PHARMACOKINETIC HERB-DRUG INTERACTION STUDY OF SELECTED TRADITIONAL MEDICINES USED AS COMPLEMENTARY AND ALTERNATIVE MEDICINE (CAM) FOR HIV/AIDS

By

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my original work, and that I have not previously in its entirety or part submitted to any University for a degree.

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ABSTRACT

Introduction

The increasing intake of traditional medicines among HIV/AIDS patients in sub-Saharan Africa needs urgent consideration by clinicians and other healthcare providers since the safety of such medications are unknown. The pharmacokinetic parameters - **A**bsorption, **D**istribution, **M**etabolism and **E**limination (ADME) play important role in the safety evaluation of drugs, thus implicating drug metabolizing enzymes and transporters as critical indicators for herb-drug interactions. The objective of this study was to evaluate the risk potential of seven herbal medicines commonly consumed by HIV/AIDS patients for drug interactions applying *in vitro* models. In this study, inhibition and induction effects of the herbal medicines on cytochrome P450s (CYPs) 1A2, 2C9, 2C19, 2D6 and 3A4 as well as P-glycoprotein (P-gp) were investigated.

Methods

Herbal medicines – *Lessertia frutescens, Hypoxis hemerocallidea, Kalanchoe integra* and *Taraxacum officinale* were sourced from Medico Herbs, South Africa were identified by experts from Compton Herbarium, South African National Biodiversity Institute, Cape Town. *Moringa oleifera*, *Echinacea purpurea* and *Kalanchoe crenata* were obtained from the repository of the National Centre for Natural Product Research (NCNPR), University of Mississippi, USA. Reversible inhibitory effect of aqueous and methanol herbal extracts were evaluated in recombinant CYPs applying the fluorescent metabolites at specified excitation/emission wavelengths; CYP1A2 (3-cyano-7-hydroxycoumarin (CHC); 405/460 nm), CYP2C9, CYP2C19 and CYP3A4 (7-hydroxy-4-(trifluoromethyl)-coumarin (HFC); 405/535 nm) and CYP2D6 (7-hydroxy-4-(aminomethyl)-coumarin (HAMC); 390/460 nm). Comparative studies in human liver microsomes (HLM) and recombinant CYPs were conducted to investigate the inhibitory effect of methanol herbal extracts and fractions on 6β testosterone hydroxylation activity. Time dependent inhibitory (TDI) effect of the herbal extracts were evaluated applying the IC_{50} shift fold, normalized ratio and the NADPH-, time- and concentration-dependent approaches. Influence of herbal extracts on metabolic clearance of testosterone was assessed in both HLM and human hepatocytes. The effects of each herbal extract on expression of CYP1A2, CYP3A4 and MDR1 genes were evaluated in activated human pregnane X receptor (PXR) co-transfected HepG2

cells. Finally, the inhibitory effect of herbal extracts on P-gp was assessed using the calcein-acetoxymethyl ester (calcein-AM) uptake and the digoxin radiolabelled substrates in MDCKII-MDRI cells.

Results

The aqueous extracts of *Moringa oleifera*, *Kalanchoe integra*, *Kalanchoe crenata*, *Echinacea purpurea* and *Lessertia frutescens* demonstrated high risk of *in vivo* inhibition on CYPs 3A4 and 1A2 with C_{max}/K_i >1.0. Methanol extracts of these herbal medicines also indicated potential risk of reversible drug interaction. The methanol extracts of *M. oleifera*, *K. crenata* and *L. frutescens* showed strong TDI effect on CYP3A4 with IC_{50} shift fold >1.5 and normalised ratio <0.7. *Moringa oleifera* intermediately reduced intrinsic clearance of testosterone in human hepatocytes ($2 \leq$ AUC ratio ≤ 5) when scaled up to humans. Methanol extracts of *Echinacea purpurea* up-regulated the expression of CYP1A2, CYP3A4 and MDR1 genes in activated PXR. *Kalanchoe crenata* and *Echinacea purpurea* indicated strong inhibition on P-gp by reducing transport of digoxin across hMDR1-MDCKII cell monolayer from basolateral to apical with IC_{50} values of 18.24 ± 2.52 µg/mL and 24.47 ± 4.97 µg/mL, respectively.

Conclusion

The herbal medicines especially *M. oleifera*, *K. integra* and *E. purpurea* have the potential to cause herb-drug interaction *in vivo* if sufficient hepatic concentration is achieved in humans.

ABSTRAK

Inleiding

Die verhoogde inname van tradisionele medisynes onder MIV/VIGS-pasiënte in sub-Sahara-Afrika verg dringend oorweging deur klinici en ander gesondheidsorgverskaffers, aangesien die veiligheid van sodanige medikasies onbekend is. Die farmakokinetiese parameters – **A**bsorpsie, **D**istribusie, **M**etabolisme en **E**liminasie (ADME) – speel ’n belangrike rol by die veiligheidsevaluering van geneesmiddels, en impliseer gevolglik geneesmiddel-metaboliserende ensieme en vervoerders as kritiese indikators vir krui-geneesmiddel-interaksies (HDI). Die oogmerk van hierdie studie is om die risikopotensiaal van sewe kruiemedisynes wat algemeen deur MIV/VIGS-pasiënte geneem word, vir geneesmiddel-interaksies te evalueer deur *in vitro*-modelle te gebruik. In hierdie studie is die inhiberings- en induseringsuitwerkings van die kruiemedisynes op sitochroom P450’s (verkort na CYP’s) 1A2, 2C9, 2C19, 2D6 en 3A4, sowel as P-glikoproteïen (P-gp), ondersoek.

Metodes

Kruiemedisynes – *Lessertia frutescens, Hypoxis hemerocallidea, Kalanchoe integra* en *Taraxacum officinale* – is van Medico Herbs, Suid-Afrika, bekom en deur kundiges van die Compton-herbarium, by die Suid-Afrikaanse Nasionale Biodiversiteitsinstituut, Kaapstad, geïdentifiseer. *Moringa oleifera*, *Echinacea purpurea* en *Kalanchoe crenata* is van die bewaarplek van die Nasionale Sentrum vir Natuurlike Produknavorsing (NCNPR) aan die Universiteit van Mississippi in die VSA verkry. Die omkeerbare inhiberende uitwerking van kruie-ekstrakte in water en metanol is in rekombinante CYP’s geëvalueer deur die gebruik van die fluoresserende metaboliete op gespesifiseerde opwekkings-/emissiegolflengtes; CYP1A2 (3-siaan-7-hidroksikumarien (CHC); 405/460 nm), CYP2C9, CYP2C19 en CYP3A4 (7-hidroksi-4-(trifluoormetiel)-kumarien (HFC); 405/535 nm) en CYP2D6 (7-hidroksi-4-(aminometiel)-kumarien (HAMC); 390/460 nm). Vergelykende studies van menslikelewermikrosome (HLM) en rekombinante CYP’s is uitgevoer om die inhiberende uitwerking van metanolkruie-ekstrakte en -fraksies op 6β-testosteroonhidroksileringsaktiwiteit te ondersoek. Die tydafhanklike inhiberende uitwerking (TDI) van die kruie-ekstrakte is geëvalueer deur gebruikmaking van die IC_{50}-verskuiwingsvou-, die genormaliseerdeverhoudings- en die NADPH-, tyd- en

konsentrasieafhanklike benaderings. Die invloed van kruie-ekstrakte op metaboliese testosteroonverheldering is in beide HLM en menslike hepatosiete geëvalueer. Die uitwerkings van elke kruie-ekstrak op die uitdrukking van CYP1A2-, CYP3A4- en MDR1-gene is in geaktiveerde menslike pregnaan-X-reseptor(PXR)-, ko-getransfekteerde HepG2-selle geëvalueer. Laastens is die inhiberende uitwerking van kruie-ekstrakte op P-gp geëvalueer, met gebruikmaking van die kalsien-asetoksimetiel-ester (kalsien-AM)-opname en die digoksien-radiogemerkte substrate in MDCKII-MDRI-selle.

Resultate

Die ekstrakte in water van *M. oleifera*, *K. integra*, *K. crenata*, *E. purpurea* en *L. frutescens* het ’n hoë risiko van *in vivo*-inhibering op CYP’s 3A4 en 1A2 met C_{maks}/K_i >1.0 getoon. Ekstrakte van hierdie kruiemedisynes in metanol het verder potensiële risiko van omkeerbare geneesmiddelinteraksie getoon. Die ekstrakte van *M. oleifera*, *K. crenata* en *L. frutescens* in metanol het sterk TDI-uitwerking op CYP3A4 met IC_{50}-verskuiwingsvou >1.5 en genormaliseerde verhouding <0.7 getoon. *M. oleifera* het intermediêre vermindering van intrinsieke testosteroonverheldering in menslike hepatosiete ($2 \leq$ AUC verhouding ≤ 5) tot gevolg wanneer die skaal na mense verhoog word. Ekstrakte van *E. purpurea* in metanol het die uitdrukking van CYP1A2-, CYP3A4- en MDR1-gene in geaktiveerde PXR opgereguleer. *K. crenata* en *E. purpurea* het sterk inhibering van P-gp getoon deur die vervoer van digoksien deur die hMDR1-MDCKII-selmonolaag van basolateraal tot apikaal met IC_{50}-waardes van onderskeidelik 18.24 ± 2.52 µg/mL en 24.47 ± 4.97 µg/mL te verminder.

Gevolgtrekking

Kruiemedisynes, veral *M. oleifera*, *K. integra* en *E. purpurea*, het die potensiaal om HDI *in vivo* te veroorsaak indien voldoende hepatiese konsentrasie by mense bereik word.

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I. **Awortwe C**, Manda VK, Avonto C, Khan SI, Khan IA, Walker LA, Bouic PJ, Rosenkranz B. In Vitro Evaluation of Reversible and Time-Dependent Inhibitory Effects of *Kalanchoe Crenata* on CYP2C19 and CYP3A4 Activities. Drug Metab Lett. 2015 Jan 18 (Appendix 1.1).

II. **Awortwe C**, Manda VK, Avonto C, Khan SI, Khan IA, Walker LA, Bouic PJ, Rosenkranz B (2014). Echinacea purpurea up-regulates CYP1A2, CYP3A4 and MDR1 gene expression by activation of pregnane X receptor pathway. *Xenobiotica*, 7:1-12 (Abstract in Appendix 1.2).

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IV. **Awortwe C**, Bouic PJ, Masimirembwa CM, Rosenkranz B **(**2014). Inhibition of major drug metabolizing CYPs by common herbal medicines used by HIV/AIDS patients in Africa- implications for herb-drug interactions. *Drug Metab Lett*, 7 (2):83-95 (Abstract in Appendix 1.4).

V. **Awortwe C**, Manda VK, Avonto C, Khan SI, Khan IA, Walker LA, Bouic PJ, Rosenkranz B (2015). *In Vitro* Evaluation of Reversible and Time-dependent Inhibitory Effects of *Kalanchoe crenata* on CYP2C19 and CYP3A4 activity. *Drug Metab Lett*, (Epub ahead of print).

VI. **Charles Awortwe**, Vamshi K. Manda, Shabana I. Khan, Larry A. Walker, Patrick J. Bouic, Bernd Rosenkranz. Modulatory effe*cts of se*lected herbal products use by HIV-infected patients in Africa on th*e* activity of cytochrome p450 enzymes and P-glycoprotein *in vitro. (Manuscript in preparation*).

AND abstract publications:

I. **Awortwe C**, Manda VK, Khan SI, Walker LA, Bouic PJ, Rosenkranz B (2014). Prediction of reversible and time-dependent inhibition potential of CYP3A4 by Lessertia frutescens fractions and possibility of herb-drug interactions with anti-HIV drugs. *Planta Med*, 80 - PU8 (Abstract in Appendix 1.5).

II. **Awortwe C**, Fasinu P, Bouic P, Masimirembwa C, Rosenkranz B (2014). Integration of parallel artificial membrane permeability assays (PAMPA) into high throughput screening of medicinal herbs for potential inhibition of drug metabolizing enzymeS: Focus on CYP1A2 and CYP 3A4 Basic & Clinical Pharmacology & Toxicology, 115 (Suppl. 1) (Abstract in Appendix 1.6).

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Articles written during, but not directly related to, this dissertation

I. Manda V, Dale OR, **Awortwe C**, Ali Z, Khan IA, Walker LA and Khan SI (2014). Evaluation of drug interaction potential of Labisia pumila (Kacip Fatimah) and its constituents. *Front Pharmacol, 5:178*.

II. **Awortwe C**, Asiedu-Gyekye IJ, Nkansah E, Adjei S (2014). Unsweetened natural cocoa has anti-asthmatic potential. *Int J Immunopathol Pharmacol*, 27(2):203-12.

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IV. Asiedu-Gyekye IJ, Antwi DA, **Awortwe C**, N'guessan BB, Nyarko AK (2014). Short-term administration of an aqueous extract of kalanchoe integra var. crenata (Andr.) Cuf

leaves produce no major organ damage in Sprague-Dawley rats. *J Ethnopharmacol*, 151(2):891-6.

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VII. Asiedu-Gyekye, I. J.; **Awortwe, C**.; Tagoe, N. A. B.; Antwi, D. A.; Adjei, S.;Edusei, D. I.; Benoit, N. B. K.; Amoateng, P.; Nkansah, E (2014). Preliminary investigation of the anti-asthmatic potential of *Kalanchoe integra* leaf extract using a model of allergic airway inflammation. *European J Medicinal Plants*, 4 (5): 542-562

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Abstract publications:

IX. Manda VK, Dale OR, **Awortwe C**, Ali Z, Khan IA, Walker LA, Khan SI (2014). *In vitro* assessment of Labisia pumila and its constituents for interactions with CYPs, P-GP and PXR. *Planta Med*, 80 - PU9

X. Fasinu P, **Awortwe C**, Bouic P, Rosenkranz B (2014). The inhibition of CYP1A2, 2C9, 2C19 and 3A4 activity by the extracts of popular medicinal herbs - implications for herb-drug interaction. *Basic & Clinical Pharmacology & Toxicology*, 115 (Suppl. 1).

XI. Fasinu P, **Awortwe C**, Bouic P, Rosenkranz B (2014). Inhibitory activity of three herbal products used in HIV/AIDS on major drug-metabolizing enzymes. *Basic & Clinical Pharmacology & Toxicology*, 115 (Suppl. 1).

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ACN	Acetonitrile
ADME	Absorption, Distribution, Metabolism and Elimination
ADR	Adverse drug reaction
AFB1	Aflatoxin B1
AFQ1	Aflatoxin Q1
AhR	Aryl hydrocarbon receptor
ARVs	Antiretroviral drugs
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BFC	7-benzyloxy-4-trifluoromethylcoumarin
BSA	Bovine serum albumin
CAR	Constitutive androstane receptor
cDNA	Complementary deoxyribonucleic acid
CEC	3-Cyano-7- ethoxycoumarin
CHC	3-Cyano-7-hydroxycoumarin
CL	Clearance
$CL_{h,b}$	Hepatic metabolic blood clearance
CL_{int}	Intrinsic clearance
Cmax	Maximum serum concentration
CYP	cytochrome P450
DDI	Drug- drug interactions
DEX	Dextromethorphan
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	Concentration of crude herbal extracts that gives 50% of maximum Response of the enzyme/transporter activity
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FDA	US Food and drug administration
GIT	Gastrointestinal tract
HAMC	3-[2-(diethylamino)ethyl]-7-hydroxy-4-methylcoumarin
HDI	Herb-drug interaction
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HepG2	Human hepatoma cell line
HFC	7-hydroxy-4-trifluoromethylcoumarin
HIV	Human immunodeficiency virus
HLM	Human liver microsomes
HPLC	High performance liquid chromatography
HPRT	Hypoxanthine-guanine phosphoribosyltransferase

HTS	High throughput screening
IC_{50}	Concentration of crude herbal extracts that causes 50% inhibition of the enzyme/transporter activity
k	Elimination rate constant
K_i	Inhibitor constant (reversible inhibition)
k_I	Inhibition constant (time-dependent inhibition)
k_{inact}	Maximal rate of enzyme inactivation (time-dependent inhibition)
k_{obs}	Inactivation rate
K_m	Substrate concentration at half maximal velocity (Michaelis-Menten constant)
KTZ	Ketoconazole
M	Molar
MAMC	7-methoxy-4-(aminomethyl)-coumarin
MBI	Mechanism-based inhibition
MDCKII	Madin-Darby canine kidney II cells
MDR1	Multidrug-resistant protein 1/ P-glycoprotein
MFC	7-methoxy-4-trifluoromethylcoumarin
MIC	Minimum inhibitory concentration
M-M	Mechaelis-Menten
mRNA	Messenger ribonucleic acid
MRP2	Multidrug Resistance Protein 2
MTT	Methyl thiazolyl tetrazolium
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCE	New chemical entity
NCNPR	National Center for Natural Products Research
NNRTI	Non-nucleoside reverse-transcriptase inhibitor
NSAID	Non-steroidal anti-inflammatory drug
nmol	Nanomolar
OATP	Organic anion-transporting polypeptides
OCT	Organic cation transporters
OMP	Omeprazole
OTC	Over-the-counter
PBS	Phosphate buffered saline
PAMPA	Parallel Artificial Membrane Permeability Assay
PCR	Polymerase chain reaction
PEPT	Peptide transporter 2
PIs	Protease inhibitors
P-gp	P-glycoprotein
pmol	Picomolar
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
rCYP	Recombinant cytochrome P450
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SJW	St. John’s wort
SLC	Solute carrier

SLCO	Solute carrier organic anion
SSRI	Selective serotonin reuptake inhibitor
TDI	Time-dependent inhibition
TEER	Transepithelial electrical resistance
THPs	Traditional Health Practitioners
$T_{1/2}$	Half- life
TRIS	Tris(hydroxymethy)aminomethane
TST	Testosterone
UNAIDS	Joint United Nations Programme on HIV/AIDS
UV	Ultraviolet
V/F	Apparent volume of distribution
V_0	Apparent (measured) uptake velocity
V_{max}	Maximum velocity
WHO	World Health Organization
µg	Microgram
µl	Microliter
µmol	Micromolar
[I]	Inhibitor concentration
[S]	Substrate concentration

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CHAPTER ONE

1. INTRODUCTION

Traditional medicines and products are used globally for treatment of diverse pathological conditions. The World Health Organisation (WHO) defines traditional medicine as herbal medicines, animal parts and minerals used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 2002). Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products, which contain active ingredients from parts of plants or other plants materials or combinations. According to a UNAIDS estimate, one-third of the adults in developed countries and over 80% of Africans depend on herbal medicines for treatment of common ailments such as cold, inflammatory disorders, heart disease, diabetes, and central nervous system diseases (UNAIDS, 2006). In developed countries, people access these medicines/health supplements through the mushrooming natural products/food supplements outlets in major cities of the world (Verma and Singh, 2008). In developing countries, people consult traditional health practitioners (THPs) who live within the communities, have been trusted over years, share identical cultural and spiritual beliefs and are ever willing to assist patients with their knowledge and skills sometimes at a reduced cost (Peltzer 2000; UNAIDS, 2006; Peltzer 2008). Other preparations of herbal medicines are available at chemical and pharmacy shops as over-the-counter (OTC), which can be consumed without the recommendation or advice of a physician (Obach, 2000). They are usually sold as nutritional supplements with implied benefits in the various disease conditions or maintenance of health without specific medicinal claims (Mathes *et al*., 2010; Serafini *et al*., 2012).

