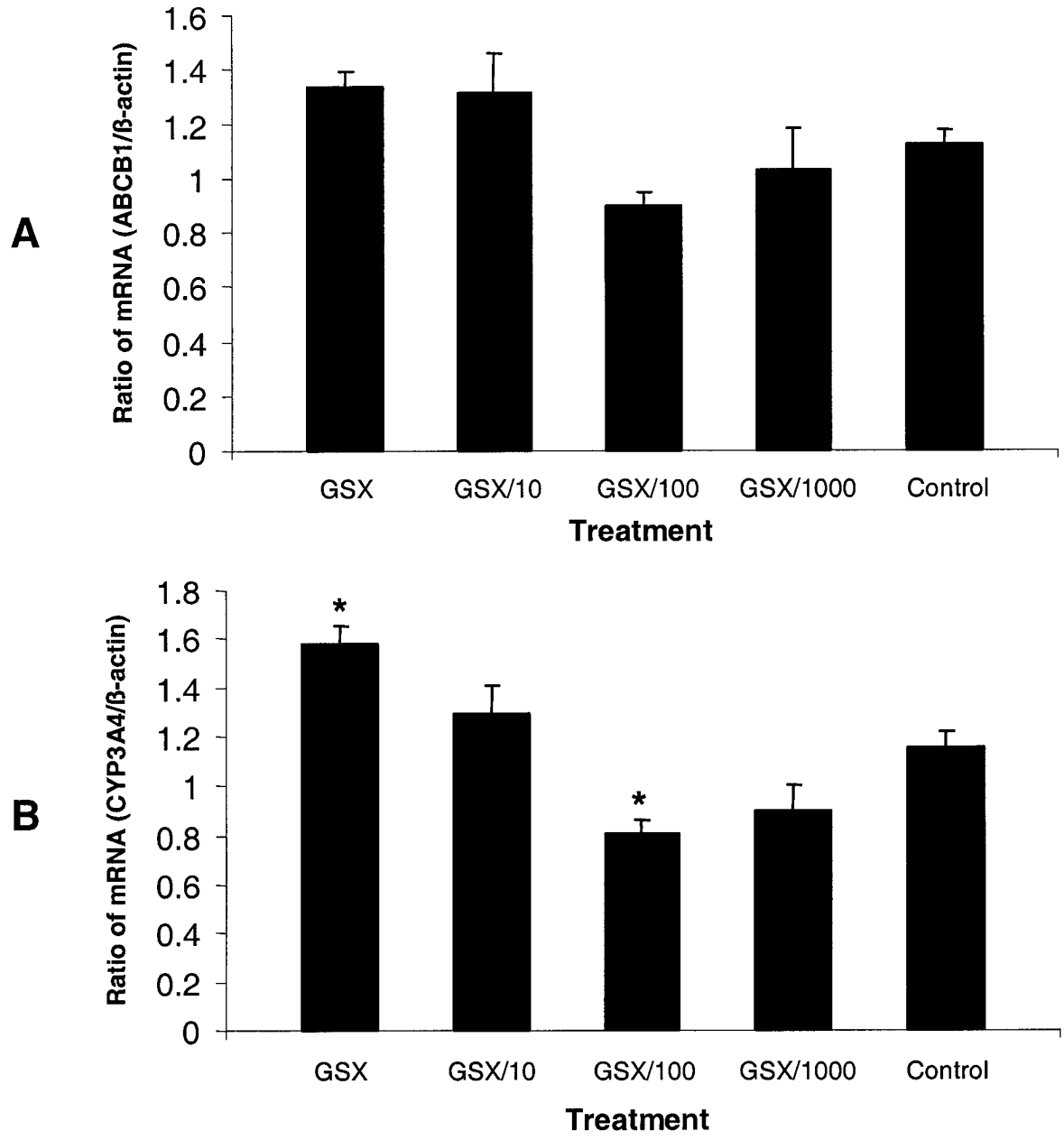


Figure 3.4: (**A**) ABCB1 and (**B**) CYP3A4 mRNA levels for caco2 cells treated with varying concentrations of aqueous extracts of Li Chung Yun goldenseal capsules (NRP121; X = 6.75 mg/ml). Data represent the mean ratio of *ABCB1* or *CYP3A4* expression relative to ß-actin ± SEM ($N = 15$). Note that all means were equal for *ABCB1* expression, while levels of *CYP3A4* were up-regulated for GS X treatments and down-regulated for GS X/100 treatments. Means were analyzed via a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