In recent years, with increased burden of HIV/AIDS especially in Africa, an overwhelming proportion of infected individuals patronize herbal medicines. Tshibangu *et al* (2004) reported that, in South Africa, herbal medicines are good supplements to antiretroviral therapies because of their immune boosting properties. Herbal medicines are used in HIV-infected patients primarily for the perceived additional anti-viral and immune boosting effects, improvement of general well-being, and feeling of control over the pathological condition. Most clinicians are unaware of the concurrent use of herbal medicines with the conventional antiretroviral drugs

(ARVs) or other treatments by their patients. Such practise has raised concern of the possible risk of adverse drug reactions (ADRs) due to the herbs alone or possible unfavorable effect on the safety and efficacy of the ARVs as a consequence of herb-drug interactions (HDIs) (Hussain, 2011).

The major pathways for HDI are via the cytochrome P450 enzymes (CYPs) and the drug transporters. Herb-induced inhibition or induction of CYPs can alter the metabolism of ARVs, leading to lack of efficacy or toxicity which could be lethal. For example, a case study in two HIV-infected patients on ritonavir indicated severe gastrointestinal toxicity after co-administration with garlic supplement for over two weeks. The symptoms however, re-occurred when the patients were given low-dose of ritonavir, suggesting possible interaction between garlic and ritonavir, through inhibition of CYP3A4 and induction of P-gp (Gallicano *et al*., 2003). Another case study in five HIV-infected patients on both nevirapine (NVP) and St. John's wort (SJW), reported reduced bioavailability of the NVP (de Maat *et al*., 2001). SJW was also demonstrated to significantly reduce exposure levels and increase the clearance of the protease inhibitor, indinavir with high risks for failed treatment in such patients (Piscitelli *et al*., 2001). These findings have led to revisions of the ARV labels where the use of SJW is not recommended in patients on ARVs (Clauson *et al*., 2008). Further pharmacokinetic and enzymological investigations indicated that SJW induces CYP3A4 (Izzo and Ernst, 2009), an enzyme responsible for metabolism of NVP and protease inhibitor.

For conventional drug discovery and development, the mechanism of many drug-drug interactions (DDI) has been shown to be through the inhibition or induction of major drug metabolising enzymes, in particular CYPs, or transporters such as P-glycoprotein. The United States Food and Drug Administration (US FDA) has therefore published guidelines on the evaluation of new chemical entities for risk of metabolism-based DDI (Zhang *et al*., 2009). With the increased use of herbal medicines in developed countries and reports of *in vivo* HDIs on mechanism similar to those of conventional DDI, research started to adopt the FDA guidelines for the *in vitro* evaluation of herbal medicines for risks of HDI (Modarai *et al*., 2007). Whilst there is a large panel of CYPs and drug transporters important for drug metabolism and potential DDI, most studies currently conduct the first screen on the 5 major CYPs (CYP1A2, 2C9, 2C19, 2D6 and 3A4) and P-glycoprotein responsible for the metabolism of over 90% of the drugs in the

market (Yang *et al*., 2004; Paine *et al*., 2006). This study is thus designed to evaluate the risk potential of selected herbal medicines (n=7) used by HIV/AIDS patients for treatment of related infections and other comorbidity to cause HDIs *in vitro*, focusing on the five major drug metabolizing enzymes and P-gp.

1.1. Overview of drug metabolism

The human body is constantly exposed to wide ranges of chemicals found in foods, air, and drugs used for treatments of diseases. The majority of these chemicals are not required for normal physiological function and may be deleterious. The body has an array of enzyme-catalyzed processes to eradicate these invading foreign substances known as xenobiotics.

There are basically two major chemical pathways for the metabolism of xenobiotics: phase I and phase II. Phase I reactions involve functionalization of lipid-soluble drug via addition of a polar group. Oxidative metabolism is considered the most important aspect of phase I although other processes such as reduction and hydrolysis do occur. The cytochrome P450 monooxygenases (CYPs) group of enzymes is principally responsible for drug oxidation which forms the crux of this study. Phase II reactions involve conjugation of endogenous substances such as glucuronic acid to the phase I generated polar compound forming water-soluble product suitable for excretion.

1.2 Cytochrome P450 enzymes (CYPs)

The capacity of mammalian tissues to oxidise lipid-soluble xenobiotics was widely recognized in the early 1950s. Nevertheless, the specific enzymes responsible for the catalysis of such reactions were unknown at that time. The main evidence of the enzyme likely to oxidize xenobiotics originated from spectroscopic studies of microsomal heme proteins. In 1955, Axelrod and Brodie *et al*., identified an enzyme in the endoplasmic reticulum of the liver capable of oxidizing xenobiotic compounds. Garfinkel and Klingenberg in 1958 detected a carbon monoxide (CO) binding pigment in the liver microsomes which had an absorption maximum at 450 nm. Later in 1964, the pigment was demonstrated to be a hemeprotein of the b-type class which was named Cytochrome P450 (CYP) after a strong feature in its absorption spectrum (Omura and Sato, 1964a and 1964b). Further investigation employing an electron spin resonance spectroscopy revealed that CYP is a low spin ferric hemeprotein with a thiol residue as an axial

heme ligand. This led to the explanation of the unusual Soret peak position and related perturbation after substrate complex formation and other chemicals in terms of charge transfer modulated by Fe-S bond (Bayer *et al*., 1969; Hill *et al*., 1970a; Hill *et al*., 1970b).

CYP enzymes are now known as a family of heme containing proteins with an iron protoporphyrin IX non-covalently bound to the apoprotein as the prosthetic group. The prosthetic group is confined between the distal and proximal helix and bound to the adjacent Cysteine-heme ligand loop. The proximal Cysteine forms double hydrogen bounds with the neighboring backbone amides. Variations in the protein components of the enzyme among members of the superfamily account for the different properties such as substrates and products specificity (Ricoux *et al*., 2007).

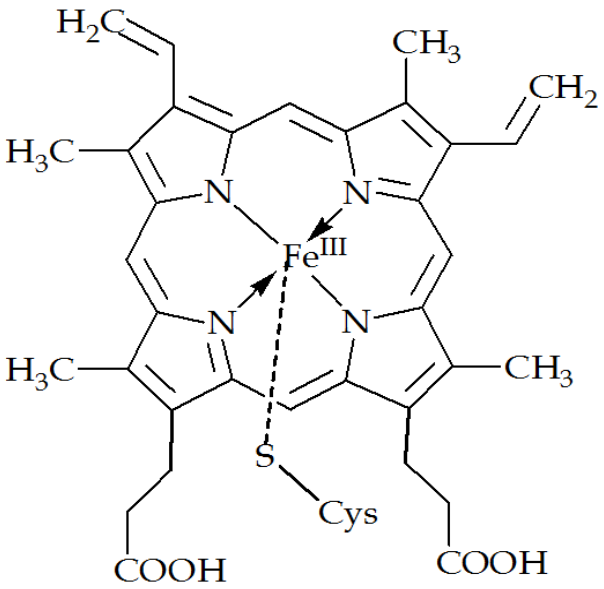


Figure 1.1 Prosthetic of cysteinato-heme enzymes**:** an iron (III) protoporphyrin-IX linked with a proximal cysteine ligand (adapted from Hu *et al*., 2011).

The mammalian CYPs are chiefly located in the endoplasmic reticulum and to a lesser extent in the mitochrondria, nuclei and lysosomes. It is predominantly found in the liver, but present in all mammalian cells except matured red blood cells and skeletal muscles. Unlike mammalian cells, bacteria CYPs are non-membrane bound and are located in the soluble fraction of the cytoplasm.

1.2.1 Cytochrome P450 nomenclature

A standardized system of CYPs nomenclature was established to name and assign individual genes into families and subfamilies (Danielson, 2002). The system of CYPs nomenclature was determined by the P450 Nomenclature Committee based on the level of amino acid sequence identity, phylogenetic association and gene organization (http://drnelson.utmem.edu/CytochromeP450). The root for all CYP genomic and cDNA sequence names is represented as CYP. The individual family is then designated by an Arabic numeral (1, 2, 3, …), the subfamily with a letter (A, B, C, …) and another Arabic numeral (1, 2, 3, …) for the respective protein. CYP sequences in the same family must display greater than 40% identical amino acid. Those belonging to the same subfamily exhibit greater than 55% identical amino acids and more than 97% identical amino acids represent the same allele except there are proof to contrary (Nebert *et al*., 1991; Nelson *et al*., 1993). An example of CYP nomenclature is illustrated in Figure 1.2.

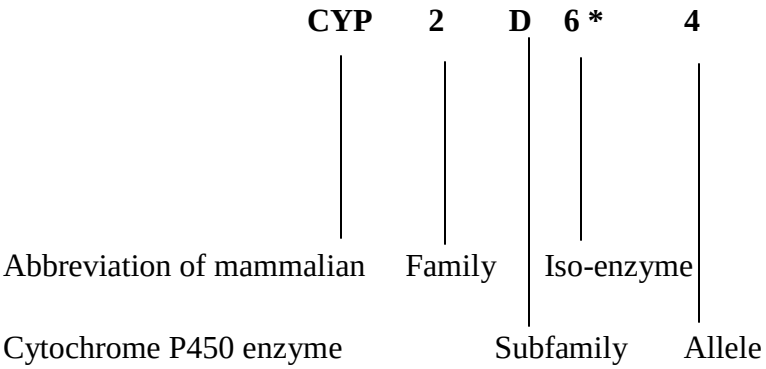


Figure 1.2 Nomenclature of CYPs (adapted from Wijnen *et al*., 2007). The asterisk (*) refers to a polymorphic gene.

1.2.2 Enteric and hepatic distribution of CYPs

The principal organ for elimination of xenobiotics, the liver, has the highest concentration of the CYPs (Lin and Lu, 2001). A systematic characterization of the five major CYPs in 60 human donor livers and anti-CYP antibodies reported the CYP3A subfamily (i.e. CYP3A4+CYP3A5) as the most abundant with 30% of the total CYP content. The CYP2C subfamily (i.e. CYP2C8+CYP2C9+CYP2C18+CYP2C19) is ranked second (18%), followed by CYP1A2

(15%), CYP2E1 (10%), CYP2A6 (5%), CYP2D6 (5%), and CYP2B6 (5%) (Paine *et al*., 2006) as depicted in Figure 1.3.

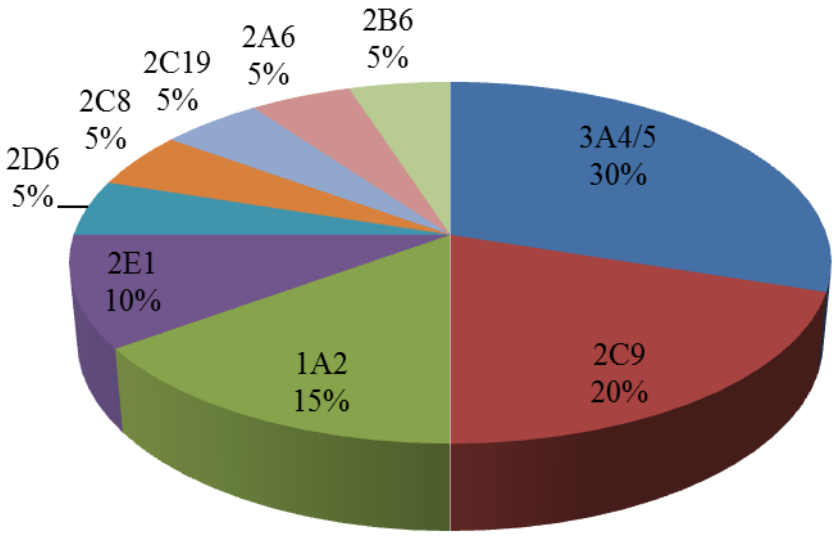


Figure 1.3 Major hepatic CYPs involved in drug metabolism.

Additionally, the CYPs are well expressed in small intestinal mucosa, lung, kidney, brain, olfactory mucosa, and skin. Nevertheless, the intestinal mucosa remains the most relevant extrahepatic site of drug biotransformation. Consequently, the presystemic biotransformation and subsequent reduction in bioavailability may occur as drugs pass sequentially through the small intestine and liver.

In the small intestine, the CYP3A4 is the most abundant CYP subfamily with 50 – 70% average specific content (Watkins, 1987; Paine, 1997). The enteric CYP3A4 is localized within the enterocytes which form the major part of the mucosa lining (Kolars, 1994). The content of enteric microsomal CYP3A and its catalytic activity decline from the proximal region towards the distal ileum (Zhang *et al*., 1999). Unlike CYP3A, the abundance and importance of other CYPs in the small intestine remain unclear. Systematic characterization of the inter-individual variation in the specific contents of CYPs in microsomal preparation from 31 human donors small intestines showed that CYP2C9 represent the second largest proportion followed, by

CYP2C19, CYP2J2 and CYP2D6 (Paine *et al*., 2006). Therapeutic effects of orally administered drugs metabolized via the major hepatic and enteric CYPs; CYP3A and CYP2C9 pathways are likely to be reduced due to presystemic biotransformation. Hence proper dose adjustment of such drugs must be done to avoid inefficacy and presystemic toxicity.

1.2.3 Role of CYPs in xenobiotic metabolism

Plants and animals are constantly engaged in biological warfare. Each develops a unique approach to defend itself against possible invasion from the opponent. Plants use three major defensive strategies including physical protection, toxic secondary metabolites and metabolic defenses. Most plant secondary metabolites act as pro-drugs in animals which need to be metabolized by CYPs to deploy their toxic effects in the body. Some plants reduce detoxification of secondary metabolites through inhibition of efflux pumps (phase III proteins) (Stermitz *et al*., 2000). Conversely, animals have developed protective mechanisms against chemical toxins produced by plants (McLean and Duncan, 2006). The metabolism of xenobiotics via CYPs to increase detoxification represent the most single effective approach used by animals to cope with toxic influences from their environment. The warfare between possum and *Eucalyptus* plants is a classic example of an effective animal detoxification system. Possums ingest doses of about 10 grams of 1, 8-cineole per day which could be quite poisonous in human beings. Compared with humans, cineole is rapidly metabolised by possum liver microsomes to less toxic compounds especially after induction through prior terpene exposure (Pass *et al*., 2001)

Human beings use a similar defensive mechanism to detoxify xenobiotics. It is estimated that approximately 66% of marketed drugs are metabolized by the CYP enzyme system (Rendic and Di Carlo, 1997; Guengerich, 2003; Williams *et al*., 2004). Five of the human genes coding for CYPs (1A2, 2C9, 2C19, 2D6 and 3A4) account for 95% metabolic clearance of drug and xenobiotics. This account however varies from the liver to the intestine and other extrahepatic metabolism as summarized in Table 1.1.

Table 1.1 Hepatic and enteric abundances of CYP isoforms. Table obtained from (Rostami-Hodjegan and Tucker, 2007; Pain, 2006).

P450 isoform	Mean abundance in liver (pmol/mg)	Mean abundance in human intestine (pmol/mg) (%)	Contribution to metabolism in marketed drug (%)
CYP1A1	ND	5.6 (7.43%)	
CYP1A2	37 (10.63%)		9
CYP2A6	29 (8.33%)		
CYP2B6	7 (2.01%)		2
CYP2C8	19 (5.46%)		
CYP2C9*	60 (17.248%)	8.4 (11.14%)	16
CYP2C19*	9 (2.59%)	1.0 (1.33%)	12
CYP2D6*	7 (2.01%)	0.5 (0.66%)	12
CYP2E1	49 (14.085%)		2
CYP2J2		0.9 (1.19%)	
CYP3A4	131 (37.64%)	43 (57.03%)	46
CYP3A5		16 (21.22%)	

CYPs with asterisk (*) are polymorphic genes and ND refers to not determined.

1.2.3.1 Role of CYP1A2 in drug metabolism

Catalytic activities of CYP1A enzymes include hydroxylation and oxidative biotransformation of polycyclic aromatic hydrocarbons and other aromatic substances. CYP1A1 prefers planar aromatic hydrocarbons whilst CYP1A2 favours metabolic clearance of aromatic amines and heterocyclic compounds. The classical biotransformation reactions catalysed by CYP1A2 include 7-ethoxyresorufin O-deethylation, phenacetin O-deethylation, and caffeine N3-demethylation (Fuhr *et al*., 2007; Zhou *et al*., 2009). The relatively high expression of CYP1A2 shows the importance of its metabolic clearance of clinically important drugs. Therapeutic agents metabolised by CYP1A2 include; analgesics and antipyretics (acetaminophen, phenacetin, lidocaine), antipsychotics (olanzapine, clozapine), antidepressants (duloxetine; Lobo *et al*., 2008), anti-inflammatory drugs (nabumetone; Turpeinen *et al*., 2009), cardiovascular drugs (propranolol, guanabenz, triamterene), the cholinesterase inhibitor tacrine used for the treatment of Alzheimer's disease, the muscle relaxant - tizanidine (Granfors *et al*., 2004), the hypnotic - zolpidem used in the short term treatment of insomnia, the drug riluzole used to treat

amyotrophic lateral sclerosis, the 5-lipoxygenase inhibitor - zileuton. Endogenous substrates of CYP1A2 comprise arachidonic acid, prostaglandins, oestrogens, melatonin and retinoic acid (Nebert & Dalton, 2006). Additionally, CYP1A2 is responsible for the bioactivation of procarcinogens like arylarenes, nitroarenes, and arylamines, found in charbroiled food and industrial combustion products. The reactive and carcinogenic products intercalates with DNA to cause permanent damage which may lead to generation of cancerous cells. Substrates of CYP1A2 are prone to drug interactions by small molecules that reversibly or irreversibly fit the active site or through the Aryl hydrocarbon receptor (AhR)-mediated gene induction. Examples of potent inhibitors are α-naphtoflavone (K_i<50 nM) and the selective serotonin reuptake inhibitor (SSRI), fluvoxamine (K_i~0.2 μM; Hiemke & Härtter, 2000).