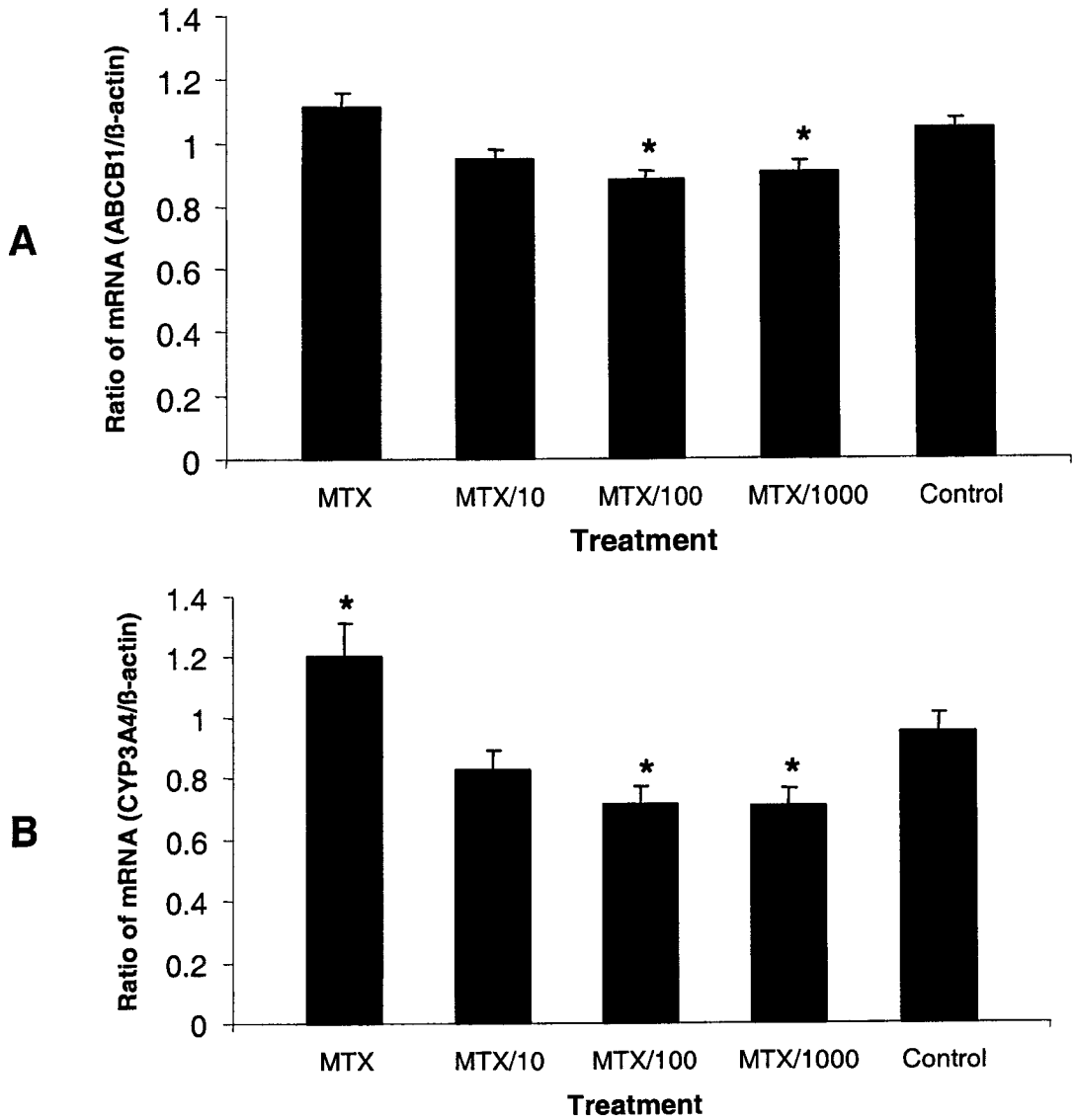


Figure 3.5: (**A**) ABCB1 and (**B**) CYP3A4 mRNA levels for caco2 cells treated with varying concentrations of aqueous extracts of Swiss Herbal Remedies milk thistle capsules (NRP126; X = 7.50 mg/ml). Data represent the mean ratio of *ABCB1* or *CYP3A4* expression relative to ß-actin ± SEM ($N = 12$). Note that the means for MT X/100 and MT X/1000 were significantly lower than control levels of *ABCB1* and *CYP3A4* expression. Levels of CYP3A4 mRNA were up-regulated by MT X treatments. Means were analysed via a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

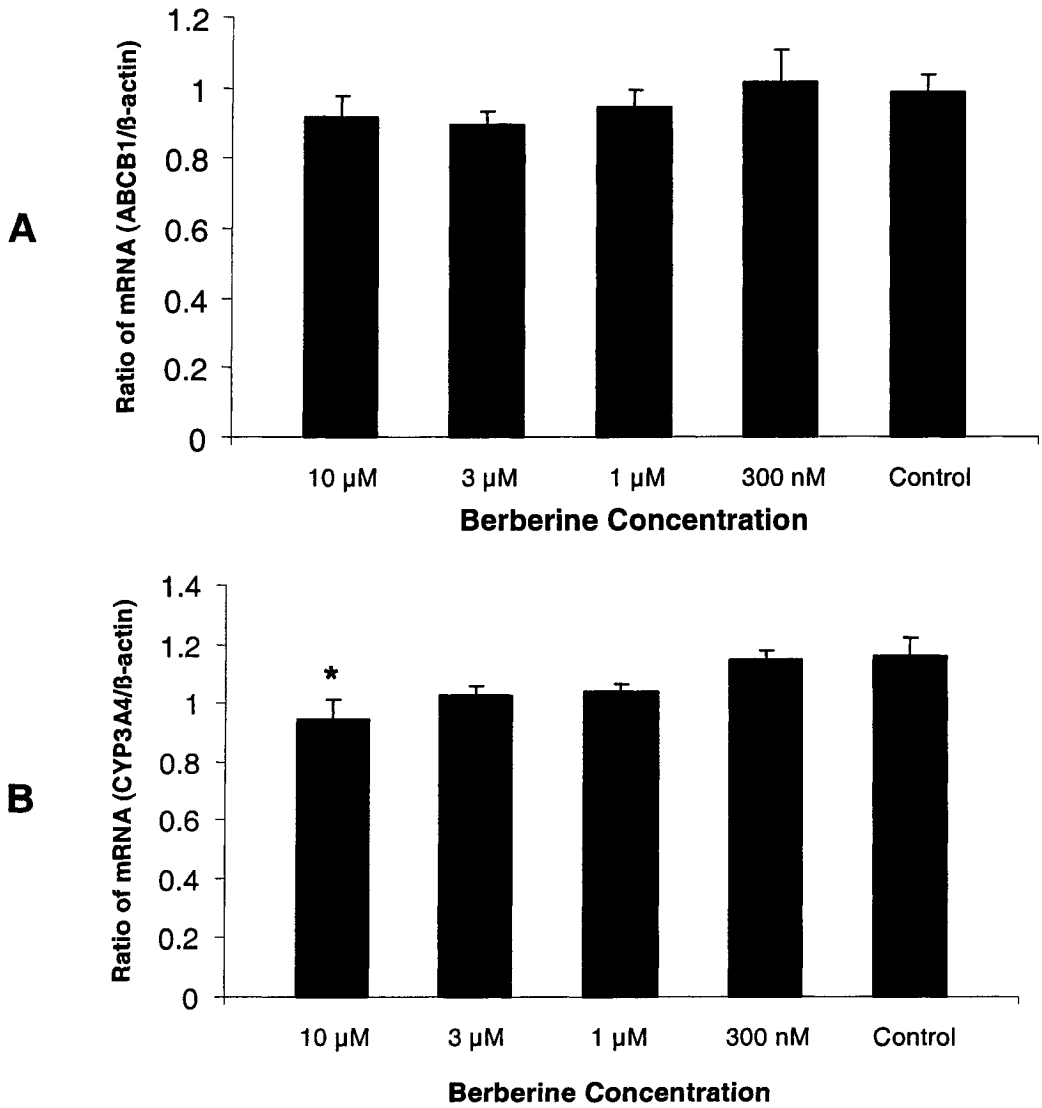


Figure 3.6: (**A**) ABCB1 and (**B**) CYP3A4 mRNA levels for caco2 cells treated with varying concentrations of berberine. Data represent the mean ratio of *ABCB1* or *CYP3A4* expression relative to ß-actin ± SEM (N = 12). Note that 10 µM berberine significantly lowered levels of *CYP3A4* expression. Means were analysed via a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

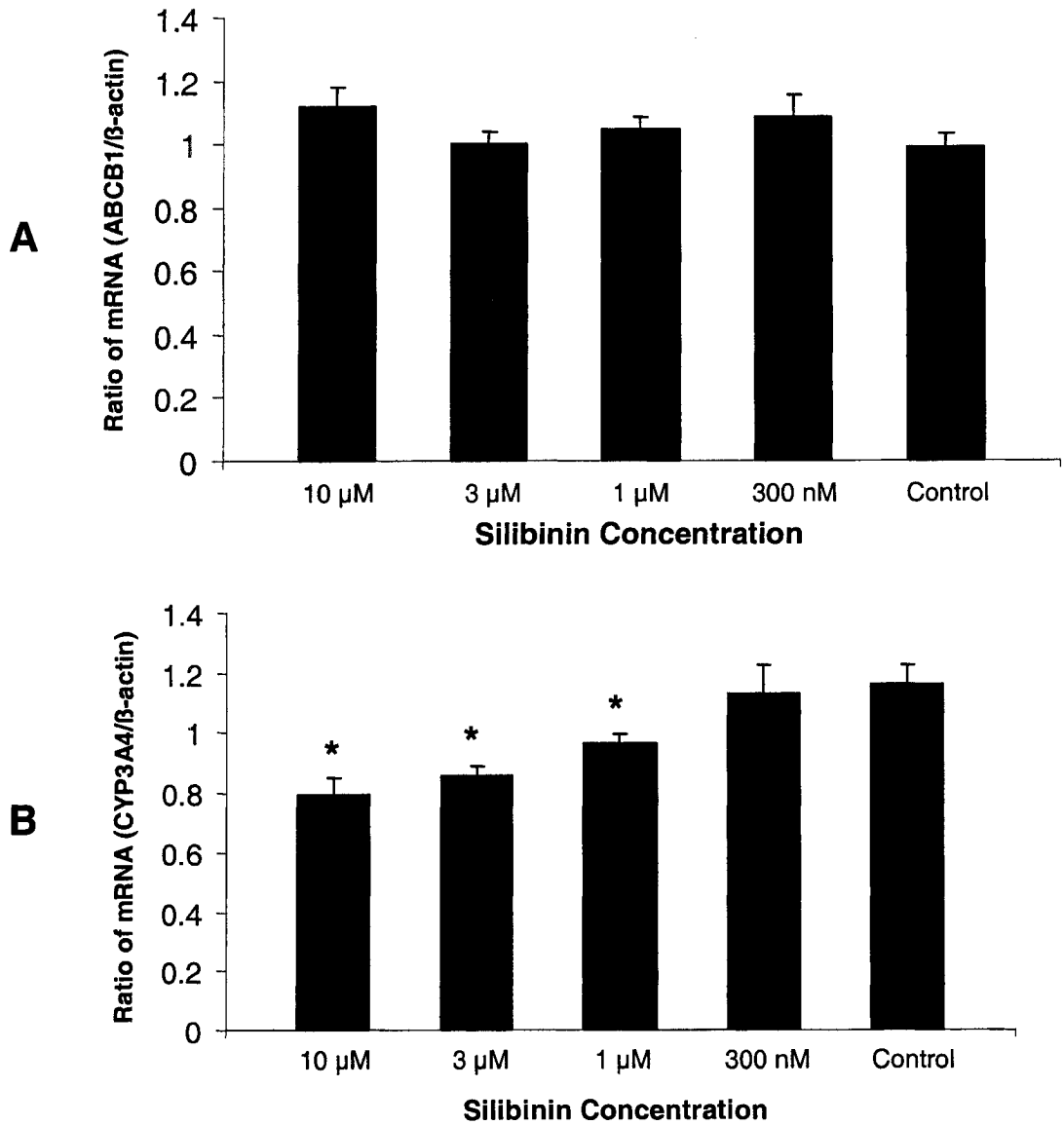


Figure 3.7: (**A**) ABCB1 and (**B**) CYP3A4 mRNA levels for caco2 cells treated with varying concentrations of silibinin. Data represent the mean ratio of *ABCB1* or *CYP3A4* expression relative to ß-actin ± SEM (N = 12). Note that levels of CYP3A4 mRNA were down-regulated by concentrations of 10 µM, 3 µM, and 1 µM silibinin, while levels of ABCB1 mRNA remained unchanged. Means were analysed via a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