1.2.3.2 Role of CYP2C9 and CYP2C19 in drug metabolism

CYP2C9, the major isoform of CYP2C accepts weakly acidic drugs including the anticoagulant warfarin, the anticonvulsants phenytoin and valproic acid, the angiotensin receptor blockers candesartan and losartan, oral antidiabetics like glibenclamide and tolbutamide, and nonsteroidal anti-inflammatory drugs (NSAIDs) (Lee *et al*., 2002). Diclofenac and tolbutamide are common substrates employed in CYP2C9 phenotyping. CYP2C9 is inhibited by several xenobiotics which can clinically influence warfarin treatment (Lu *et al*., 2008).

The discovery of CYP2C19 as genetic polymorphic CYP enzymes has led to the classification of S-mephenytoin phenotype into poor and extensive metabolizers (Küpfer and Preisig, 1984). It is the primary enzyme for the metabolic inactivation of proton pump inhibitors including omeprazole and pantoprazole, and for the metabolic activation of the anticoagulant clopidogrel to the active 2-oxo metabolite (Hulot *et al*., 2006; Kazui *et al*., 2010; Dansette *et al*., 2011; Boulenc *et al*., 2012). CYP2C19 also plays an important role in the metabolism of several antidepressants of the first and second generation (Brøsen, 2004).

1.2.3.3 Role of CYP2D6 in drug metabolism

Although CYP2D6 has a low relative abundance in the liver, it is responsible for the metabolism of large number of drugs including approximately 15-25% of clinically used drugs. Therapeutic

classes of drugs metabolised by CYP2D6 include; antiarrhythmics (e.g. propafenone, mexiletine, flecainide), tricyclic and second generation antidepressants (e.g. amitriptyline, paroxetine, venlafaxin), antipsychotics (aripiprazole, risperidone), β-blockers (bufuralol, metroprolol), anti-cancer drugs (selective estrogen receptor modifier (SERM) tamoxifen), opioid analgesics (codeine and tramadol) (Zanger *et al*., 2008; Stingl *et al*., 2012). Clinically, the phenotype of CYP2D6 is determined using selective test drugs such as debrisoquine, dextromethorphan, metoprolol, sparteine, and tramadol (Frank *et al*., 2007).

Two independent scientists Eichelbaum *et al*., (1979) and Smith, (1986) discovered the genetic deficiency of debrisoquine and spartein metabolism. The study on debrisoquine, a sympatholytic antihypertensive drug observed a pronounced hypotensive response in one group compared to others. This was found to be due to impaired 4-hydroxylation of debrisoquine (Smith, 1986). An independent research conducted by a team of physicians in Bonn around the same time observed increased side effects associated with decreased oxidative metabolism of sparteine, an oxytocic and antiarrythmic alkaloid (Eichelbaum *et al*., 1979; Smith, 1986). Population studies were conducted for both debrisoquine and spartein with determination of the urinary metabolic ratio (MR). Individuals with MR greater than 12.6 and 20 were categorised as poor metabolisers of debrisoquine and spartein, respectively for ratio of debrisoquine to 4-OH-debrisoquine and of sparteine to 2- and 5-dehydrospartein (Evans *et al*., 1980; Eichelbaum *et al*., 1986).

These two studies gave recognition to CYP2D6 as a polymorphic enzyme with different phenotypes in a given population. The frequency of phenotype of poor metabolisers differs among the various ethnic groups. Less than 1% of Asians, 2-5% of African-Americans, and 6-10% of Caucasians are poor metabolisers of CYP2D6 (Kalow *et al*., 1980). The CYP2D6*3, *4, *5 and *6 are the common variant alleles found in Caucasians which accounts for about 98% of poor metabolisers.

The Asian and African-American populations tend to have reduced CYP2D6 activity compared with Caucasians due to a lower occurrence of non-functional alleles (e.g., *3, *4, *5,*6), but a higher frequency of alleles associated with reduced activity (e.g., *10, *17) (Wang *et al*., 1993).

In *in vitro* assays, bufuralol is commonly used as probe substrates of CYP2D6. Quinidine and methadone with high affinity for CYP2D6 inhibit the enzyme even though they are not substrates (Gelston *et al*., 2012).

1.2.3.4 Role of CYP3A in drug metabolism

The CYP3A subfamily enzymes metabolize ~30% of clinically used therapeutic drugs (Bu, 2006; Liu *et al*., 2007; Zanger *et al*., 2008). This is attributed to the large and flexible active site of CYP3A4 capable of accommodating and metabolizing many lipophilic compounds with comparatively large structures (Scott and Halpert, 2005; Hendrychová *et al*., 2011). Typical large substrates are immunosuppressants like cyclosporine A and tacrolimus, macrolide antibiotics like erythromycin, and anticancer drugs including taxol. However, smaller molecules including ifosfamide, tamoxifen, benzodiazepines, several statins, antidepressants, opioids are also substrates. CYP3A4 is also known as an efficient steroid hydroxylase which catabolizes several endogenous steroids namely testosterone, progesterone, androstenedione, cortisol and bile acids. Typical probe drugs used to measure general CYP3A activity include; midazolam, testosterone, erythromycin, alprazolam, and dextromethorphan (Fuhr *et al*., 2007; Liu *et al*., 2007).

Because CYP3A4 and CYP3A5 share > 85% identical primary amino acid sequence, there is high similarity in substrate selectivity between the isoforms (Williams *et al*., 2002). However, few substrate variations have been reported. Aflatoxin B_1 (AFB1) 3α- hydroxylation to AFQ1 is solely catalyzed by CYP3A4 and results in detoxification and subsequent elimination of AFB_1, whereas CYP3A5 converts it to the genotoxic aflatoxin B(1)-8, 9-epoxide (AFBO) (Kamdem *et al*., 2006).

1.2.4 CYP catalytic cycle

The CYP cycle usually starts with P450 iron in the ferric state. Although the catalytic cycle has been presented here in stages, it is noteworthy to mention that these reactions do not necessarily occur in the order discussed. In some cases, substrate binding and dissociation can occur with some ferrous P450. Also, some CYPs assume the ferrous resting state in the highly reducing

environment in cells with low oxygen tension. The general stepwise catalytic cycle for the CYP reactions are presented from step 1-9:

Step 1: Substrates bind to the active or peripheral site of the CYPs. The rate of substrate binding is considered to be a relatively rapid, univalent process especially for CYPs with relatively small binding sites such as CYP2A6 in humans. For such enzymes, the CYP protein undergoes rapid conformational changes allowing for substrate binding. The CYP2A6 substrate imbeds deeply into the active site, therefore the protein must be opening and closing rapidly. The rate of substrate binding to CYPs with large active sites like CYP3A4 and CYP1A2 is comparatively slow. Here, the substrate binds to the peripheral sites of the CYP followed by a conformational change that moves the substrate closer to the heme. The substrate displaces axial water molecule upon binding to the CYP active site. Consequently, the displacement of the iron from the plane of porphyrin ring at the resting state increases. This makes the heme better electron sink and activates an electron transfer from the reductase protein.

Step 2: After substrate binding, the iron (III) reduces to the ferrous state via electron transfer. NADPH donates electron to CYP through the accessory flavoprotein NADPH-P450 reductase, and progressive electron flow from the reductase prosthetic group FAD to FMN to the CYP.

Step 3: Triplet dioxygen reacts with ferrous CYP to produce a stable dioxygen adduct. Iron (III)-oxygen bond formation occurs via donation of an electron each from the iron (III) center and the triplet oxygen, respectively. Although the iron (III)-oxygen complex is relatively stable, it can dissociate to an iron (III) and superoxide anion at room temperature. The release of superoxide within the enzyme is accompanied by decoupling (uncoupling) reaction characterized by disproportionation and generation of a harmful radical, hydrogen peroxide.

Step 4: It is a relatively slow step which generates negative charge iron (III)-peroxo complex which undergoes rapid deprotonation to generate iron (III)-hydroperoxo complex. The unprotonated iron (III)-peroxo complex is a stronger nucleophile than the corresponding iron (III)-hydroperoxo complex. However, both entities participate in CYP-catalysed reactions involving generation of nucleophilic oxidant intermediates. Oxidative decarbonylation of

aldehydes and the final stage of the aromatization of the cycle A of androstenedione in the biosynthesis of estrone are classical examples of such intermediate nucleophilic oxidants.

Step 5: Iron (III)-peroxo is protonated to produce P450-Fe^{III}-OOH intermediate which behaves as nucleophile.

Step 6: The terminal oxygen atom of Fe^{III}-OOH entity is further protonated to produce iron (V)-oxo species and water molecule. The O-O bond in Fe^{III}-OOH undergoes heterolytic cleavage by protonation to generate compound I or iron (V)-oxo which is considered as the main reactive species of the catalytic cycle.

Step 7-9: Abstraction of hydrogen or electron occurs. Oxygen is inserted to generate hydroxylated species through the oxygen rebound mechanism takes place. The substrate is finally released. The catalytic cycle for CYP activity is summarized in Figure 1.4.

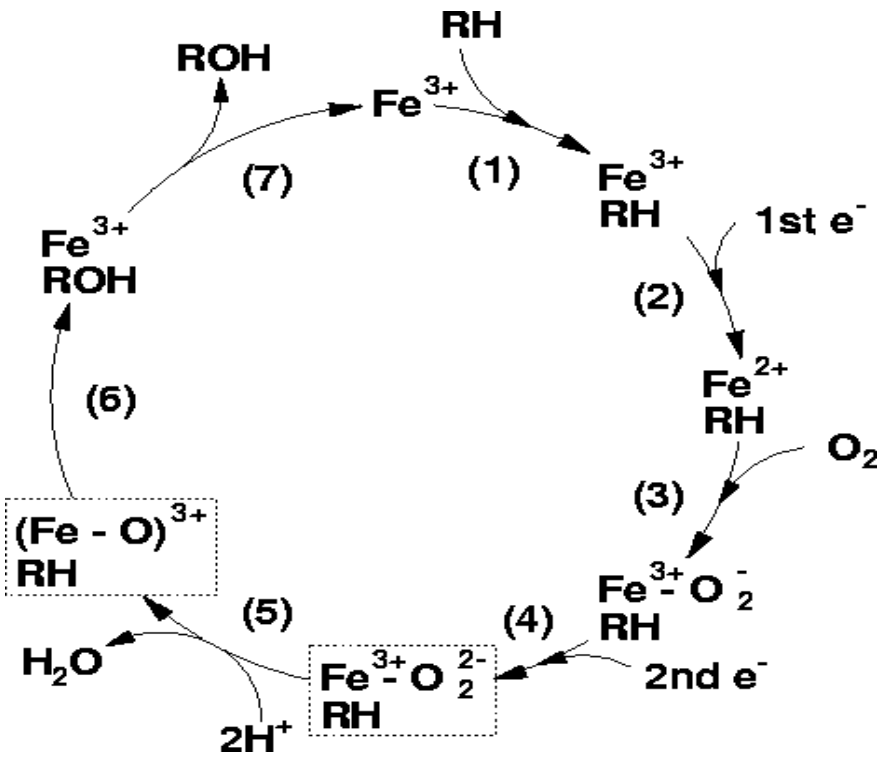
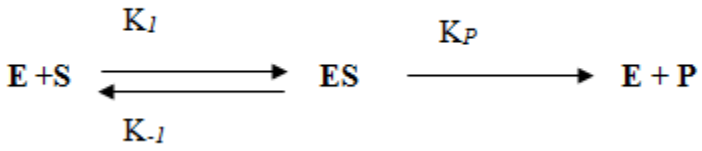


Figure 1.4 The general scheme for CYP catalysis (adapted from Guengerich 2001). Where the numbers refer to steps 1-9 of the catalytic cycle.

1.2.5 Enzyme kinetics of CYP-mediated reactions

The use of multiple drugs for the treatment of chronic diseases such as HIV/AIDS, diabetes and hypertension is routine in clinical practice. However, multiple administrations of drugs in patients have the tendency to cause DDI with significant clinical consequences ranging from drug toxicity to inefficacy. In DDI, one drug (inhibitor or inducer) influences the activity of the CYP on the metabolism of the second drug (substrate). Inhibition and induction are the main mechanisms responsible for such interactions. The apoprotein structures in different CYPs determine the variations in the substrates specificity and kinetic properties for each isoform.

Generally, most CYP-mediated reactions follow simple Michaelis-Menten (M-M) kinetics. The kinetic parameters derived from M-M kinetics based on *in vitro* data can be employed to predict *in vivo* pharmacokinetic DDI. The M-M kinetic assumes that only a single binding site is available at the active site of the enzyme where catalytic activity occurs and the reaction velocity can be characterized as a hyperbolic saturating profile as illustrated in Scheme 1.1 and Figure 1.5, respectively (Segel, 1975). The reaction velocity is plotted against the range of substrate concentrations used.



Scheme 1.1: The classical enzyme catalytic process.

E, S, ES and P are the concentrations of the enzymes, substrates, enzyme-substrate complex and the products (metabolites), respectively, K_1 is the association rate constant; K_{-1} and Kp are the dissociation rate constant from ES to E and S, and E and P, respectively.

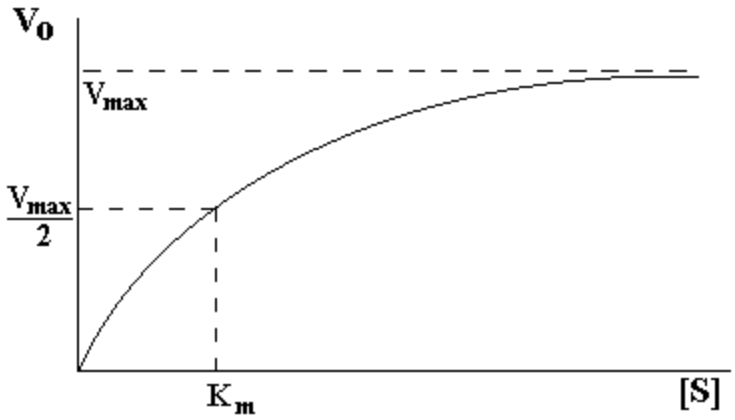


Figure 1.5 Michaelis-Menten relationship for substrate metabolism.

$v = Vmax. [S]/K_m + [S]$ Equation 1.1

The M-M constant (K_m) is defined as;

$K_m = (kp + k\text{-}_1) / K_1$ and $V = (k_p/K_m)*E*[S]$ Equation 1.2

Where v is the reaction velocity; V_{max}, maximum velocity; [S], substrate concentration; K_m, Michaelis constant; *kp*, rate of dissociation and E, free enzyme concentration.

Hence the reaction velocity (V) depends on the concentration of both free enzyme and the substrate in the steady state.

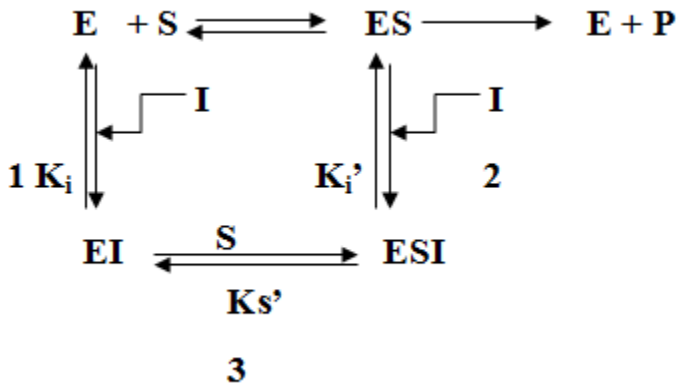
The enzyme catalytic rate reaches its maximum (V_{max}) when the substrate saturates the active site. At this point, the rate of catalytic activity becomes substrate concentration-independent. The dissociation rate constant *Kp* becomes the maximum catalytic rate constant (K_{cat}). To predict CYP-mediated DDI in clinic, the intrinsic clearance of specific drug is determined as $CL_{int} = V_{max}/K_m$ (equation 1.3), an important parameter which bridges enzyme kinetics and pharmacokinetics (Shou *et al*., 2001).

1.3 Inhibition of CYP enzymes

CYP inhibition has been implicated in most clinically relevant DDI. The mechanism of CYP inhibition is broadly classified into: (I) reversible inhibition and (II) irreversible inhibition also referred as time-dependent inhibition (TDI).

1.3.1 Reversible inhibition

Reversible inhibition involves rapid association and dissociation between drugs and enzymes. It can further be divided into competitive, uncompetitive, noncompetitive and mixed inhibition as demonstrated in Scheme 1.2. Competitive inhibition is the most common and uncompetitive inhibition the rarest event.



Scheme 1.2: Reversible enzyme inhibition. Figure adapted from (Shou *et al.*, 2001)

Pathway 1 (competitive inhibition), pathway 2 (uncompetitive inhibition), pathway 1 and 2 (noncompetitive inhibition) and pathways 1, 2 and 3 (mixed inhibition).

It is noteworthy to mention that reversible inhibition follows M-M kinetics.

1.3.1.1 Competitive inhibition

It occurs when the inhibitor (I) competes with the substrate (S) for the same binding site within the enzyme apoprotein. For competitive inhibition, the metabolic rate (v) can be expressed as:

$$v = V_{max}.[S]/K_m\ (1+[I]/K_i)+[S] \qquad \text{Equation 1.4}$$

V_{max} is the maximum metabolic rate and K_i, inhibition constant for an inhibitor (I). Based on the equation above, the substrate concentration [S] determines the strength of inhibition. The inhibition by a specific concentration of an inhibitor is more pronounced when the substrate concentration is low and less when the substrate concentration is high (Nelson and Cox, 2008).

1.3.1.2 Noncompetitive inhibition

The inhibitor binds to both free and substrate-bound enzyme at site(s) different from the substrate-binding site resulting in a conformational change in the protein. In noncompetitive

inhibition, it is assumed that inhibitor binds to the unbound enzyme and ES complex with similar affinity. Metabolic rate for noncompetitive inhibition is expressed as:

$v = \{V_{max}/(1+[I]/K_i)\}.[S]/K_m + [S]$ Equation 1.5

Thus, the substrate concentration does not determine the degree of inhibition.

1.3.1.3 Uncompetitive inhibition

The inhibitor binds to the enzyme only when it has formed a complex with the substrate. However, unlike competitive and noncompetitive inhibition, the inhibitor cannot bind to the unbound enzyme. The metabolic rate of uncompetitive inhibition can be expressed as:

$v = \{V_{max}/(1 +[I]/K_i)\} . [S]/ K_m/(1 +[I]/K_i) +[S]$ Equation 1.6

In this case, the strength of inhibition depends on the substrate concentration.

1.3.1.4 Mixed inhibition

The inhibitor binds identical at site(s) that overlap with substrate-binding site, the free and the substrate-bound enzyme with different affinities. The metabolic rate for mixed inhibition is expressed as:

$v = V_{max}/(1 +[I]/K_i) +(1 + Ks/[S]) \times (1 + [I]/K_i)$ Equation 1.7

Ks is the dissociation constant of the enzyme-substrate complex. It is approximately equivalent to Km (K3 <<K2).

1.3.2 Prediction of *in vivo* DDI based on *in vitro* reversible inhibition

The influence of a test drug (inhibitor) on a specific CYP probe substrate in a test system (HLMs or recombinant CYP) can be assessed by monitoring different concentrations of the inhibitor. The IC_{50} or K_i values determined are used to rank the risk of inhibition *in vivo*. Before the conduct of *in vitro* DDI studies, the concentrations of the probe substrate (K_m, V_{max}) and the inhibitor (IC_{50} or K_i) must be established. IC_{50} is the inhibitor concentration at which the reaction rate is reduced by 50%. The K_m value is determined by nonlinear regression plot of enzyme activity against substrate concentration. Optimization of substrate turnover by a given test system remains key procedure for determination of the kinetic parameters for specific probe substrate. It

is important to aim a turnover which is linearly dependent on time and less than 20% substrate consumption. Preferably, the lowest amount of protein that yields quantifiable metabolite concentrations in the incubation mixture should be utilized. The relationship between IC_{50} and K_i (inhibition constant) can be established for the various categories of reversible inhibition as illustrated in equation 1.8.

Competitive: $IC_{50} = K_i*(1 + [S]/K_m)$

Uncompetitive: $IC_{50} = K*(1 =K_m/[S])$

Noncompetitive: $IC_{50} = K_i$

Mixed: $IC_{50} = K*(1 + [S]/K_m)/(1 + (K_i/K')*([S]/K_m))$

If the concentration of probe substrate is equivalent to the K_m, inhibitor concentration capable of decreasing the enzyme activity by 50% (IC_{50}) will be the same as the K_i value when the type of inhibition is noncompetitive ($IC_{50} = K_i$) or almost twice the K_i for competitive inhibitor or an uncompetitive inhibitor ($IC_{50} = 2K_i$).

The criteria for ranking the potential risk of an inhibitor to cause DDI according to FDA draft guidance for drug interaction (2012) is based on $[I]/K_i$ ratio where [I] is the concentration of the inhibitor an individual is exposed to and the K_i, the inhibition constant for a pure compound. For $[I]/K_i$ ratio to be meaningful in the estimation of HDI for herbal supplements consisting of multiple constituents, certain assumptions must be established. First, the amount of an inhibitor [I] used is based on the assumption that the aqueous or methanol soluble extracts of the herbs to be absorbed from the GIT and to interact with intestinal CYPs and P-glycoprotein was completely soluble in the 250 mL GIT fluid (Fasinu *et al.*, 2012). Secondly, for an herbal medicines consisting of multiple phytochemical constituents, it is unlikely to determine the K_i since most of the constituents are unknown. It is therefore assumed that at least one phytochemical constituent of the herb is responsible for the inhibitory effect observed on CYPs. Furthermore, based on the design of the *in vitro* assay, substrate concentration at K_m was used to determine the IC_{50} value. It is therefore assumed from the experimental design that the speculated phytochemical constituent exhibits competitive inhibition which will result to a higher inhibition index. The herbal extracts were subsequently risk ranked based on FDA guidelines where, [I]/K*i*

>1.0 is associated with high risk for DDI, [I]/K*i* = 0.1-1 is associated with intermediate risk for DDI and [I]/K*i*<0.1 is unlikely to result in DDI (Zhou *et al*., 2005; Huang *et al*., 2008; Prueksaritanont *et al*., 2013).

Furthermore, to estimate the phytochemical constituents of the herb interacting with the hepatic CYPs and transporters, the maximum hepatic input amount of the extract entering the portal vein from the GIT and systemic circulation was determined using the equation: $[I]_{in} = [I]_{gut} + K_aF_aD/Q_h$. where, K_a (absorption rate constant), F_a (fraction absorbed from the gut into the portal vein), D (dose of herb used in humans) and Q_h (hepatic blood flow rate). To make the above equation useful, it was assumed that there is complete absorption of the soluble herbal extract from the GIT into the portal vein (F_a = 1.0). The values of K_a and Q_h were also assumed to be 0.1 min^{-1} and 1610 mL/min, respectively (Kanamitsu *et al*., 2000)

1.3.3 Time dependent inhibition

Time dependent inhibition (TDI) is a kinetically defined phenomenon whereby inhibitors display an increased intensity of enzyme inhibition upon prolonged duration of pre-incubation with the enzyme and NADPH. Mechanism-based inhibition (MBI) is a subset of TDI which involves inactivation of the enzyme via the formation of metabolic intermediates that bind tightly and irreversibly to the enzyme. Hence TDI is also referred as irreversible inhibition. MBI is characterized by NADPH-, time- and concentration-dependent enzyme inactivation and substrate protection. Often, MBI results in unfavourable DDI as the inactivated enzyme has to be replaced by *de novo* synthesis to recover its *in vivo* activity. MBI can be broadly classified into two major subdivisions, namely (i) quasi-irreversible and (ii) true irreversible inactivation (Lin and Lu, 1998).

1.3.3.1 Quasi-irreversible inactivation

During quasi-irreversible inactivation, the inhibitors undergo metabolic activation by the CYP enzyme to generate reactive intermediates which coordinate with the ferrous form of the CYP heme in a tight noncovalent complex resulting to the formation of metabolic intermediate complex (MIC). The MIC is catalytically inactive and cannot bind to carbon monoxide which is used as a diagnostic test to assess whether the CYP TDI happens through MIC formation. Many

of heme-ligand complexes are reversible *in vitro* after dialysis, hence the term 'quasi-irreversible'. *In vitro* treatment of microsomes or recombinant CYPs with $K_3Fe(CN)_6$ oxidizes the iron to the ferric states Fe(III) and displaces the metabolic intermediate from the active site leading to reactivation of the CYP activity. However, in *in vivo* MIC are very stable to the extent that the inactivated CYP is generally unavailable for drug metabolism. Consequently, DDIs due to quasi-irreversible inhibitors are prominent after multiple dosing compared to the reversible inhibitors (Lin and Lu, 1998).

Common quasi-irreversible CYP inactivators include primary amines and 1, 3-benzodioxole (methylenedioxyphenyl) derivatives. Primary amines are oxidized to nitroso metabolites via the intermediate hydroxylamine metabolites capable of forming MIC with the ferrous form of the heme iron atom. The nitroso-heme complexes can be decoupled *in vitro* by treating the inactivated CYP with potassium ferricyanide. The potassium ferricyanide oxidizes iron (II) to ferric state (III) and liberates the active carbon monoxide binding CYP enzyme. The transition from ferrous to ferric weakens the complex, an indication that the metabolic intermediate only strongly coordinates to the ferrous iron (Murray, 2000).

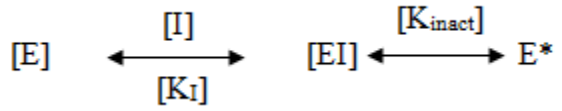
1.3.3.2 True irreversible inactivation of CYP

True irreversible inactivation of CYP involves formation of covalent bond between the metabolite and the CYP. At times, irreversible inactivation of CYP can generate hapten formation which triggers an autoimmune-response leading to acute hepatotoxicity (Li, 2002). Such an immune toxicity depends on a number of factors such as competing detoxification pathways, daily dose, dose regime, genetic factors and the intended target population. Reactive metabolite formed within the CYP active site can cause true irreversible inhibition via two different mechanisms: (i) covalent modification of heme prosthetic group and/or apoprotein in CYP enzymes and (ii) covalent modification of CYP by heme fragments.

1.3.3.2 Prediction of *in vivo* DDI based on *in vitro* TDI

The effect of a mechanism based inactivator *in vivo* is a combined function of its ability to inactivate the CYP and the synthesis rate of the enzyme (Mayhew *et al*., 2000). Hence, to assess the potential impact of MBI *in vivo*, it is important to determine the inactivation parameters of

the inactivator *in vitro*. The critical characteristics of mechanism-based enzyme inactivation are illustrated in the scheme below:



Scheme 1.3: Kinetic for mechanism-based inactivation.

Upon binding to an enzyme, an inactivator has three possible fates: (i) release from the enzyme through reversible binding, (ii) conversion to a product (P) through productive catalytic cycle or (iii) inactivation of enzyme by forming an irreversible complex (EI). E* is the concentration of free enzyme at equilibrium.

Based on the scheme, the rate of enzyme inactivation can be defined by:

$$d/dt[E]t = -K_{inact} \times [I] \times [E]_t/[I] + K_I \qquad \text{Equation 1.9}$$

$[E]_t$ is the active enzyme concentration at time t, [I] is the inactivator concentration, and K_I and K_{inact} are estimated by the nonlinear regression. For the K_{inact} and K_I parameters to be meaningful, two assumptions are made: (i) the binding of inactivator to the enzyme is in a rapid equilibrium and (ii) the catalytic steps common to both productive catalysis and irreversible inactivation are rapid and non-rate limiting hence can be ignored. The rate of enzyme inactivation at a given inactivator concentration is termed as the apparent inactivation rate constant, which is commonly denoted by k_{obs} or λ (Tudela *et al*., 1987) (Figure 1.6A):

$$\lambda = [I] \times K_{inact}/[I] + K_I \qquad \text{Equation 1.10}$$

Linear and nonlinear methods are used to obtain K_{inact} and K_I values. Taking reciprocal of each sides of the above equation gives

$$1/\lambda = 1/K_{inact} + K_I/K_{inact}.1/[I] \qquad \text{Equation 1.11}$$

A plot of $1/\lambda$ against 1/[I] generates a trend of line which has y-intercept of $1/K_{inact}$ and an x-intercept of $-1/K_I$. This is also referred to as double reciprocal or Kitz-Wilson plot (Kitz and

Wilson 1962) (Figure 1.6B). For a nonlinear regression, the hyperbolic relationship between λ and the [I] are fitted to derive estimated K_{inact} and K_I (Figure 1.6C).

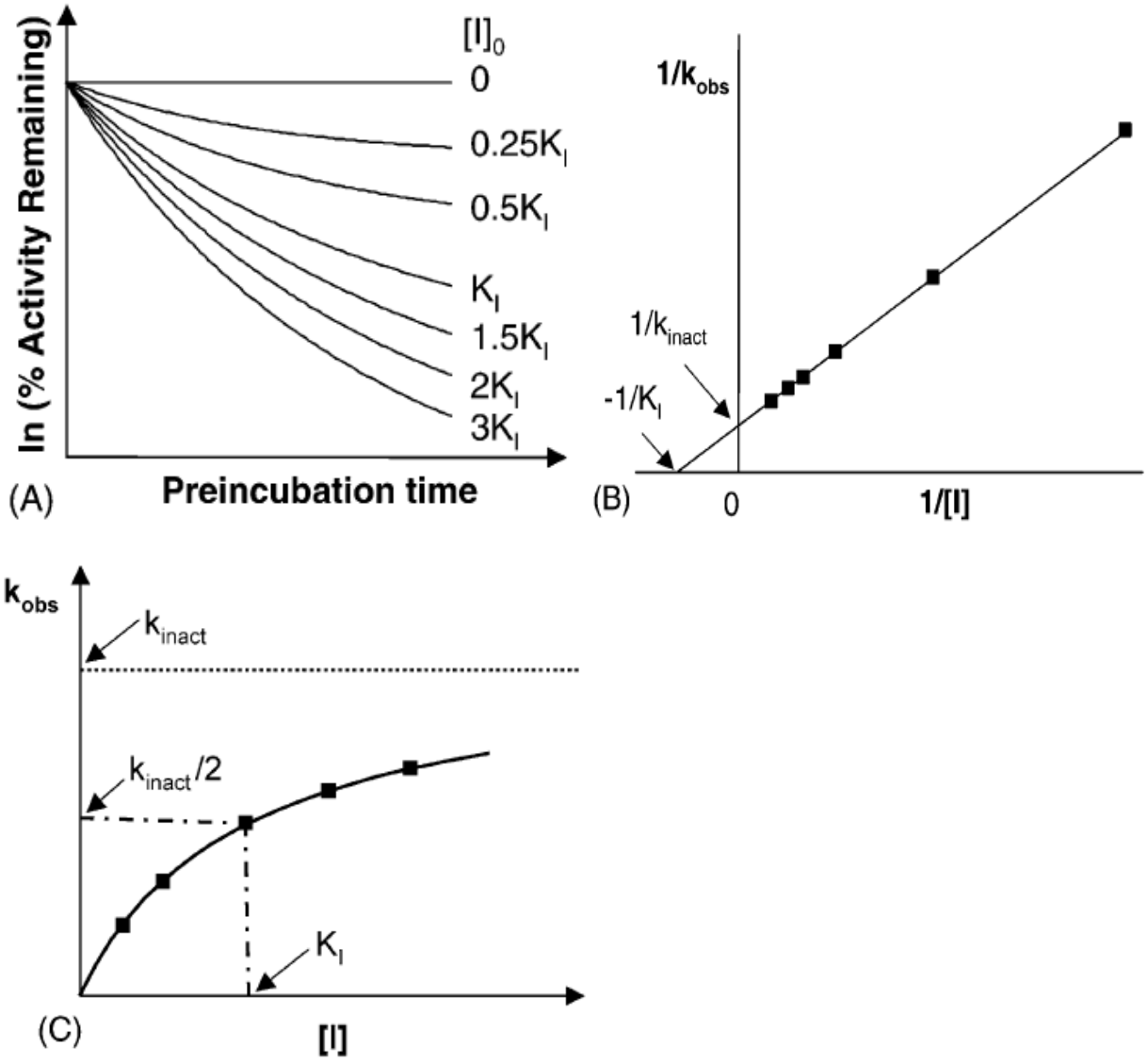


Figure 1.6 Graphical representations relevant to conventional experimental procedure for chracterising MBI. (A) time- and concentration-dependent inactivation of enzyme by MBI. (B) The Kitz-Wilson plot, a double reciprocal plot of the observed inactivation constant (k_{obs}) as function of MBI concentration [I]. (C) Relationship between the observed inactivation rate constant (K_{obs}) and the MBI constant [I]. Figure adapted from (Yang *et al*., 2005).

1.4 Induction of CYPs

Induction of CYPs occurs as a consequence of a drug or xenobiotic triggering an increase in gene transcription to generate protein activity above the normal biological range. Increase hepatic clearance of drugs metabolized by the induced CYPs is an indicative of elevated levels of enzyme gene expression. Pharmacokinetically, the AUC of the co-administered drug are reduced in the presence of an inducer. Interaction between midazolam (CYP3A4 substrate) and antidepressant herbal product, St. John's wort (CYP3A4 inducer) is an example where the AUC of midazolam declined by 79% when the two products were administered in healthy subjects (Mueller *et al*., 2006). Additionally, certain drugs, for example carbamazepine and artemisinin which are known substrates for CYP3A4 and CYP2B6, respectively cause autoinduction by both acting as inducer and thus reduce plasma levels of the affected drugs (Bertilsson *et al*., 1980; Simonsoun *et al*., 2003) (Table 1.2). Study conducted in healthy volunteers indicated that concomitant administration of rifampicin and oral contraceptives could lead to failure of the antifertility effects of the latter (Reimers and Jezek, 1971). Such effect was attributed to the induction of estrogen-2-hydroxylation pathway which is catalyzed primarily by CYP3A4 in the presence of rifampicin (Lee *et al*., 2003).

Induction of CYPs can have serious consequences: 1) reduction in the intrinsic pharmacological activity of the target drug or a coadministered drug due to rapid hepatic clearance, and 2) increased toxicity of primary drug due to elevated levels of metabolites in plasma as the expression of CYP increases in the presence of the co-administered drug.

1.4.1 Mechanism of induction

There are two primary mechanisms of enzyme induction: 1) stabilization of the mRNA or enzyme and 2) increased gene transcription. Transcriptional gene activation is however regarded as the common mechanism for induction. Transcriptional gene activation of drug metabolizing CYPs is mediated by nuclear receptors which function as transcription factors. These transcription factors include aromatic hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR). This portion of the review will however focus on detailed mechanism of PXR mediated induction which is the most extensively studied nuclear receptor.

Table 1.2 Commonly used *in vivo* probes for clinical studies and CYP inducers. Table adapted from (FDA, 2012 and Tucker *et al*., 2001).