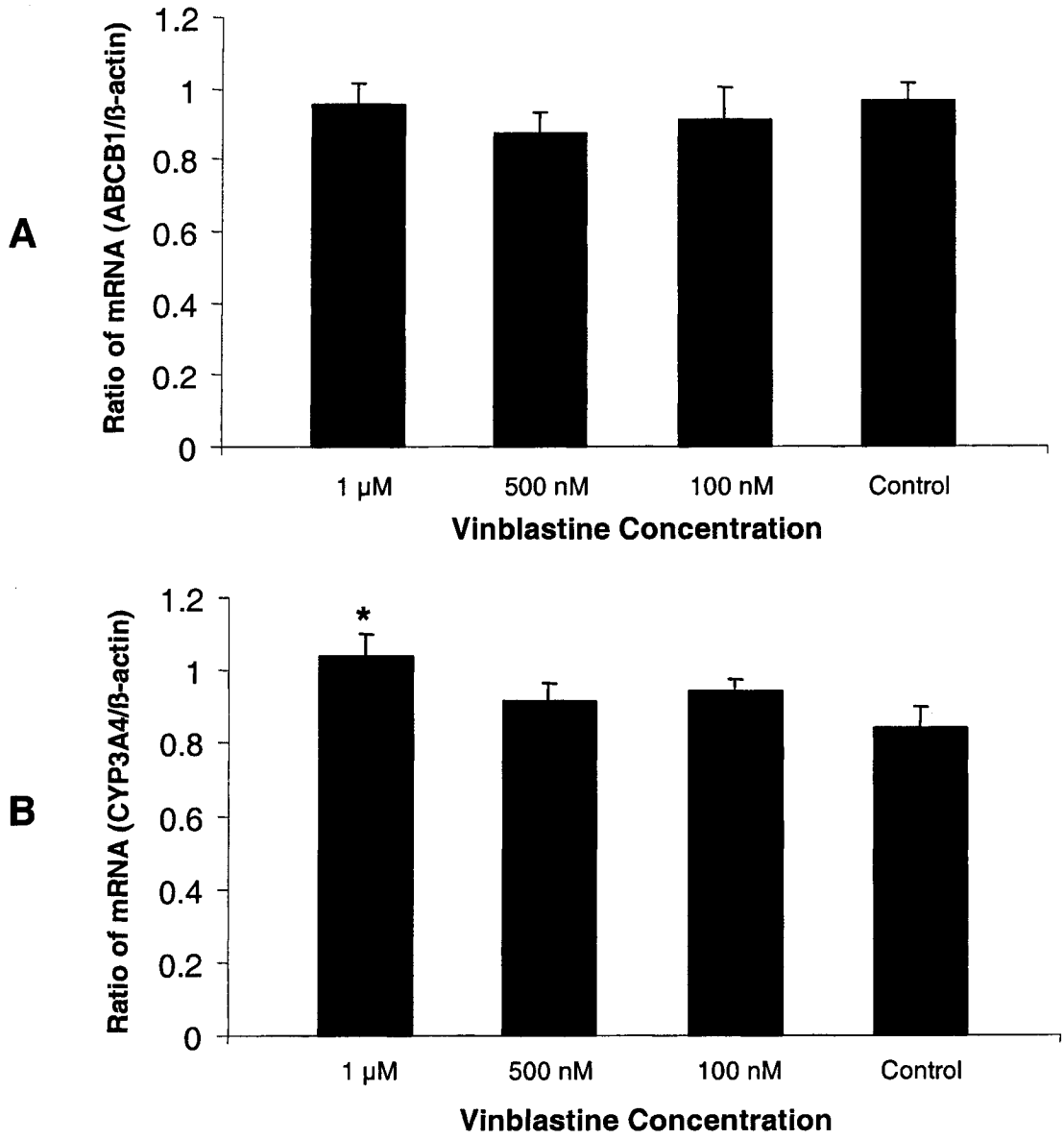


Figure 3.8: (**A**) ABCB1 and (**B**) CYP3A4 mRNA levels for caco2 cells treated with varying concentrations of vinblastine. Data represent the mean ratio of *ABCB1* or *CYP3A4* expression relative to ß-actin ± SEM ($N = 12$). Note that 1 µM vinblastine significantly increased levels of *CYP3A4* expression. Means were analysed via a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

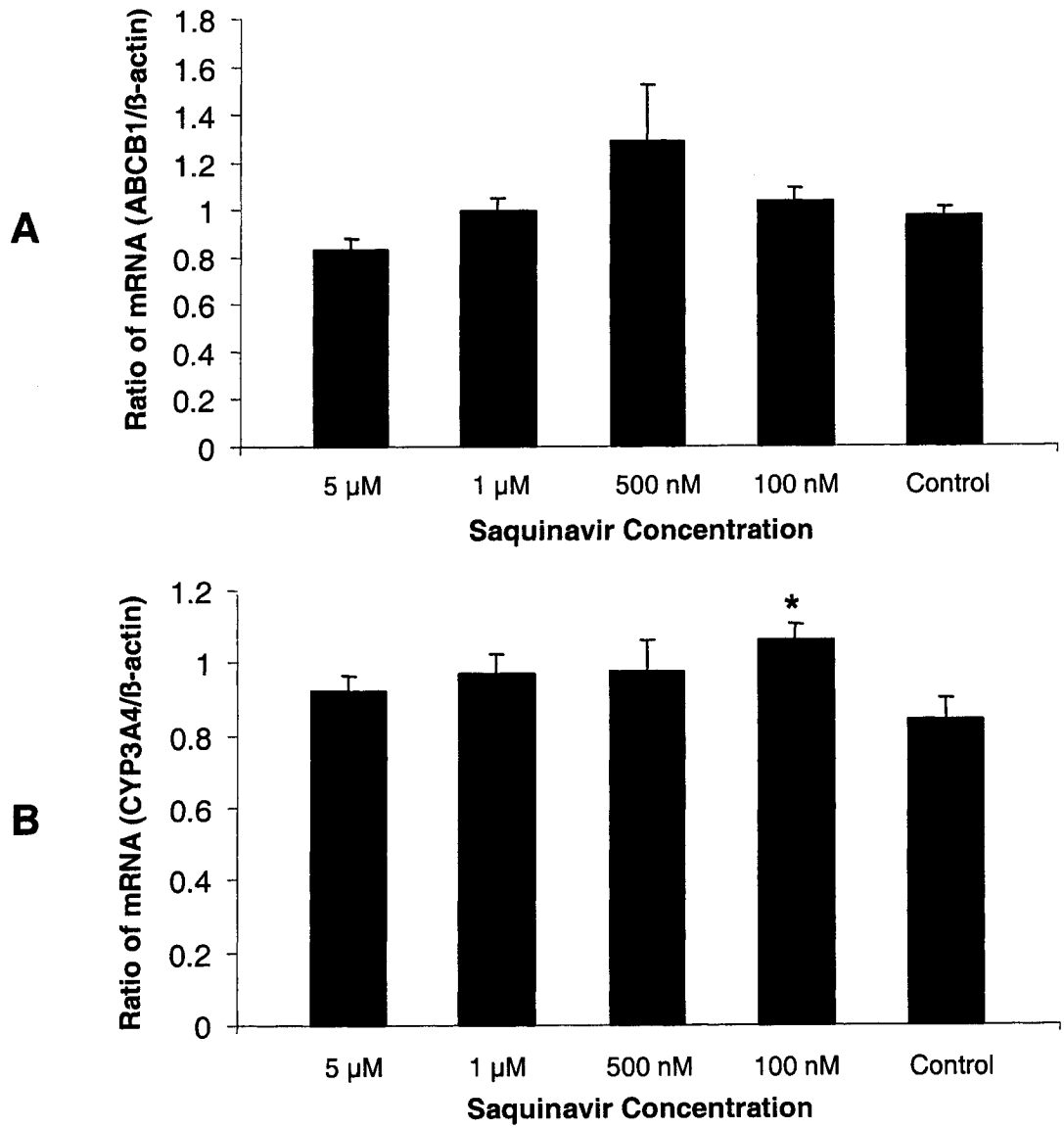


Figure 3.9: (**A**) ABCB1 and (**B**) CYP3A4 mRNA levels for caco2 cells treated with varying concentrations of saquinavir. Data represent the mean ratio of *ABCB1* or *CYP3A4* expression relative to ß-actin ± SEM (N = 12). Note that the lowest tested concentration of saquinavir (100 nM) significantly increased levels of *CYP3A4* expression. Means were analysed via a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

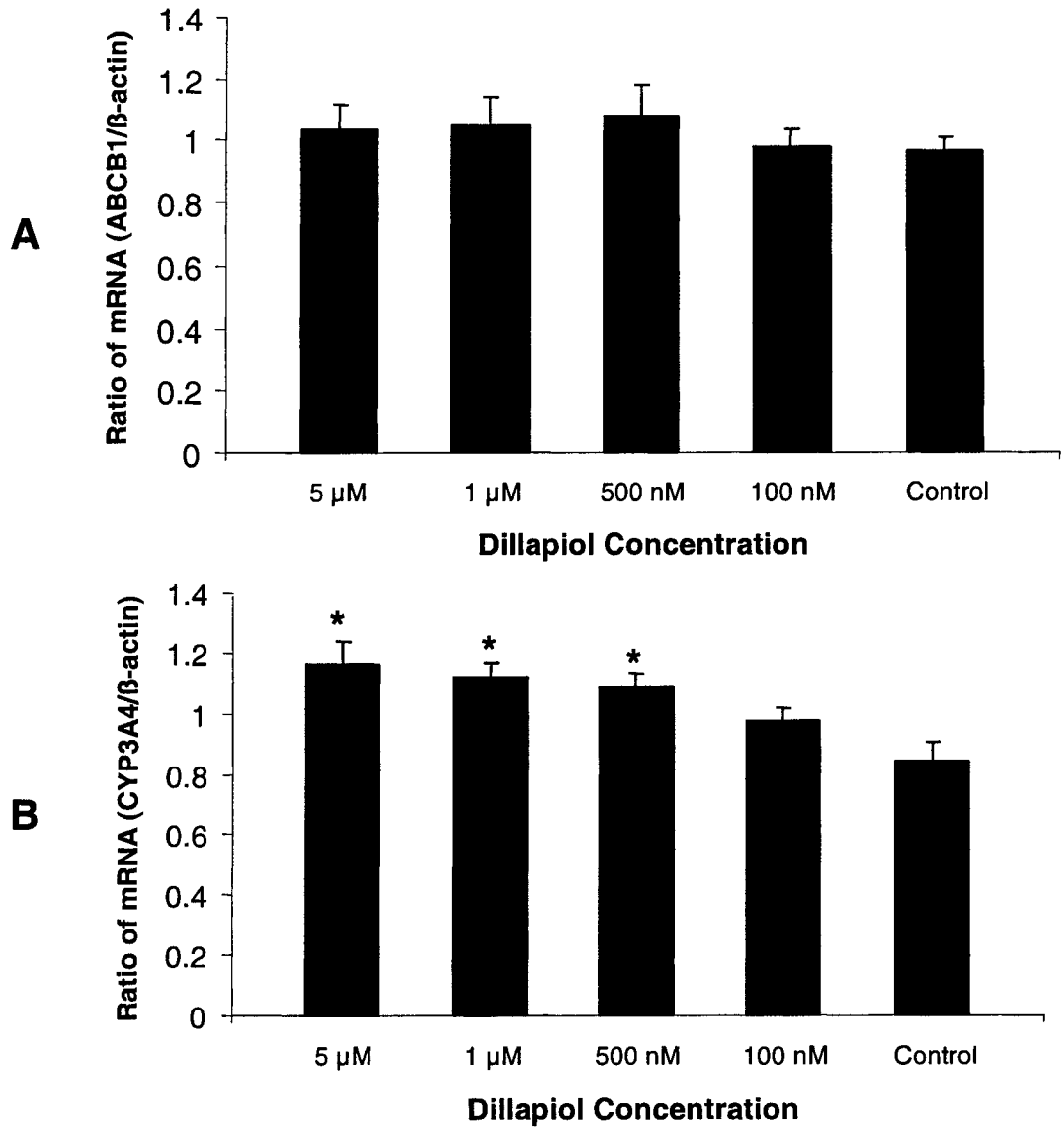


Figure 3.10: (**A**) ABCB1 and (**B**) CYP3A4 mRNA levels for caco2 cells treated with varying concentrations of dillapiol. Data represent the mean ratio of *ABCB1* or *CYP3A4* expression relative to ß-actin ± SEM ($N = 12$). Note that levels of CYP3A4 mRNA were up-regulated by concentrations of 5 µM, 1 µM, and 500 nM dillapiol, while levels of ABCB1 mRNA remained unchanged. Means were analysed via a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison ($p < 0.05$).