P450	Substrates/Probes	Inducers
CYP1A2	Theophylline, caffeine, acetaminophen, aromatic amines, phenacetin	Cigarette smoke, omeprazole charbroiled meats, cruciferous vegetables
CYP2A6	Coumarin, butadiene, nicotine	Barbiturates
CYP2B6	Efavirenz	Rifampin
CYP2C8	Repaglinide, rosiglitazone	Rifampin
CYP2C9	S-warfarin, tolbutamide, phenytoin, NSAIDs	Rifampin, barbiturates
CYP2C19	Omeprazole, esoprazole, lansoprazole, pantoprazole, citalopram, diazepam, hexobarbital, imipramine, proguanil, propanolol	Rifampin, barbiturates
CYP2D6	Antidepressants, neuroleptics, beta-blockers, antiarrhytmics, codeine, etylmorphine, desipramine, dextromethorphan, atomoxetine, nicotine	Not identified
CYP2E1	Chlorzoxazone, acetaminophen, alcohols, caffeine, dapsone, enflurane, theophylline	Ethanol, isoniazid
CYP3A4	Midazolam, buspirone, felodipine, lovastatin, eletriptan, sildenafil, simavastatin, triazolam, acetaminophen, carbamezapine, cyclosporin, digitoxin, diazepam, erythromycin, fluoxetine, nifedipine, quinidine, saquinavir, terfenadine, verapamil, warfarin	Rifampin, carbamazepine, dexamethasone, phenytoin, troleandomycin

1.4.2 PXR mediated induction of CYP3A

This nuclear receptor is closely related to the vitamin D receptor (VDR) and complex with VDR DNA elements as a heterodimer with a retinoid X receptor alpha (RXRα). PXR element has been identified in human CYP3A4 and rat CYP3A23 promoter sites. X-ray crystallography showed that PXR has the largest ligand-binding pocket compared to other nuclear receptors, enabling it to be more promiscuous (Watkins *et al*., 2001). Upon activation by ligand binding, PXR translocates into the nucleus to form heterodimer with RXR. The PXR/RXR dimer binds to response elements upstream of the target gene causing chromatin remolding and transcriptional activation **(**Meijerman *et al*., 2006). Once the transcription of target genes is activated, CYP3A4 expression level increases as depicted in Figure 1.7.

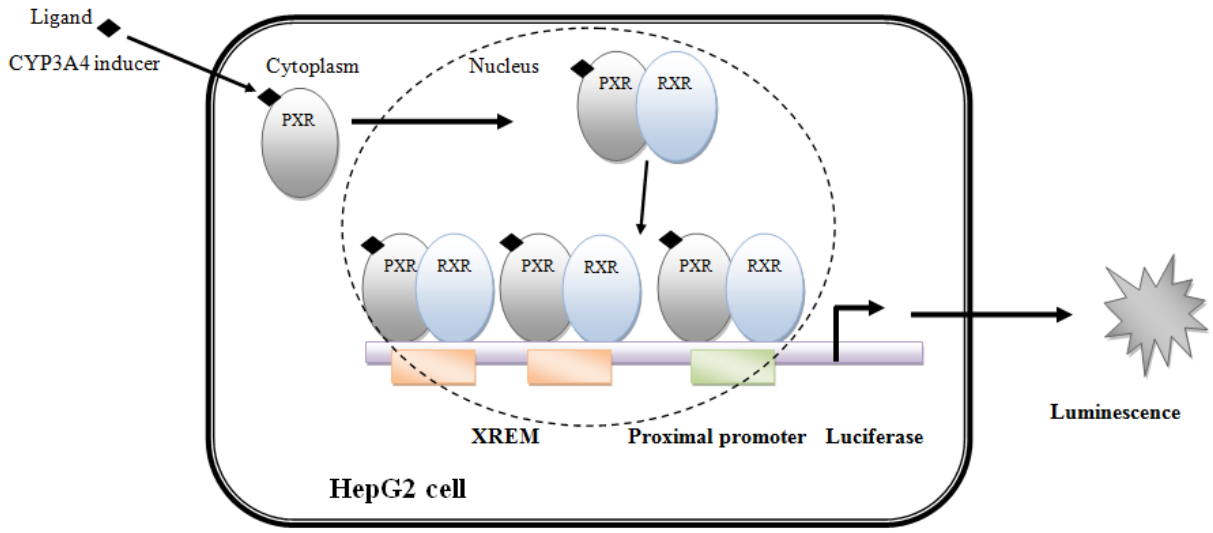


Figure 1.7 Mechanism of PXR activation via luciferase reporter gene assay (adapted from Meijerman *et al*., 2006**).**

1.4.3 Clinical relevance of CYP induction

Unlike enzyme inhibition which is rapid and reproducible *in vitro*, induction is time-dependent. There is therefore the probability of ignoring enzyme induction by compounds which has resulted in serious implications. For example, some protease inhibitors have been found to interact with SJW. A study showed that intake of 900 mg/day of SJW standardized to 0.3% hypericin for two weeks led to a decrease in plasma concentration of indinavir (Piscitelli *et al*., 2000). SJW is reported to cause induction of hepatic CYP3A4 responsible for metabolism of indinavir. Low plasma concentration of the protease inhibitor leads to development of drug resistance and treatment failure in HIV-infected patients. The immunosuppressive agent, cyclosporine is a substrate for P-gp and CYP3A4 and is widely used after transplantation with cases of allograft rejection reported when the plasma concentration decline below the effective level in patients taking SJW (Akhlaghi and Trull, 2002). Case reports of patients receiving transplantation of heart, liver, kidney and pancreas have shown that a decline in blood trough concentration of cyclosporine resulted in transplant graft rejection within 3-4 weeks of using SJW (Barone *et al.*, 2000; Ruschitzka *et al.*, 2000).

1.4.4 *In vitro* models for CYP inhibition and induction studies

1.4.4.1 Recombinant CYPs

These are cDNA CYPs expressed in baculovirus-insect cell or an *Escherichia coli* (*E. coli*) based expression system. Only single enzyme is expressed in such systems, hence resulting to higher activity when a specific substrate is applied (Foti *et al*., 2010). Recombinant enzymes are usually employed in high throughput screening (HTS) of new candidate drugs at early stages of drug development. The absence of contribution of other metabolic enzyme in recombinant CYP assays is a major setback associated with this system (Kramer and Tracy, 2008).

1.4.4.2 Human liver microsomes

It is regarded as the gold standard enzyme source for most *in vitro* inhibition studies. Human liver microsomes (HLM) are very convenient, easy to use and affordable. HLM contains multiple phase I hepatic drug metabolizing enzymes compared to the recombinant CYPs. Microsomes obtained from liver has vesicles of the hepatocytes endoplasmic reticulum collected from liver preparation such as fresh human liver, liver slices, liver cell lines and primary

hepatocytes at specific differential centrifugation. HLMs do not represent complete *in vivo* situation since only CYP and UGT enzymes are present in HLM (Brendon *et al*., 2003).

1.4.4.2 Human liver S9 fractions

These are subcellular fractions containing drug metabolism enzymes such as CYPs, flavin monooxygenases and UDP-glucuronyltransferases. S9 fractions depict a complete representation of metabolic profile compare to HLM since they contain both phase I and phase II drug-metabolism enzymes. However, the enzyme activity is relatively lower than that of HLM (Brendon *et al*., 2003).

1.4.4.3 Liver slices

The liver slices have been developed as an *in vitro* model for assessment of hepatic drug metabolism. The tissue slices maintain the cytoarchitecture of the liver which can be easily examined when needed. Phase II enzymes, albumin production, and gluconeogenesis have been shown to decrease slightly but remain fairly stable for up to 20-96 hours in cultured liver slices (Toutain *et al*., 1998). Major challenges with liver slices include erratic supply of the liver, maintenance of slice cultures with quality viability over the study period and the high interindividual variability (Thohan and Rosen, 2002).

1.4.4.4 Primary hepatocytes

Cultured human hepatocyte is currently the gold standard for studying CYP induction (Lecluyse, 2001). Availability of tissue is a major concern and restricts the use of human hepatocytes *for in vitro* applications. Furthermore, there is time-dependent decline in the expression of mRNA for the major CYPs resulting in reduced enzyme activities. To overcome this flaw, dishes and plates for human hepatocytes assays are coated with an extracellular matrix such as Matrigel or collagen which is termed as sandwich culturing to mimic physiological condition. Human hepatocytes in sandwich culture have cytoarchitecture similar to that found *in vivo*.

1.4.4.5 Cell lines

Fa2N-4, a non-tumorigenic hepatic cell line, is the most utilized cell system by the pharmaceutical industry (Sinz *et al*., 2008). It shows inducible CYP1A1/2, CYP3A4, CYP2C9, UGT1A and MDR1 mRNA expression, as well as increased enzymatic activity of CYP1A2, CYP2C9 and CYP3A4 when treated with prototypical inducers. Fa2N-4 is however flawed with

low basal enzyme activity; hence sensitive analytical methods are required to measure enzyme activity. Because of the low basal enzyme activity, mRNA detection is often used as an end point when using this cell line.

Human hepatoma cell lines such as HepG2, HepaRG and BC2 have been employed for induction studies. HepaRG and HepG2 cells have been shown to respond differently to CYP1A1/2 and 3A4 inducers whilst BC2 cells have been reported to respond to CYP1A inducers (Aninat *et al*., 2006; Guillouzo *et al*., 2007). These cell lines however do not maintain the complete phenotypic characteristics of human hepatocytes including enzyme, receptor function or expression.

1.4.4.6 Reporter gene

In the reporter gene assay, plasmids containing elements of the target gene promoter of interest fused into the reporter gene expression vector is transiently or stably transfected into cell lines. HepG2 cell line transfected with two plasmids containing 5'-regulatory elements (PCR5) of CYP3A4 linked to a gene expressing luciferase and PXR expression vector is used for the assessment of PXR activation or CYP3A4 induction in most research laboratories (Sinz *et al*., 2006). The cells are treated with the test samples and PXR activation or CYP3A4 induction measure using luciferase activity as depicted in Figure 1.7 above. Cells used in reporter gene assays are easy to maintain, and the luciferase measurement applicably for high throughput screenings. However, the levels of target genes and receptor expression are not at physiological levels and the cell lines may not contain all the regulatory factors present in hepatocytes. Hence reporter gene assays are used only for screening purposes to flag compounds as potential inducers.

1.4.4.7 Transgenic/Chimeric animals

Humanized nuclear receptor transgenic mice, knock out mice and chimeric mice with human transplanted hepatocytes have emerged as useful models for investigating the regulation of drug metabolizing enzymes by potential drug candidates. These *in vivo* models provide an advantage over the above-mentioned *in vitro* models. Transgenic animals have dynamic systems where drug absorption, distribution, metabolism, and elimination occur simultaneously. Animals can be administered with drug candidates at doses equivalent to those expected in human plasma to improve assessment of induction or inhibition potential of such drugs. The major flaw with the transgenic/knock out systems are that only single or double genes are "humanized" and the

cross-talk between nuclear receptors/enzymes/transporters may be compromised as the human and mouse systems may or may not be working in a similar fashion (Sinz *et al*., 2008).

1.5 Drug transporters

Drug transporters play key part in drug disposition, efficacy and adverse effects. Studies resulting to clinically significant DDIs and HDIs have suggested that transporters work together with drug metabolizing enzymes in drug disposition and elimination. Uptake and efflux transporters are localized at both the apical side and basolateral membranes of the intestinal enterocytes. Efflux transporters commonly expressed in the intestinal membranes include P-glycoprotein (P-gp), multidrug resistance proteins (MRP 1–6), and breast cancer resistance protein (BCRP). These are members of the ATP-binding cassette (ABC) transport proteins which utilise ATP as energy source to transport substrates against a concentration gradient from the cytoplasm of intestinal cells back to the intestinal lumen or to the blood (Ho and Kim, 2005; Oostendorp *et al*., 2009; Shugarts *et al*., 2009).

Primary active efflux transporters notably P-gp, MRP2, and BCRP are well expressed on brushborder apical membrane of the enterocytes and reported to form major hindrance to intestinal absorption of drugs (Chan *et al*., 2004; Yu *et al*., 2007). These have the tendency to reduce oral bioavailability of specific drugs by expulsion from the enterocytes into intestinal lumen. P-gp is a well-recognized efflux transporter which influences absorption and excretion of several drugs due to its wider range of substrate specificity (Troutman *et al*., 2001). BCRP is capable to extrude glucuronides and sulphate conjugates formed in the enterocytes into the lumen, although its main function relates to excretion of drugs and xenobiotics into breast milk and a defensive role at the placental barrier (Adachi *et al*., 2005). MRP2 plays an important role in detoxification of drug and xenobiotics which have undergone phase II metabolism to facilitate excretion (Russel *et al*., 2002). Unlike efflux transporters, apical membrane uptake transporters increase absorption of drugs from the lumen into the enterocytes (Kullak-Ublick *et al*., 2001). Finally, receptor mediated endocytosis can also play a role in drug absorption.

Uptake transporters e.g., peptide transporter (PEPT), organic anion transporter (OAT), organic anion transporting polypeptide (OATP), organic cation transporter (OCT) are members of solute

carrier (SLC and SLCO) superfamilies and function independent of ATP but rather transport drugs according to their concentration gradient (Figure 1.8).

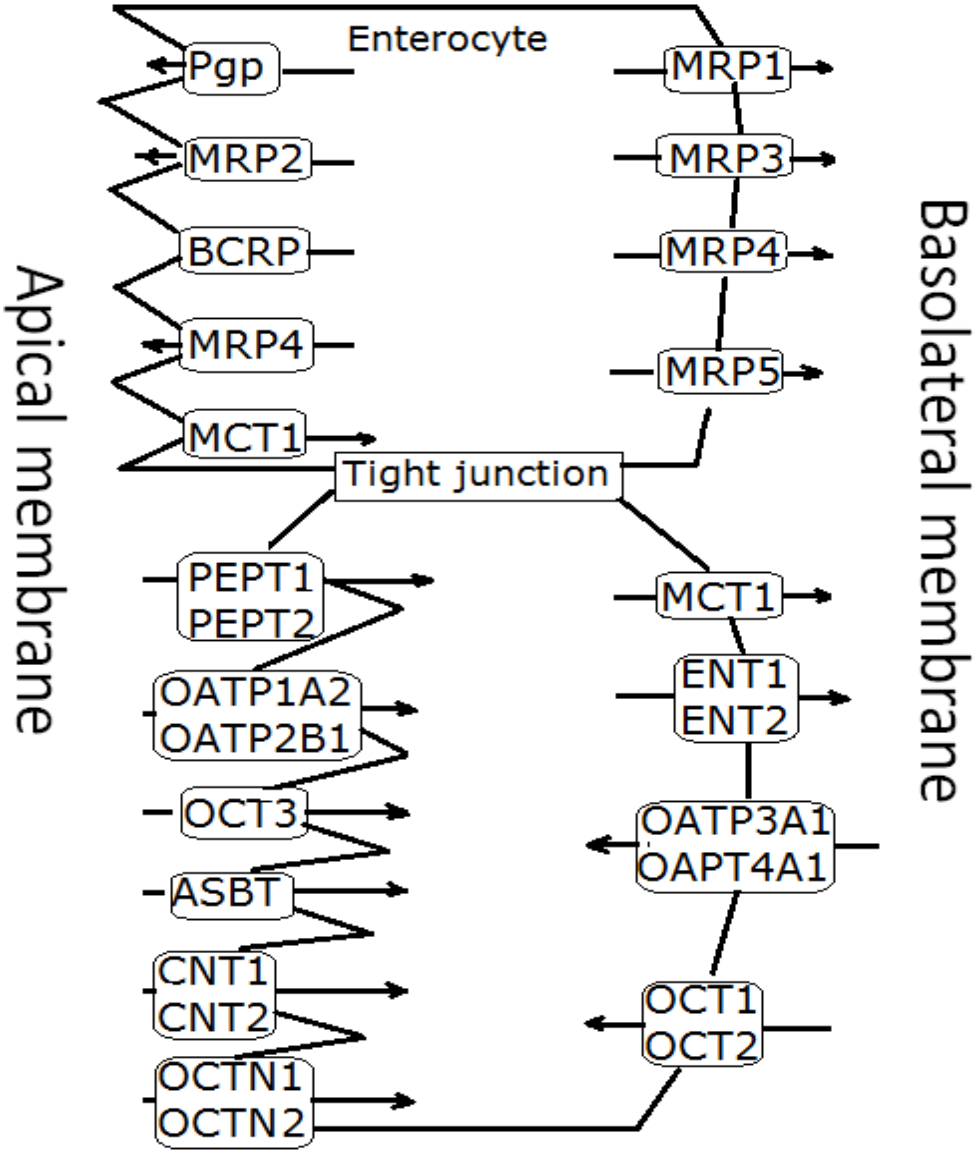


Figure 1.8 Localization of ABC and SLC transporters in human small intestine (adapted from Artursson, 1991; Awortwe *et al*., 2014a).

The various abbreviations represent: BCRP (breast cancer reistance protein); Pgp (p-glycoprotein); MRP (multi-drug resistance associated proteins); MCT (monocarboxylate transporters); PEPT (peptide transporters); OATP (organic anion-transporting polypeptide); OCT (organic cation transporter); OCTN (organic zwitterions/cation transporters); CNT (concentrative nucleoside transporter); ENT (equilibrative nucleoside transporter); and ASBT (apical sodium dependent bile acid transporters).

1.5.1 *In vitro* models for evaluation of P-glycoprotein

1.5.1.1 Caco - 2 cell line

The Caco - 2 cell line is the most common and extensively characterized cell-based model for the assessment of absorption of drugs via the intestinal membrane enterocytes (Artursson, 1991; Artursson and Karlsson, 1991; Rubas *et al*., 1996). It is derived from heterogenous human epithelial colorectal adenocarcinoma cells, developed through research by Jorgen Fogh (1975) at Sloan-Kettering Institute for Cancer Research (Fogh and Trempe, 1975). The Caco - 2 cells have the intrinsic ability to initiate spontaneous differentiation on reaching confluence in the presence of normal culture conditions. At confluence, there is progressive development of brush border. The surface occupied by each cell gradually reduces from 5 to 20 days post-confluence and intimate intercellular junctions are developed. During the same period, the length and density of microvilli increase. The Caco - 2 cells form well - developed tight junctions after 21 days of culturing. At day - 30 post-confluence, the cell surface reaches minimum with typical enterocytes-like morphology. Most cells develop complete brush borders with tall and regular microvilli when the full structural polarization is achieved after 30 days (Vachon and Beaulieu, 1992). The complete polarized Caco - 2 cells resemble human small intestinal mucosa cells expressing brush borders, tight junctions and, efflux and uptake transporters at both apical and basolateral compartments.