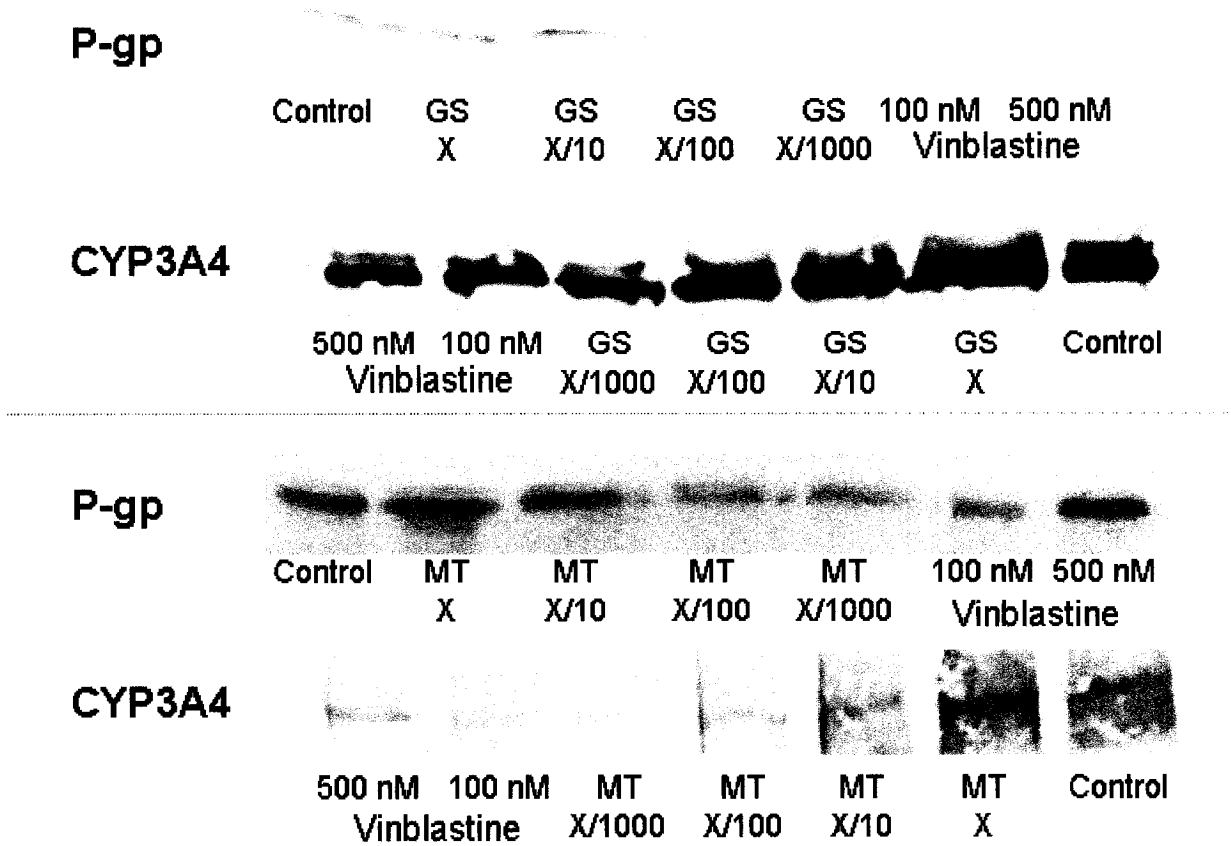


Figure 3.11: Representative Western blot membranes showing protein levels of P-gp and CYP3A4. Bands correspond to the pooled total protein ($N = 1$) recovered from the organic phase leftover during the total RNA isolation protocol. Bands correspond to caco2 cell treatments with aqueous extracts of Li Chung Yun goldenseal capsules (GS: NRP121; X = 6.75 mg/ml) or Swiss Herbal Remedies milk thistle capsules (MT: NRP126; X = 7.50 mg/ml).

Note: The experimental data derived from the Western blot analyses was done in collaboration with our laboratory's post-doctoral researcher Dr. Mitra Panahi. I provided her with the organic fractions collected during the various total RNA isolations and she performed the subsequent protein purification, immuno-blotting techniques, and densitometrical analysis. I use this data with her permission.

Table 3.1: Relative protein levels of P-glycoprotein and CYP3A4 isolated from caco2 cell treated with various herbal extracts or pure compounds. Protein levels from each experiment were pooled ($n = 6$; $N = 1$) and are compared to control values.

Product	Treatment Concentration	Relative Protein Level vs Control	
		P-glycoprotein	CYP3A4
Solaray goldenseal (X = 3.15 mg/ml)	GS X	0.18	0.32
	GS X/10	0.28	0.43
	GS X/100	0.37	0.5
	GS X/1000	2.62	0.46
Li Chung Yun goldenseal (X = 6.75 mg/ml)	GS X	1.65	1.26
	GS X/10	1.38	1.16
	GS X/100	0.79	0.88
	GS X/1000	0.59	0.72
Solaray milk thistle (X = 1.75 mg/ml)	MT X	2	0.38
	MT X/10	1.57	0.1
	MT X/100	1.92	0.56
	MT X/1000	3	1.08
Swiss Herbal Remedies milk thistle (X = 7.50 mg/ml)	MT X	1.16	1.7
	MT X/10	0.8	0.84
	MT X/100	0.58	0.7
	MT X/1000	0.5	0.49
Berberine	10 μM	1.02×10^{1}	1.64
	3 μM	1.26	1.14
	1 μM	4.32	0.42
	0.3 μM	1.12	0.47
Silibinin	10 μM	1.69	0.91
	3 μM	1.04×10^{1}	1.6
	1 μM	1.99	2.25
	0.3 μM	4.58	1.01

Table 3.1: continued...

Product	Treatment Concentration	Relative Protein Level vs Control	
		P-glycoprotein	CYP3A4
Vinblastine	1 μM	0.42	0.3
	0.5 μM	0.38	0.21
	0.1 μM	0.29	0.19
Saquinavir	5 μM	2.99	3.49
	1 μM	0.79	2.65
	0.5 μM	0.53	2.21
	0.1 μM	0.32	1.58
Dillapiol	5 μM	1.52	0.91
	1 μM	0.31	0.81
	0.5 μM	2.85	0.86
	0.1 μM	0.76	0.45

Note: The experimental data derived from the Western blot analyses was done in collaboration with our laboratory's post-doctoral researcher Dr. Mitra Panahi. I provided her with the organic fractions collected during the various total RNA isolations and she performed the subsequent protein purification, immuno-blotting techniques, and densitometrical analysis. I use this data with her permission.

Table 3.2: A qualitative summary relating mRNA expression for *ABCB1* and *CYP3A4* to basal levels (control), as well as their corresponding protein (p-glycoprotein and CYP3A4) for all experiments. Note: since protein levels were derived from pooled samples (n = 6; N = 1), "no change" (NC) was assigned to treatments falling within 25% of control levels.

Product	Treatment Concentration	mRNA Levels		Protein Levels	
		ABCB1	CYP3A4	P-glycoprotein	CYP3A4
Solaray goldenseal (X = 3.15 mg/ml)	GS X	NC	NC	decreased	decreased
	GS X/10	NC	NC	decreased	decreased
	GS X/100	NC	NC	decreased	decreased
	GS X/1000	NC	NC	increased	decreased
Li Chung Yun goldenseal (X = 6.75 mg/ml)	GS X	NC	increased	increased	NC
	GS X/10	NC	NC	increased	NC
	GS X/100	NC	decreased	decreased	NC
	GS X/1000	NC	NC	decreased	decreased
Solaray milk thistle (X = 1.75 mg/ml)	MT X	NC	NC	increased	decreased
	MT X/10	NC	decreased	increased	decreased
	MT X/100	NC	decreased	increased	decreased
	MT X/1000	NC	NC	increased	NC
Swiss Herbal Remedies milk thistle (X = 7.50 mg/ml)	MT X	NC	increased	NC	increased
	MT X/10	NC	NC	NC	NC
	MT X/100	decreased	decreased	decreased	decreased
	MT X/1000	decreased	decreased	decreased	decreased
Berberine	10 μM	NC	decreased	increased	increased
	3 μM	NC	NC	NC	NC
	1 μM	NC	NC	increased	decreased
	0.3 μM	NC	NC	NC	decreased

Table 3.2: continued...

Product	Treatment Concentration	mRNA Levels		Protein Levels	
		ABCB1	CYP3A4	P-glycoprotein	CYP3A4
Silibinin	10 μM	NC	decreased	increased	NC
	3 μM	NC	decreased	increased	increased
	1 μM	NC	decreased	increased	increased
	0.3 μM	NC	NC	increased	NC
Vinblastine	1 μM	NC	increased	decreased	decreased
	0.5 μM	NC	NC	decreased	decreased
	0.1 μM	NC	NC	decreased	decreased
Saquinavir	5 μM	NC	NC	increased	increased
	1 μM	NC	NC	decreased	increased
	0.5 μM	NC	NC	decreased	increased
	0.1 μM	NC	increased	decreased	increased
Dillapiol	5 μM	NC	increased	increased	NC
	1 μM	NC	increased	decreased	NC
	0.5 μM	NC	increased	increased	NC
	0.1 μM	NC	NC	decreased	decreased

CHAPTER 4: GENERAL DISCUSSION

4.1 Biological Relevance of The Preceding Studies

Berberine Concentrations

In the first study, several concentrations of aqueous goldenseal extracts (LCY-GS and Sol-GS) were analysed for their ability to inhibit the CYP3A4-mediated metabolism of the fluorescent substrate 7-RBE. Stock solution concentrations of goldenseal in this assay ranged from 100 mg/ml - 1.56 mg/ml, and the relative berberine content of these aqueous goldenseal extracts varied considerably (Table 2.3). In order to determine whether or not the berberine content in this experimental design is reflective of typical berberine plasma concentrations, one must compare the berberine content in the total reaction volume with other known experimental values. Consider the following calculations: (1) the IC_{50} values for LCY-GS and Sol-GS aqueous extracts were found to be 3.23 mg/ml and 3.03 mg/ml respectively; (2) in the overall reaction, each extract represented 5% of the total volume and therefore, the corresponding goldenseal concentration for each IC_{50} value would be 0.162 mg/ml (LCY-GS) and 0.152 mg/ml (Sol-GS); (3) HPLC analysis of aqueous LCY-GS extracts revealed a berberine content of 0.97 %, while Sol-GS aqueous extracts had a berberine content of 15.47% (Table 2.3); (4) therefore, the estimated corresponding berberine concentration in the reaction volume would be 1.57 μg/ml for LCY-GS aqueous extracts and 23.5 μg/ml for Sol-GS aqueous extracts. In their study involving patients with congestive heart failure, Zeng and Zeng (1999) found that the berberine plasma concentration in patients varied from 0.07 μg/ml to 27.1 μg/ml across a variety of conditions. Therefore, the berberine concentration in the extracts used in the CYP3A4 studies are reflective of typical plasma concentrations.

In the second study, caco2 cell monolayers were treated with various dilutions of aqueous

goldenseal extracts. The goldenseal extract concentrations ranged from 6.75 mg/ml (X) to 6.75 μg/ml (X/1000) for LCY-GS, and 3.15 mg/ml (X) to 3.15 μg/ml (X/1000) for Sol-GS. When using a similar calculation as outlined above, one can easily estimate the berberine content in the media for both extracts. LCY-GS berberine concentrations ranged from 65.5 μg/ml (X) to 65.5 ng/ml (X/1000), while Sol-GS berberine concentrations ranged from 487.3 μg/ml (X) to 487.3 ng/ml (X/1000). Therefore, both sets of extract dilutions encompass biologically relevant berberine concentrations as evidenced by the plasma concentrations determined by Zeng and Zeng (1999).

Silibinin Concentrations

Using similar calculations as those outlined for berberine determination, estimates of silibinin concentrations in the various studies are as follows: (1) for the CYP3A4 inhibition assays, aqueous SHR-MT extracts had an IC_{50} of 33.0 mg/ml, while aqueous Sol-MT extracts had an IC_{50} of 24.5 mg/ml. These correspond to silibinin concentrations of 1.65 μg/ml and 1.47 μg/ml respectively. (2) In the caco2 studies, the milk thistle extract concentrations ranged from 7.50 mg/ml (X) to 7.50 μg/ml (X/1000) for SHR-MT, and 1.75 mg/ml (X) to 1.75 μg/ml (X/1000) for Sol-MT. Therefore, estimates of media silibinin concentrations ranged from 7.50 μg/ml (X) to 7.50 ng/ml (X/1000) for SHR-MT, and 2.1 μg/ml (X) to 2.1 ng/ml (X/1000) for Sol-MT. In their review of milk thistle usage as a therapy for the treatment of liver disease, Flora *et al.* (1998) report typical maximal silibinin plasma concentrations ranging from 0.12 μg/ml to 0.34 μg/ml, dependent on the specific study and milk thistle dosage administered. Therefore, the extract dilutions utilized in the preceding studies encompassed biologically relevant silibinin concentrations.

NHPs and Drug Disposition

As demonstrated in the preceding studies, NHPs and protease inhibitors can influence the

same drug disposition pathways. However, the reason for the incidence of adverse drug reactions occurring with HIV/AIDS patients is multifactorial. Whereas many of these factors are strictly pharmacologic interactions or expected toxicity inherent in the drug classes, some are related to altered immunity (Dasgupta and Okhuysen, 2001). As well, numerous other factors can also influence the nature of these NHP-drug interactions. Some of these factors include: (1) variation in the quality of the product available. Herbal product crops can often vary dramatically in their levels of biomarkers and other secondary metabolites (which may include other active ingredients) due to genetic factors within the plant, environmental factors (Büter *et al.*, 1998) such as precipitation and growing conditions, and seasonal variations (Southwell and Bourke, 2001); (2) extrinsic factors influencing a patient, including such things as environmental influence (stress, climate, pollution, culture, socioeconomic factors, education, comedication, and nutrition); (3) intrinsic factors such as the physiological and pathological condition of a patient (age, cardiovascular function, presence of diseases, drug compliance, alcohol consumption, nutrition, smoking, and comedication); and (4) intrinsic factors within the patient related to differences in clinical and metabolic pharmacogenetics. For example, allelic variants exist for numerous detoxification isozymes and drug transporters. These allelic variants can have either an increased or decreased sensitivity to substrates resulting from changes in receptor sensitivity, receptor number, and signal transduction (Zühlsdorf, 1998). As well, the resorption, metabolism, and elimination of xenobiotics is mediated by numerous detoxification mechanisms acting simultaneously. It is important to remember that each drug disposition mechanism is part of a dynamic interplay in the *in vivo* state, and a xenobiotic may be a substrate for more than one metabolic enzyme or transporter. Thus, xenobiotics metabolized or transported by the same enzyme may interact with each other at the molecular level, potentially resulting in