The Caco - 2 cell lines is considered as a good model which mimics the intestinal biochemical barrier due to expression of membrane efflux proteins (P-gp, MRP 1-3), CYP isoenzymes and phase II conjugating enzymes such as sulfotransferase, UDP-glucuronyltransferase, glutathione S-transferase (Prueksaritanont *et al*., 1996; Hunter and Hirst, 1997). Table 1.3 summarizes the characteristics of a parental Caco - 2 cell line. Permeability of substrate via the Caco - 2 cell monolayer has proven to correlate closely with oral absorption in humans compared with other parental cell-based models. Currently, Caco - 2 monolayers are applied for screening the potential effects of herbs and new chemical entities (NCE) on absorption of drugs.

Table 1.3 Characteristics of Caco-2 cell line

Parameter	Description
Origin	Human colorectal adenocarcinoma
Growth in culture	Monolayer epithelial cells
Differentiation	14-21 days after confluence in standard culture medium
Morphology	Polarized cells, with tight junctions, apical brush border
Electrical resistance	High transepithelial electrical resistance (TEER)
Digestive enzymes	Typical membranous peptidases and disaccharidases of the small intestine

1.5.1.2 MDCK

The Madin-Darby canine kidney (MDCK) is considered as an alternative to Caco - 2 cells for permeability studies. The MDCK cell line is derived from the normal dog kidney. When cultured in an appropriate media, MDCK differentiate into columnar epithelial cells with clear tight junctions similar to Caco - 2 formed within on a semi-permeable membrane. The differentiated MDCK cell maintains characteristics of kidney epithelial cells such as asymmetric distribution of enzymes and vectorial transport of sodium and water from the apical to the basolateral membrane (Balcarova-Stander *et al*., 1984). The MDCK has a shorter cultivation period (3 - 5 days) compared to 21 days of Caco - 2 cell lines. Furthermore, the transepithelial electrical resistance (TEER) of MDCK cells is lower and closer to that of small intestine compared to Caco - 2 cells. This often results in a higher permeability coefficient of hydrophilic compounds in MDCK cells than Caco - 2 cells. The shorter culture duration is advantageous since it reduces cost, time, and enhances high throughput. The MDCK cell line appears to be more robust and

need less rigorous nutritional schedule over a short incubation period relative to Caco - 2 cells. However, these cells are derived from dog kidney and therefore there is a high propensity of differences in the expression levels of some transporters and metabolic activity as compared to Caco - 2 cells (Braun *et al*., 2000).

1.5.1.3 LLC-PK1

LLC-PK1 cells derived from pig kidney epithelial have been employed as an alternative model to Caco - 2 cells for assessing permeability of test compounds. Studies have reported the use of LLC-PK1 for characterization of passive absorption of test compounds (Thwaites *et al*., 1993; Li *et al*., 2002; Adachi *et al*., 2003). LLC-PK1 stably transfected with P-gp/MDR1 has been reported as an important model for studying bidirectional transport of compounds.

1.6 Evidence of herbal medicines interactions with antiretrovirals drugs

Several pharmacokinetic ARV-herb interactions and case studies have been reported. These have been summarised in Tables 1.4a and 1.4b.

St. Johns wort (SJW) is widely used for the management of clinical depression, which is a common co-morbidity associated with HIV/AIDS. Clinically pharmacokinetic drug interaction study between indinavir and SJW was conducted to determine the effect of 2-week regimen of SJW on the AUC_{0-8} and the concentration 8 h post-dose (C_8) of indinavir, at steady state using crossover study design involving eight healthy subjects (Piscitelli *et al*., 2000). The result showed significant reduction in the systemic exposure of indinavir in the presence of SJW. However, since this was a crossover study, it is unknown what influence duration had on the effect observed. Additionally, *in vitro* studies indicated that SJW or its major component, hyperforin, has a dual effect on CYP3A4 as a potent inducer and inhibitor which is the primary enzyme for the metabolism of indinavir (Krusekopf *et al*., 2003; Lee *et al*., 2006). In 2001, a case study in five HIV/AIDs patients who were concurrently taken nevirapine and SJW was reported. The study showed that plasma concentrations of nevirapine in patients taking SJW were lower than those who took only nevirapine (de Maat *et al*., 2001).

Garlic (*Allium sativum*) supplements are ranked among top ten best patronized herbal products worldwide with confounding reported incidence of garlic-drug interactions (Bianchini and

Vainio, 2001). Extracts from garlic are commonly used in HIV-infected patients to fight opportunistic infections and improve health due to its antihyperlipidemic, antioxidant and antimicrobial activities (Standish *et al*., 2001). A case study in two HIV-infected patients on ritonavir showed severe gastrointestinal toxicity after administration of garlic supplement for over two weeks (Tattelman, 2005). The symptoms re-occurred when patients were given low-dose of ritonavir. This result suggested dose independent interaction between garlic and ritonavir, through inhibition of CYP3A4 and induction of P-gp. In another study, 2 × 5 mg daily intake of garlic showed reduction in plasma concentration of ritonavir after day-4 (Foster *et al*., 2001).

A population pharmacokinetic study using allicin, an isolated phytochemical compound from garlic also showed a reduction in plasma levels of saquinavir (Piscitelli *et al*., 2002a). Interestingly, an *in vitro* study of saquinavir as a probe substrate induced expression of P-gp (Dupuis *et al*., 2003). Therefore, the reduced plasma levels of saquinavir reported previously was attributed to auto-induction of P-gp by saquinavir. Other HDI studies on garlic oil using midazolam and alprazolam as probe substrates for CYP3A4/5, showed no changes in the metabolism of either probe substrates (Gurley *et al*., 2005).

The hepatoprotective effect of *Silybum marianum* renders its useful in HIV-infected patients for management of possible hepatotoxicity associated with some ARVs (Cohen *et al*., 2001). Independent studies conducted in human subjects on indinavir and milk thistle showed non-significant effects of the herb on pharmacokinetic parameters of the PI (Dicenzo *et al*., 2003). This result agreed with an earlier *in vitro* study which showed that; therapeutic dose of milk thistle has no effect on inhibition or induction of both CYP3A4 and P-gp (Doehmer *et al*., 2005). However, caution must be exercised towards the use of such herbal products with non-significant interactions on monotherapeutic ARVs since the current treatment of HIV-infected patients employs combination therapy.

Table 1.4a Case reports on ARVs and herb drug interactions

Medicinal plants	Concomitant ARV	Sample size	Possible Mode of interactions	Clinical outcome	References
Cat's claw *Uncaria tomentosa*	atazanavir, ritonavir and saquinavir	45 year old woman with cirrhosis	Inhibition of CYP3A4	↑ Cmin (serum trough concentration)	Lopez Galera *et al.*, 2008
St. John's wort *Hypericum perforatum*	Lamivudine	1 HIV-infected patient	unknown	Increased HIV RNA viral load	Zhou *et al.*, 2007
St. John's wort *Hypericum perforatum*	Nevirapine	5 HIV-infected patients	Induction of CYP3A4	↑CL/F	De Maat *et al.*, 2001
Garlic *Allium sativum*	Ritonavir	2 HIV-infected patients	Inhibition of CYP3A4 or Pgp	Severe GI Toxicity	Laroche *et al.*, 1998

Garlic	Ritonavir	10 HIV seronegative	Non-significant CYP3A4/P-gp inhibition	↓AUC by 17%, ↓ Cmax, by -1%	Gallicano *et al*., 2003
	Saquinavir	10 healthy volunteers	Induction of CYP3A4 & Pgp	↓AUC by 51%, ↓C_{8h} by 49%, ↓Cmax by 54%	Piscitelli *et al*., 2002
St. John's wort	Indinavir	8 HIV seronegative	CYP3A4 induction	↓ AUC by 57%, ↓C trough by 81%	Piscitelli *et al*., 2000
Milk thistle	Indinavir	10 HIV-infected patients	Modulation of CYP3A4 and P-gp	↓AUC by 9%, ↓trough level (C_{8h}) by 25%	Piscitelli *et al*., 2002
	darunavir-ritonavir	15 HIV-infected patients	NA	↓C_{max} and AUC_{0-12} by 15%	Molto *et al*., 2012
Ginkgo	Lopinavir/ ritonavir	14 healthy volunteers	NA	⇳C_{max} and AUC_{0-12}	Robertson *et al*., 2008
Goldenseal	Indinavir	10 healthy volunteers,	CYP3A4 inhibition	↑Cmax by 7% , ↓ CL/F by 7%	Sandhu *et al*., 2003

Table 1.4b: *In vitro* screening of herbs for effect on CYP3A4, P-gp and PXR

Medicinal plants	Effect on CYPs	Effect on P-gp	Effect on PXR	References
Catharanthus roseus	Inhibits of CYP2D6 and CYP3A4 in HLM	Not determined	Not determined	Usia *et al*., 2005
Hypoxis hemerocallidea	Inhibits CYP3A4, 3A5 and 2C19 in CYP transfected microsomes	Inducers in Caco-2	Inducer in HepG2	Nair *et al*., 2007
Lessertia frutescens	Inhibits CYP3A4 in CYP-transfected microsomes	Inhibitor in P-gp transfected membranes	Inducer in HepG2 transfected PXR	Mills *et al*., 2005
Hypoxis obtusa	Inhibits CYP1A2, 2C9, 2C19, 2D6 and 3A4 in recombinant CYPs	No effect in Caco-2	Not determined	Gwaza *et al*., 2009

1.7 Selected herbal products use by HIV/AIDS patients

The herb-drug interaction Unit of the Division of Clinical Pharmacology, Stellenbosch University in collaboration with HOPE Cape Town invited two traditional health practitioners (THPs) who practice in the Mfuleni and Delft communities in Cape Town, Western Cape Province. The two THPs identified *Hypoxis hemerocallidea* and *Lessertia frutescens* as popular herbs patronised by their clients. Exotic herbal products such as *Moringa oleifera*, *Echinacea purpurea*, *Taraxacum officinale*, *Kalanchoe crenata* and *Kalanchoe integra* were identified through the help of THPs who own herbal products shops in Bellville, Cape Town and Harare Zimbabwe. All the plants were purported to be taken orally for management of HIV/AIDS and its related co-morbidities. Fresh samples of *Lessertia frutescens*, *Moringa oleifera*, *Echinacea purpurea* and *Kalanchoe crenata* were obtained from the repository of the National Centre for Natural Products Research (NCNPR), University of Mississippi, Oxford, USA. Specimens of each sample with voucher numbers are available at the NCNPR. Capsules and powdered roots of *Hypoxis hemerocallidea*, and roots of *Taraxacum officinale* with certificate of identification and batch numbers were sourced from Medico Herbs, Somerset West, Western Cape. Tablets of *Lessertia frutescens* were obtained from Pyto Nova Natural Medicines, Kwazulu Natal. The capsules of *Echinacea purpurea* were sourced from Herbal Soultions, Harare, Zimbabwe.

1.7.1 *Moringa oleifera*

It is the most cultivated species of the monogeneric family Moringaceae (Ramachandran *et al*., 1980). *Moringa oleifera* is widely distributed in sub-Himalayan ranges of Arabia, India, Maadagascar, North East and South Western Africa and Sri Lanka (Fahey, 2005). The plant is known as the ‘drumstick tree’ or the ‘horse radish tree’ in some part of the world whilst others referred to it as the kelor tree (Anwar and Bhanger, 2003). In the Nile valley *M. oleifera* is popular known as “Shagara al Rauwaq’ which means ‘tree for purifying’ (Anwar *et al*., 2007). In Ghana, *M. oleifera* is referred to as the ‘miraculous tree’.

Moringa oleifera has had enormous attention as the natural nutrition of the tropics. Special websites and radio programs have been dedicated to promoting the sale of *Moringa* in certain

part of West Africa (http://www.africamoringa.co.za, http://www.moringasource.com, http://miracletrees.org). Many countries use the various parts of *M. oleifera* as a nutritional vegetable particularly in India, Pakistan, Philippines, Hawaii and several African countries (Anwar 2003). The leaves have been documented to be a rich source of β-carotene, protein, vitamin C, calcium and potassium which are purported to stimulate immune system in HIV/AIDS patients. It is also used as preservative to improve shelf-life of fat containing foods due to high contents of natural antioxidant such as ascorbic acid, flavonoids, phenolic acid and carotenoids (Siddhuraju and Becker, 2003). A cross-sectional study conducted in HIV/AIDS patients attending a referral hospital in Harare, the capital city of Zimbabwe, indicated that 68% of the participants reported taken *M. oleifera* while on various ARVs (Monera *et al*., 2012). The study participants attributed the use of *M. oleifera* to its immune boosting effect. In a similar study carried out at the Family Care Centre antiretroviral therapy clinic in Harare, Zimbabwe, *M. Oleifera* was reported as a common herbal medicine consumed by HIV-infected patients (Mudzviti *et al*., 2012).

Each part of the plant has been used for treatment of many diseases in the folkloric medicine including cardiovascular, gastrointestinal, hematological and hepatorenal disorders. The leaf juice of *M.oleifera* is known to exhibit stabilizing effect on blood pressure (Dahot, 1988). Aqueous infusion of the roots, leaves, flowers, and seed have been documented to possess diuretic activity (Caceres *et al*., 1992). Experiments done in a rat model showed that methanolic extract of *Moringa* leaves has antiulcerogenic and hepatoprotective effects (Pal *et al*., 1995).

Preliminary study on aqueous and methanolic extracts of *M. oleifera* leaves indicated a strong inhibitory effect on CYP3A4 (Menora *et al*., 2008). However, the study was based on reversible inhibition of CYP3A4 in HLM using testosterone as a probe substrate. HIV/AIDS patients often consume *M. oleifera* for chronic purposes; hence TDI investigation is required to ascertain the effect of prolonged use of the herb on CYP3A4 activity in both HLM and hepatocytes. Additionally, pharmacokinetic studies conducted in Swiss albino mice indicated that *M. oleifera* increased plasma concentration of rifampicin when administered concurrently (Pal *et al*., 2011). Therefore *in vitro* systems such as recombinant CYPs, HLM and human hepatocytes which are widely recommended for HDI studies will be employed to further investigate the effect inhibitory effect of *M. oleifera* on CYP3A4.

1.7.2 *Kalanchoe crenata*

Kalanchoe crenata is a freshly herbaceous plant belonging to the family of Crassulaceae also known as the Bryophyllum. It is commonly known as “never die” or “dog’s liver”. Though *K. crenata* originated from Madagascar, it is now found in many tropical regions of Africa (Adjanohoun *et al*., 1996). Traditional health practitioners (THPs) extensively use *K. crenata* for treatment of several diseases. Juice from fresh leaves is employed to treat asthma, headache, convulsion, abdominal pain and smallpox. In West Province of Cameroon, the juice is widely used by THPs to treat diabetes mellitus (Kamgang *et al*., 2008). The use of *K. crenata* as antidiabetic herbal product in HIV/AIDS patients has been attributed to an increased in blood sugar levels reported as an adverse effect associated with the comsumption of some ARVs. Anecdotal cases of transient insulin-dependent diabetes mellitus have been reported in patients on didanosine (Garcia-Benayas *et al*., 2006). Thus, *K. crenata* may affect the metabolism of such ARVs when administered concurrently. However, no study has been reported on the potential of *K. crenata* to interact with drugs.

1.7.3 *Kalanchoe integra*

Kalanchoe integra belongs to the family "Crassulaceae" and is widely used in folklore medicine for cerebrovascular disorders, especially stroke (Dokosi, 1998). Species can be found in tropical and subtropical countries and is mostly referred to as the "life-plant" (Boyle, 1995). HIV infection can result to stroke through several mechanisms, including opportunistic infection, vasculopathy, cardioembolism, and coagulopathy (Benjamin *et al*., 2012). *K.integra* is also reported to be effective in the management of depression. Anecdoctal reports have indicated the use of *K. integra* in HIV/AIDS patients. This may be attributed to the benefits of *K. integra* in the management of cerebrovascular disorders and depression which are co-mordities associated with HIV/AIDS. However, no study on the potential of *K. integra* to cause HDI has been documented.

1.7.4 *Lessertia frutescens*

Lessertia frutescens, popularly known as Cancer bush (Sutherlandia) has been used as part of traditional medicine system in South Africa for many years. It is widely consumed by HIV-infected patients in South Africa as an immune booster and to improve well-being (Gericke 2001). Concoctions of *L. frutescens* are used for the treatment of diseases such as diabetes, rheumatoid arthritis and gastrointestinal disorders (van Wyk *et al*., 2008). Tablets of *L. frutescens* are sold in several Pharmacy shops and Supermarkets in South Africa as an adaptogenic tonic to stimulate appetite and to counteract the muscular dystrophy in HIV/AIDS patients. A study on phytochemical constituents of *L. frutescens* showed that neither the plant nor its constituents inhibited atazanavir transport and metabolism in Caco-2 cell lines and HLM, respectively (Muller *et al*., 2012). However, this study failed to isolate individual triterpenoids (Sutherlandioside A-D) and quercetin glycoside (suthelandin A-D) constituents of *L. frutescens*. Extensive *in vitro* studies conducted to investigate the influence of crude methanol extract of *L. frutescens* on the 5 major CYPs activities also did not incorporate the isolated compounds (Fasinu *et al*., 2012). Another study conducted in rats indicated that chronic administration of *L. frutescens* reduced AUC and C_{max} values of nevirapine significantly (Minocha *et al*., 2011). The current study, therefore takes into an account the reversible and irreversible inhibitory effects of sutherlandioside A-D and sutherlandin A-D on metabolic activities of the 5 major CYPs.