complications when 2 or more substrates concomitantly compete for the same enzyme(s) or transporter(s) (Ameer and Weintraub, 1997). The result of such an interaction may be reduced metabolism by one enzyme, with a subsequent increase in metabolism or transport by another enzyme, thus altering the subsequent metabolic profile. Such interactions could potentially build up toxic levels of one or more of the products, or result in the shunting of the xenobiotics or their metabolites through alternate isozyme/transport pathways, thus adversely affecting the safety and efficacy of these compounds. Hence, a product that is GRAS and effective in the short term may eventually become toxic and/or ineffective with repeated administration over time, resulting in serious and deleterious clinical consequences. For example, if the change in efficacy results from decreased plasma and cellular levels in a person living with HIV, there will be a decreased therapeutic pressure on the virus that could ultimately lead to the development of viral resistance and increased viral load.

4.2 Hypotheses Validation and Future Research

For the first study (Chapter 2), I had hypothesized that extracts from commercial NHPs of goldenseal and milk thistle would be highly inhibitory of CYP3A4-mediated metabolism and active stimulators of P-gp, since the two plants are known to have constituents that affect CYP3A4 metabolism and are substrates for P-gp transport. These extracts were validated for the presence of these constituent marker compounds by HPLC and were subsequently found to exhibit moderate to high levels of CYP3A4 inhibition (the ranked IC_{50}s (Table 2.5) were as follow: [1] Sol-GS; [2] LCY-GS; [6] Sol-MT; and [7] SHR-MT). Similarly, both goldenseal and milk thistle teas had significantly higher P-gp ATPase activities than five other herbal teas and the positive control verapamil (20 μM) (Figure 2.4).

For the second study, I had hypothesized that the extracts of GS and MT would increase transcript levels for both genes. Clearly this was not the case. CYP3A4 expression was largely modulated by extracts of both NHPs, since both upregulation and downregulation was observed (Fig. 3.4 and Fig. 3.5). *ABCB1* was largely unresponsive in this cellular model (most likely because it is constitutive) and appears to be of no value as a biomarker under our experimental conditions.

In vitro models such as the ones described in this thesis are clearly useful for generating large amounts of high throughput data for determining the potential interaction of NHPs and therapeutic products at the level of drug disposition. Clearly, the important markers are (1) stimulation of P-gp ATPase activity; (2) inhibition of CYP3A4 activity, and (3) modulation of *CYP3A4* gene expression. However, selected products showing both high and low activity in any one of these assays need to be advanced to more detailed *in vivo* pharmacokinetic clinical studies in order to determine if the current *in vitro* results correlate to the *in vivo* state.

4.3 Summary

NHPs may contain potent mixtures of biologically active compounds which may individually or synergistically have significant levels of pharmacological activity and thus generate beneficial therapeutic effects and/or adverse reactions. Thus, based on their pharmacological actions, NHPs could either reinforce or weaken the effects of coadministered drugs (or vice versa) (Ernst, 2000). For example, a recent study conducted by Stermitz *et al.* (2000) highlighted the synergistic potential of berberine (isolated from *Berberis fremontii* in this case rather than *Hydrastis canadensis*). Briefly, they examined the antimicrobial potential of 5'-methoxyhydnocarpin (5'-MHC), a minor component of the traditional Native American remedy for leprosy called chaulmoogra oil. Alone, 5'-MHC had no antimicrobial activity but strongly potentiated the action of berberine against *Staphylococcus*

aureus. The multidrug resistance pump-dependent efflux of berberine from *S. aureus* was completely inhibited by 5'-MHC, and the resultant level of berberine in the cells was strongly increased in its presence. This indicated that the synergistic reaction of berberine with 5'-MHC effectively disabled the bacterial resistance mechanism. Other studies have revealed that on its own, berberine has photogenotoxic properties. Berberine is photosensitive and can intercalate with DNA, causing cytogenetic damage via the production of singlet oxygen radicals (Philogène et al., 1984).

However, despite issues such as these, the use of NHPs for medical purposes is certainly warranted. Plants contain numerous bioactive secondary metabolites and many drugs currently used today have been developed from plant sources. In fact, modern pharmacological medicine originally derived numerous beneficial and widely used drugs from medicinal plants, fungi, and microoorganisms. Examples include aspirin (salicylic acid; an anti-inflammatory and antipyretic from meadowsweet [*Filipendula ulmaria*]), digoxin (derived from foxglove [*Digitalis purpurea*] used in treating congestive heart failure, quinine (an antimalarial from Peruvian bark trees [*Cinchona* spp.], reserpine (an antihypertensive and antipsychotic from Indian snakeroot [*Rauwolfia serpentina*]), morphine (an analgesic from the opium poppy [*Papaver somniferum*]), vinblastine (an antineoplastic from the Madagascar periwinkle [*Catharanthus roseus*]), and verapamil (a calcium channel blocker from the opium poppy [*Papaver somniferum*]), to name but a few (Yarnell, 2000).

The goal of studies like the ones outlined in this thesis is risk assessment. It is important to predict which NHPs may lead to the occurrence of adverse events when administered concomitantly with other NHPs or therapeutics in order to maximize benefit of NHP use and minimize their risk Competent and intelligent herbal use can be extremely efficacious and effective, as evidenced by there long use in ethnopharmacologically-based traditional medicine. However, when considering

using NHPs, other forms of CAM, or therapeutic products, it is always important to remember the words of the Greek scholar Paracelsus (1493 -1541): "All substances are poisons: There is none which is not a poison. The right dose differentiates a poison and a remedy.".

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