1.7.5 *Hypoxis hemerocallidea*

Hypoxis hemerocallidea is widely distributed in the southern Africa sub-region. The corm of the plant is commonly referred to as ‘African potato’ due to its potato-like shape. African potato extracts, powders, infusions and decoctions have been used for centuries as traditional medicine for treatment and management of cancer, immune-related diseases, urinary tract infections and heart weaknesses (Singh, 1999). In Swaziland, *Hypoxis* is popularly known as “*zifozonke*” referring to its application for treatment of diverse diseases (Amusan *et al*., 2007). Folkloric reports have documented the use of *Hypoxis* for treatment of prostate hyperplasia (Singh, 1999). Scientific investigations into immunostimulant properties of *Hypoxis* have gain attention in both academia and research laboratories in South Africa due to the purported benefit in HIV-infected patients. *Hypoxis* tablets and plant materials are available as supplements for immune-related illness such as cold, flu and arthritis in the pharmacy shops and mushroom stores (Mill *et al*.,

2005). A prospective study to access HIV patients' use of traditional, complementary and alternative medicines (TCAM) and its effect on ARV adherence at three public hospitals in Kwazulu-Natal, South Africa, implicated *H. hemerocallidea* as a major herb consumed by these patients prior to the introduction of ARV (Peltzer *et al*., 2011). However, there is no clinical or experimental evidence to support its immunostimulant claim.

Substantial numbers of studies on the influence of *H. hermerocallidea* on *in vitro* metabolism of probe substrates of CYPs and antiretroviral drugs have been conducted (Brown *et al*., 2008; Mogatle *et al*., 2008). Study conducted by Fasinu *et al*., (2013) implicated *H. hemerocallidea* as mild inhibitor of major CYPs, OATP1B1 and OATP1B3. Also, investigation employing pure compounds of *H. hemerocallidea* implicated stigmasterol and norlignans as inhibitors of CYP3A4/5 and CYP2C19 mediated metabolism (Nair *et al*., 2007). However, with introduction of different formulations of this plant material into the market, there is likelihood of variations in the strength of inhibitory effect on the activities of the CYPs. Such effect may produce different levels of systemic toxicity or inefficacy when administered together with ARVs. Also, the effect of *H. hemerocallidea* in HIV/AIDS patients on both ARVs and anti-tuberculosis medications like rifampicin is unknown. Hence capsules and roots of *H. hemerocallidea* have been included in this study to investigate their effects on CYPs and HepG2 transfected PXR treated with rifampicin.

1.7.6 *Echinacea purpurea*

Echinacea species are examples of herbal products with an overwhelming popularity in both developed and developing countries. *Echinacea* preparations are ranked as one of the important herbal medications used globally for the management of upper respiratory infections, influenza and common cold. In the United States, the sale of *Echinacea* is ranked number 2 with its sale reaching above $36 million in the year 2006 (SPINS, 2007). Though there are nine different species of *Echinacea*, most preparations are made from three namely *Echinacea purpurea*, *Echinacea angustifolia* and *Echnacea pallida*. Liquid extracts, capsules, tablets, dried roots, creams, gels and teas are commercially available in both pharmacy shops and mushroom stores as over-the-counter preparations. It is however estimated that about 80% of *Echinacea* products currently in the markets are prepared from *E. purpurea* (Li, 1998). In HIV-infected patients, *E.*

purpurea preparations are predominantly consumed for its immunomodulatory and antiviral properties (Behm *et al*., 2004). The practice of *E. purpurea* consumption for its purported benefit is gaining popularity in HIV-patients resident in some sub-Saharan African countries.

In vivo study in fifteen HIV-infected patients on boosted protease inhibitor darunavir-ritonavir showed that concurrent consumption of *E. purpurea* together with the ARV is safe (Moltó *et al*., 2011). Another study on the inhibitory effect of *E. purpurea* employing fluorescent probe substrates implicated the plant material as mild inhibitor of CYP3A4 (Yale and Glurich, 2005). However, comparative *in vitro* studies on *E. purpurea* indicated lower inhibitory effect on CYP3A4 when testosterone was used as probe substrate compared to the fluorescent substrates benzyloxy-trifluoromethylcoumarin (BFC) and 7-benzyloxyquinoline (BQ) (Hansen and Nilsen, 2008). Although *E. purpurea* demonstrated weak inhibitory effect on CYP3A4 activity, only human recombinant CYP3A4 was investigated. The metabolic clearance of testosterone might be affected by transcellular diffusion and presence of other drug metabolizing enzymes in human hepatoytes. This study therefore employs hepatocytes in addition to HLM to investigate the effect of *E. purpurea* on metabolic clearance of testosterone.

E. purpurea has been reported in both *in vitro* and *in vivo* studies as an inducer of CYP3A4 (Gorski *et al*., 2004; Mrozikiewicz *et al*., 2010; Penzak *et al*., 2010; Modarai *et al*., 2011). However, the underlying mechanism for CYP3A4 induction remains unclear. The PXR has been implicated as the nuclear receptor gene responsible for induction of CYP3A4 **(**Meijerman *et al*., 2006). The current study will attempt to investigate the underlying mechanism for CYP3A4-mediated induction by *E. purpurea* in HepG2 transfected with human PXR.

1.7.7 *Taraxacum officinale*

Taraxacum officinale is an herbaceous perennial plant. A first reference to its application is reflected in its name, which is derived from the Greek words “taraxis” for inflammation and “akeomai” for curative. In English speaking countries, *T. officinale* is commonly known as dandelion, from the French word - dent-de-lion. This refers to the serrated leaves of the plant (Schütz *et al*., 2005). The first evidence for its therapeutic use by Arabian physicians‘dates back 10 and 11th centuries for treatment of liver and spleen ailments (Sweeney *et al*., 2005). The

German physician and botanist Leonhard Fuchs (1543) described its use, among others, to medicate gout, diarrhoea, blister, spleen and liver complaints. In North American aboriginal medicine, infusions and decoctions of the root and herb were applied to remedy kidney disease, dyspepsia and heart-burn (Sweeney *et al*., 2005). Other folklore uses of the plant include arthritis, eczema, diabetes mellitus and bronchitis. Drug-induced diabetes associated with didanosine in HIV/AIDS paitents might account for the use of *T. officinale* by these patients (Garcia-Benayas *et al*., 2006). Also, diarrhoea is a major opportunistic co-morbidity in HIV/AIDS patients due to increase availability of enteric parasites (Kulkarni *et al*., 2009). Thus, the use of *T. officinale* in such immunocompromised patients may be attributed to its potential to reduce diarrhoea. An *in vitro* investigation to determine the anti-viral property of *T. officinale* aqueous extract indicated potent inhibitory effect against HIV-1 replication and reverse transcriptase activity (Han *et al*., 2011). In traditional Chinese medicines, *T. officinale* extract is added to herbal preparations to boost immune system in immunocompromised patients. A study conducted in Wistar rats indicated that *T. officinale* inhibits CYP3A4 (Maliakal and Wanwimolruk, 2001). However, the effect of *T. officinale* on other intestinal CYPs and P-glycoprotein is unknown.

1.8 Justification of study

The consumption of herbal medicines has become popular in most developed and developing countries. These medicines are well distributed throughout several outlets including pharmacy shops, chemical sellers and even at the local markets. Advertisement of herbal medicines frequently stream on the internet with attractive purported pharmacological effects convincing patients to patronize them. Majority of such medicines are used for the treatments and managements of chronic diseases such as diabetes, hypertension, migraine and sexual impotence. The use of herbal medicines among HIV/AIDS patients has received attention in the clinics and research institutions as their potential to cause inefficacy and/or systemic toxicity can be detrimental to the patient. Surveys and clinical studies have indicated that a significant proportion of HIV/AIDs patients acquire herbal medicines purported to have antiviral properties and to ameliorate idiosyncratic ADRs associated with some of the antiretrovirals. Such practice might lead to potential interactions between the antiretroviral drugs and the active ingredients of

the herbal medicines, further endangering the health of these patients. Unlike herbal medicines found in Westernized countries with extensive information on their potential to cause HDIs, safety information for most herbal medicines in sub-Saharan Africa is scanty.

In most pharmaceutical companies, the *in vitro* methods employed in this study are routinely used at drug discovery and development processes to screen new chemical entities with the potential to cause drug interactions (Figure 1.9). Early screening process using this approach facilitates attrition of drugs and prevents postmarket withdrawal of medications with DDI potential. For herbal medications already being used by consumers, such *in vitro* investigations are conducted by pharmaceutical companies and other dedicated laboratories to highlight the likely risk associated with these medications once concurrently used with orthodox drugs. Studies of this nature offer the possibility of drug re-labeling or insertion of blackbox warning as reported in the case of SJW and indinavir and further serves as the basis for justification of studies in healthy subjects during ethics approval.

This study aims to investigate the likely risk of drug interactions between popular herbal medicines used by HIV/AIDS patients and probe substrates of CYPs and P-glycoproteins using *in vitro* models, and to further make an *in vivo-in vitro* estimation. CYPs and P-gp play a critical role in the metabolism and absorption of antiretroviral drugs. The CYP3A4 and CYP2B6 are responsible for the metabolism of efavirenz and nevirapine. Efavirenz also inhibits CYP2C9 and 2C19 to a less extent. The protease inhibitors (PIs) are substrates primarily for CYP3A4. For example ritonavir is known as the most potent inhibitor whilst lopinavir and tipranavir are inducers of CYP3A4. A case study reported HIV RNA resistance to indinavir and lamivudine in a patient using SJW, a potential cause of therapeutic failure (Chiba *et al*., 1996). Also drugs such as amitriptyline, acetaminophen and diazepam metabolized by CYP1A2 are used for the management of peripheral neuropathy in HIV/AIDS patients.

Most herbal medicines are consumed orally. The GIT conditions such as the low pH (pH 1-4), intestinal motility and the barrier influence the physico-chemical properties and absorption of an administered drug. The routine HDI studies *in vitro* introduce the herbal medicines or the active ingredients directly unto the CYPs. Such approach might lead to false positives and negatives predictions. Hence, introduction of absorption models in the prediction of HDI might increase the accuracy of such predictions.

Parallel artificial membrane permeability assay (PAMPA) is an absorption model employed in most pharmaceutical and research laboratories to monitor the passive permeability properties of new candidate drugs. Such parameters help medicinal chemist and the pharmacologist to make necessary modification to the functional groups of a drug candidate, improving its permeation through the intestinal membrane and also enhance the intrinsic activity. Herbal medicines contain several active ingredients with some likely to be impermeable via the intestinal membrane barrier. Though passive and active permeation regulate the absorption of herbal medicines through the intestinal membrane, the use of PAMPA as a passive permeability model to monitor the inhibitory effects of herbal medicines on CYPs activity will reduce the underestimation or overestimation of HDI observed in the conventional approach. Thus integration of PAMPA into CYPs screening assays in this study offers a paradigm shift in the conduct of HDI, reducing the tendency for false positive and negative predictions.

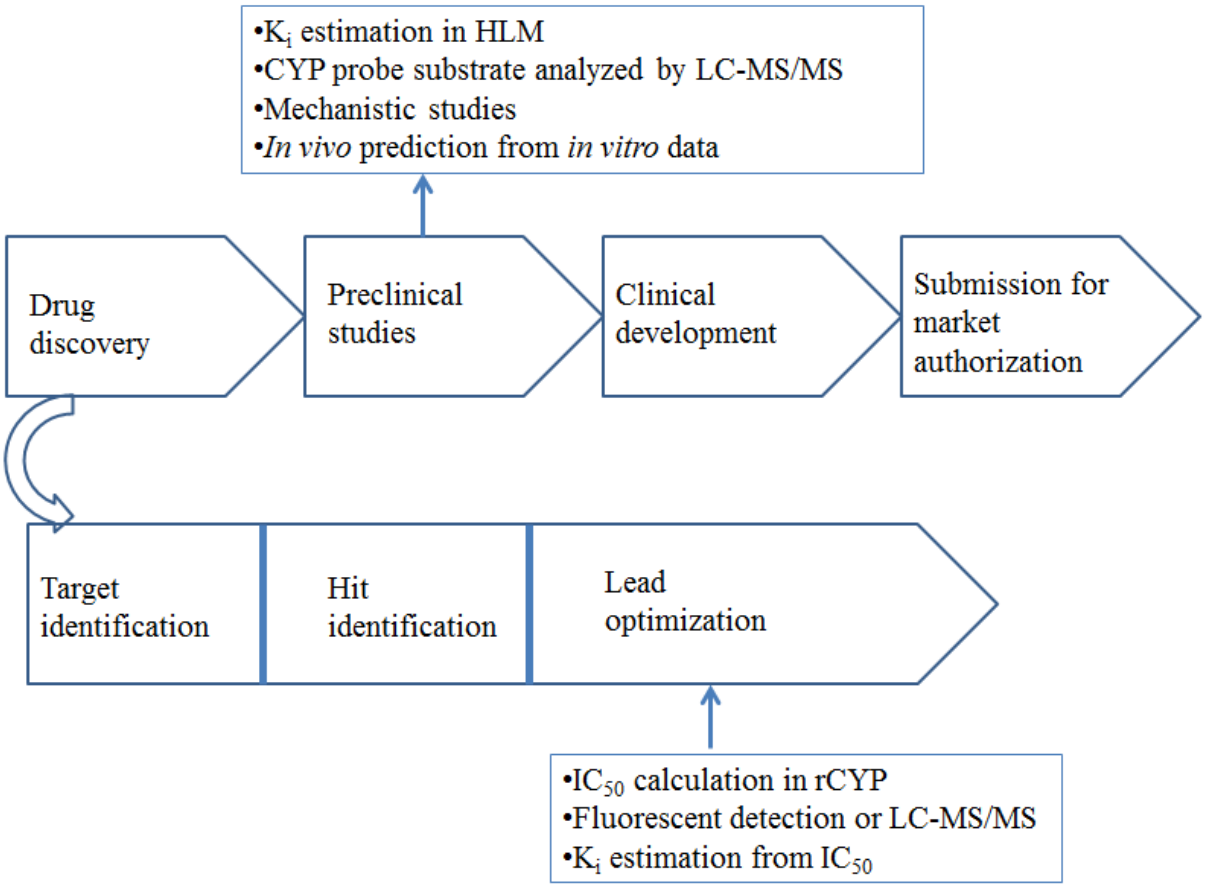


Figure 1.9 Timing of *in vitro* metabolism studies during drug discovery and development (adapted from Wienkers and Heath, 2005)

1.9 Aim of the thesis

The primary aim of the thesis was to apply the *in vitro* models used in DDI studies to investigate interactions of selected herbal medicines with conventional probe substrates of the five major drug metabolizing enzymes and P-glycoproteins.

The specific aims were to:

1. investigate inhibition of CYPs and P-glycoprotein by herbal medicines namely; *Moringa oleifera*, *Kalanchoe integra*, *Kalanchoe crenata*, *Echinacea purpurea*, *Lessertia frutescens*, *Hypoxis hemercallidea* and *Taraxacum officinale* in recombinant CYPs, HLM, hepatocytes and MDCKII-MDR1 cells.
2. rank the potential risk of each herb to cause HDI employing FDA draft guideline for DDI (2012)
3. investigate the degree of inhibition of CYP by the above mentioned herbal medicines after permeation through PAMPA.
4. investigate the modulation of PXR and regulation of CYP3A4, CYP1A2 and P-gp genes in activated PXR by the selected herbal medicines.

1.10 Ethical consideration

This study was approved by the University of Stellenbosch Health Research Ethics Committee with Ethics Reference number S12/09/249 (Apeendix 2).

CHAPTER TWO

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Chemicals

3-Cyano- 7-ethoxycoumarin, sulfaphenazole, ketoconazole, quinidine, troleandomycin, caffeine and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). 7-Benzyloxy-4-trifluoromethylcoumarin, 7-methoxy-4-trifluoromethylcoumarin, and 7-methoxy-4-aminomethylcoumarin and PAMPA were obtained from BD Gentest (Woburn, MA). Cimetidine was obtained from Research Institute of Smithkline and French Laboratories Ltd. Tranylcypromine, ketoconazole and 0.5M potassium phosphate buffer (pH 7.4) were from BD Gentest (Woburn, MA, USA). Troleandomycin was from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Testosterone, 6β-hydroxytestosterone, magnesium chloride, and corticosterone were from Sigma-Aldrich (St Louis, MO, USA). NADPH regeneration solution A and B were purchased from Corning Discovery Labware (Woburn, MA, USA). HPLC grade acetonitrile, methanol and formic acid were from Fisher Scientific (Waltham, MA, USA). Solid Phase Extraction (SPE) cartridges (silica gel 10 g/60 mL, code 57134) were purchased from Supelco (Bellefonte, PA, USA). Organic solvents (chloroform and methanol) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). CellTiter 96® AQueous One Solution Reagent containing tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS and an electron coupling reagent (phenazine ethosulfate; PES) were obtained from Promega, Wisconsin, USA.

2.1.2 Biologicals

2.1.2.1 Drug metabolizing enzymes

Bactosomes prepared from *Escherichia coli* cells coexpressing recombinant human NADPH-P450 reductase and individual human P450s (CYP1A2, 2C9, 2C19, 2D6 and 3A4) were purchased from BD Gentest (Woburn, MA, USA). Mixed gender pooled human liver microsomes prepared from 25 donors with protein concentration (20-26 mg/mL) and total P450 concentration (≥0.320 nmol/mg) were obtained from BioreclamationIVT (Baltimore, USA).

2.1.2.2 Cell cultures

Pooled, mixed-gender cryopreserved hepatocytes prepared from 20 donors and Hepatozyme media were obtained from BD Gentest (Woburn, MA), stored in a liquid nitrogen tank (until use), and thawed according to supplier instructions before use. MDCK-II (parental) and hMDR1-MDCK-II (transfected) cell lines were a gift from Dr. Gottesman (NIH, Bethesda, USA). The pSG5-hPXR expression vector was provided generously by Dr. Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX, USA; Lehmann *et al*., 1998) and the reporter plasmid CYP3A4-PXR response element (PXRE)-LUC (containing the proximal 0/–362 and distal 7208/7797 PXRE regions fused upstream of luciferase; Goodwin *et al*., 1999) was a kind gift from Dr. Christopher Liddle (University of Sydney, Westmead, NSW, Australia).

2.1.2.3 Other reagents

Dulbecco's Modified Eagle Medium (DMEM-F12), Minimal Essential Medium (MEM), Hanks Balanced Salt Solution (HBSS), HEPES, Trypsin EDTA, Penicillin-streptomycin and Sodium Pyruvate were purchased from GIBCO BRL (Invitrogen Corp., Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Hyclone Lab Inc. (Logan, UT, USA). All plastic-wares for mammalian cell culture were purchased from Corning Costar Corp. (Corning, NY, USA). Qiagen Rneasy® mini and micro kit were from Qiagen Ltd (Austin, TX, USA). TaqMan® Universal PCR Master Mix Reagents, SYBR® Green PCR Master Mix Reagents and TaqMan® probes were obtained from Bio-Rad laboratories (Hercules, CA, USA).

2.1.3 Herbal medicines and products

The herbal medicines and products were obtained from traditional medicines suppliers and pharmacy shops. A total of seven (7) raw herbal medicines, two capsules and one tablet were obtained from different suppliers (Table 2.1). *Taraxacum officinale* and *Hypoxis hemerocallidea* sourced from Medico Herbs, Somerset West, Western Cape, were identified and authenticated with the assistance of experts in the Compton Herbarium, South African National Biodiversity Institute, Cape Town and samples kept at the Division of Clinical Pharmacology, Stellenbosch University. The following voucher numbers were assigned to these plant materials: *L. frutescens* (#3222), *E. purpurea* (#13117), *K. crenata* (#2865) and *M. oleifera* (#11695) obtained from the repository of the National Centre for Natural Product Research (NCNPR), University of Mississippi, USA. Information on the mode of use, dose, and specific HIV/AIDS related indications were indicated on the various labels.

2.1.4 Pure compounds of *L. frutescens*

Pure compounds of Sutherlandioside B and D, and combination of Sutherlandin A &B, C &D from *L. frutescens* were obtained from Dr. Xing-con Li, National Center for Natural Products Research, the University of Mississippi, Mississippi, USA (Figure 2.1A and B).

Table 2.1 Herbal medicines commonly used by HIV/AIDS patients in Africa.

Preparation	Manufacturer	Plant species	Voucher number	Code for sample preparation
Lessertia (herbal powder, tablets)	Capsule - Phyto nova natural medicines [KwaZulu Natal, South Africa], Herbal powder - NCNPR	*Lessertia frutescens* subspecies microphylla (SU1)	NCNPR 3222	Aqueous: T50 (tablets), T79 (tea cuts). Methanol: LFT (tablet), LFC (tea cut) Methanol: 39SF (whole plant), LFT (tablets)
Hypoxis (capsules and powdered tubers)	Medico Herbs [Cape Town, South Africa]	*Hypoxis hemerocallidea*	-[N]	Aqueous: T41 (capsule), T30 (powdered leaves). Methanol: HHC (capsules), HHL (leaves)
Dandelion (herbal roots)	Medico Herbs [Cape Town, South Africa]	*Taraxacum officinale*	-[N]	Aqueous (T66), methanol (TO)
Echinacea (capsules) and whole plant	Capsule - Herbal solutions [Harare, Zimbabwe] Whole plant - NCNPR	*Echinacea purpurea*	NCNPR 13117	Aqueous: capsule (T60) Methanol: EP (whole plant material), 2660 (capsules)
Moringa (powdered leaves)	Herbal solutions [Harare, Zimbabwe] and NCNPR	*Moringa oleifera*	NCNPR 11695	Aqueous (2680), Methanol (MO)
Kalanchoe (whole plant)	Whole plant -NCNPR	*Kalanchoe integra* *Kalanchoe crenata*	-[N] NCNPR2865	Aqueous (T61), Methanol (2600). Aqueous (T65), Methanol (39KC)

-[N] refers to no voucher number for herbs identified by the assistance of experts in the Compton Herbarium, South African National Biodiversity Institute, Cape Town.

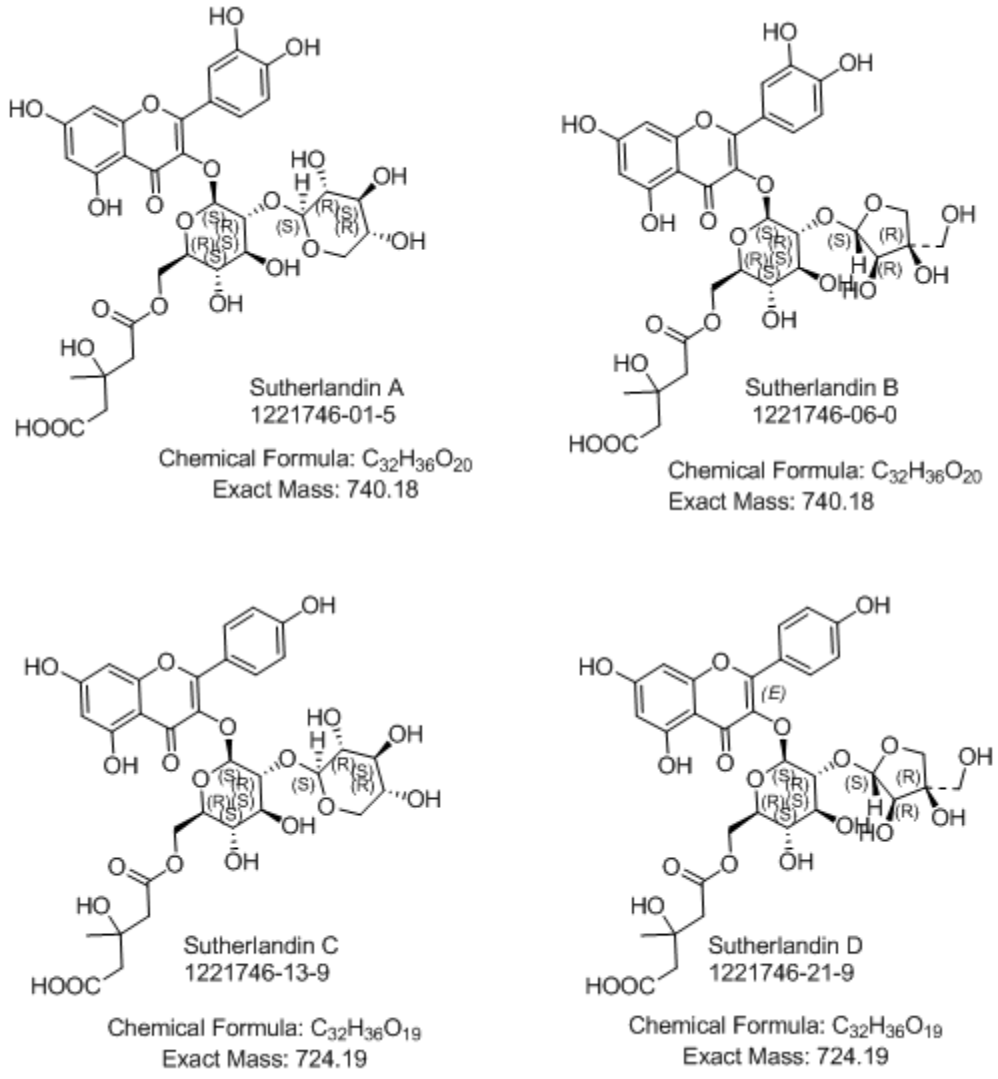


Figure 2.1A Structures of flavonoids in *L. frutescens* used in this study (adapted from Bharathi *et al*., 2010).

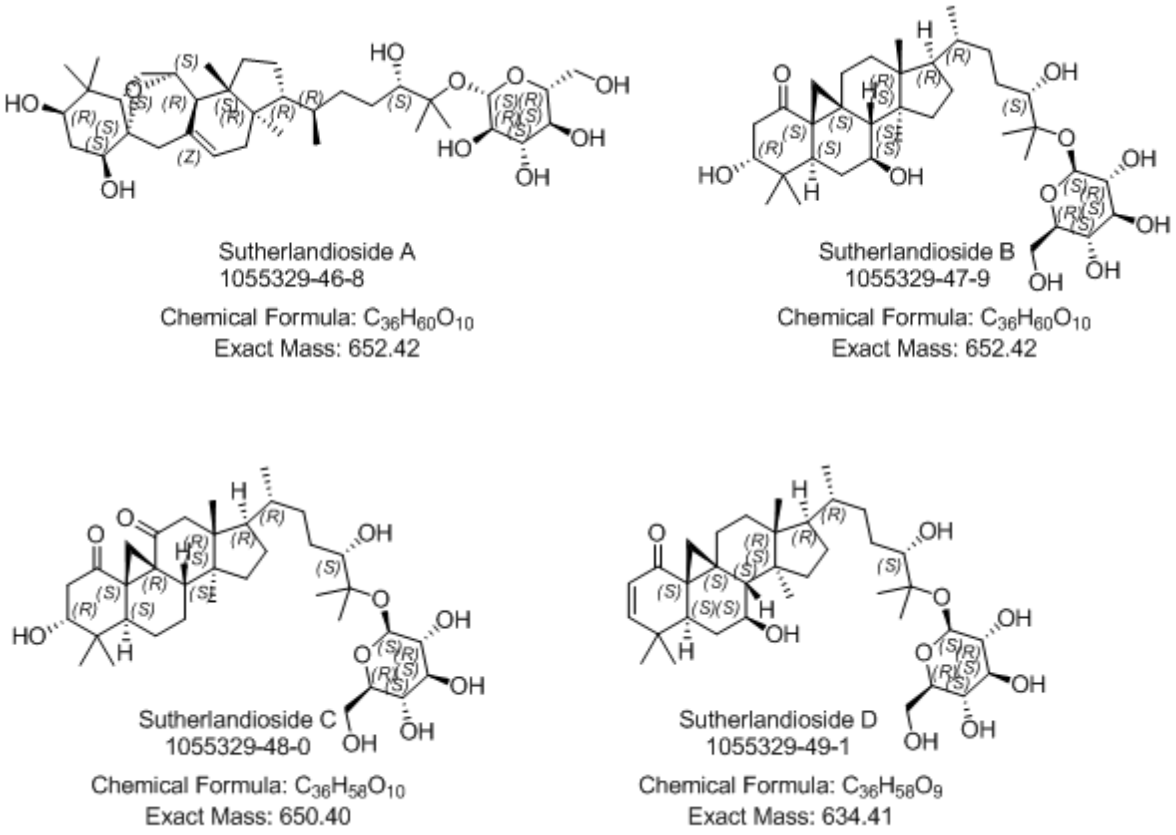


Figure 2.1B Structures of saponins in *L. frutescens* used in this study (adapted from Bharathi *et al*., 2010).

2.2 Methods

2.2.1 Preparation of herbal medicines

2.2.1.1 Aqueous extraction of herbal medicines

Powdered plant materials (10.0 g) were extracted with hot water in a round bottom flask. The flasks were placed in a water bath at 60 ^{o}C with constant shaking for 1 hour to mimic the indigenous mode of extraction. The mixture was removed from the water bath and allowed to extract for 72 hours at room temperature, decanted, and centrifuged at 13000 x g for 5 mins. The supernatant was filtered (0.45 μm; Whatman International LTD, Maidstone, England) and freeze dried. The dried aqueous extracts were put into plastic bottles, labeled as *H. hemerocallidea* capsule (T41) and powdered leaves (T30), *L. frutescens* tablets (T50) and tea cut (T79), *T. officinale* root (T66), *E. purpurea* capsules (T60), *M. oleifera* powdered leaves (T80) and stored at -20 ^{o}C for further use throughout the study.

2.2.1.2 Methanol extracts and fractions of herbal medicines

Each dried plant material (10.0 g) was extracted four times with 100 mL of methanol by sonication (30 min), centrifuged at 13000 x g for 5 minutes at 25^{o}C and filtered. The solvent was evaporated under reduced pressure in a vacuum dryer at 37 °C to give a dried methanolic crude extract. The crude extract (820.0 mg) was then suspended in methanol (7.0 mL) and adsorbed on celite (1.0 g). The solvent was evaporated and the powdered residual material was loaded onto a 10 g solid phase extraction (SPE) silica gel cartridge and fractionated using $CHCl_3$ (100 mL), $CHCl_3$:MeOH 9:1 (100 mL), $CHCl_3$:MeOH:H_2O 8:2:0.5 (150 mL), and methanol (150 mL). Fractions of 25 mL were collected and combined according to their TLC profile to give different fractions (Figure 2.2A-D). Samples of crude methanolic extract and fractions were dissolved in methanol (10.0 mg/mL) and stored at -20 ^{o}C for further use throughout the study.

2.2.1.4 Extraction of herbal capsule

200 mg capsule preparations of *Echinacea*, *Lessertia*, and *Moringa* were transferred into separate Eppendorf tubes and each extracted four times with 1.0 mL of water or methanol. The tubes containing appropriate solvent and capsules were first shaken by hand and sonicated for 30 mins. The extracts were centrifuged at 13000 x g for 5 minutes at 25 ^{o}C. The supernatants were transferred into clean Eppendorf tubes and re-centrifuged as described above. The final

supernatants were dried, dissolved in aqueous or methanol (10 mg/mL) and stored at -20^{o}C for further use throughout the study.

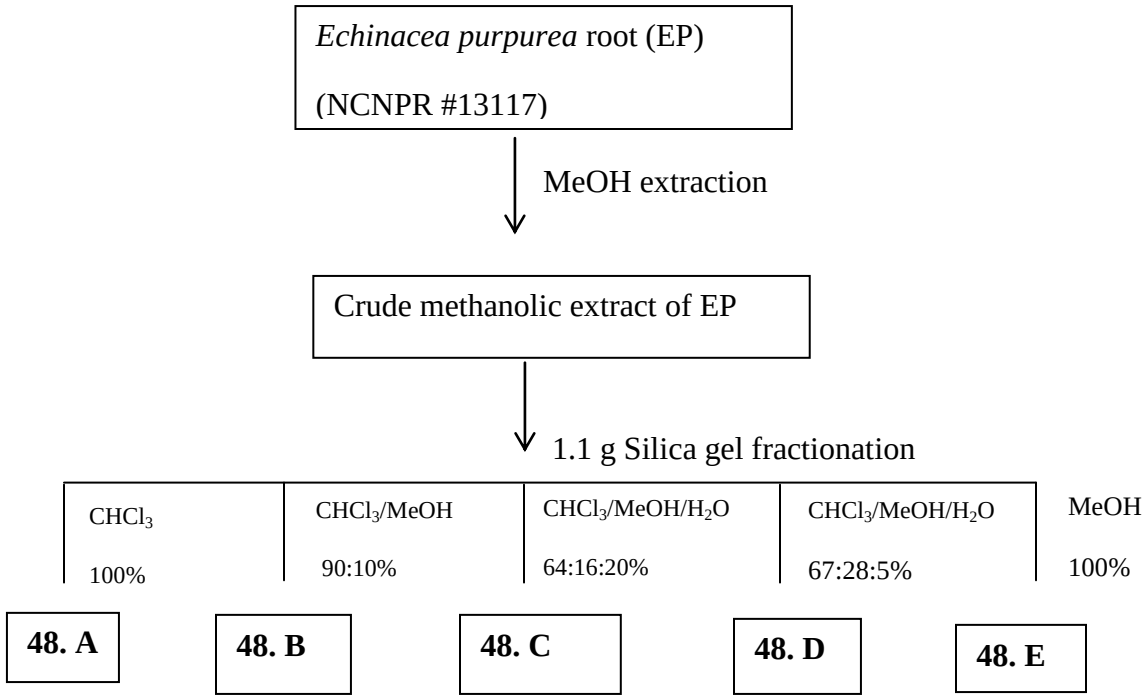


Figure 2.2A Schematic representation of fractionation of methanolic crude extract of *E. purpurea*

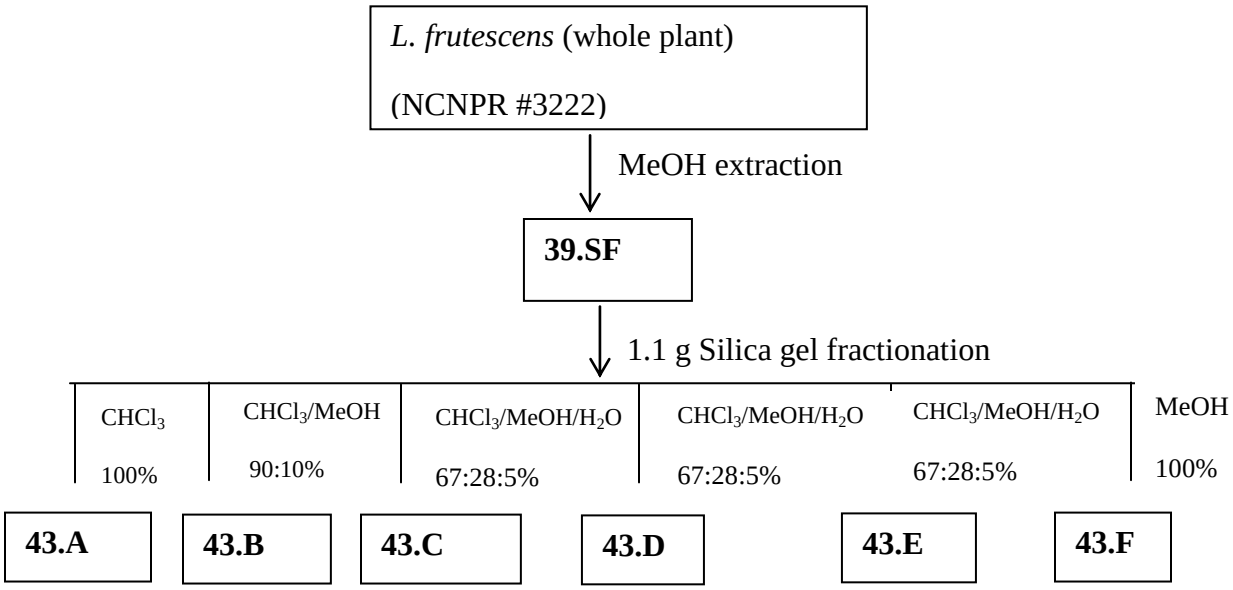


Figure 2.2B: Schematic representation of fractionation of methanolic crude extract of *L. frutescens*

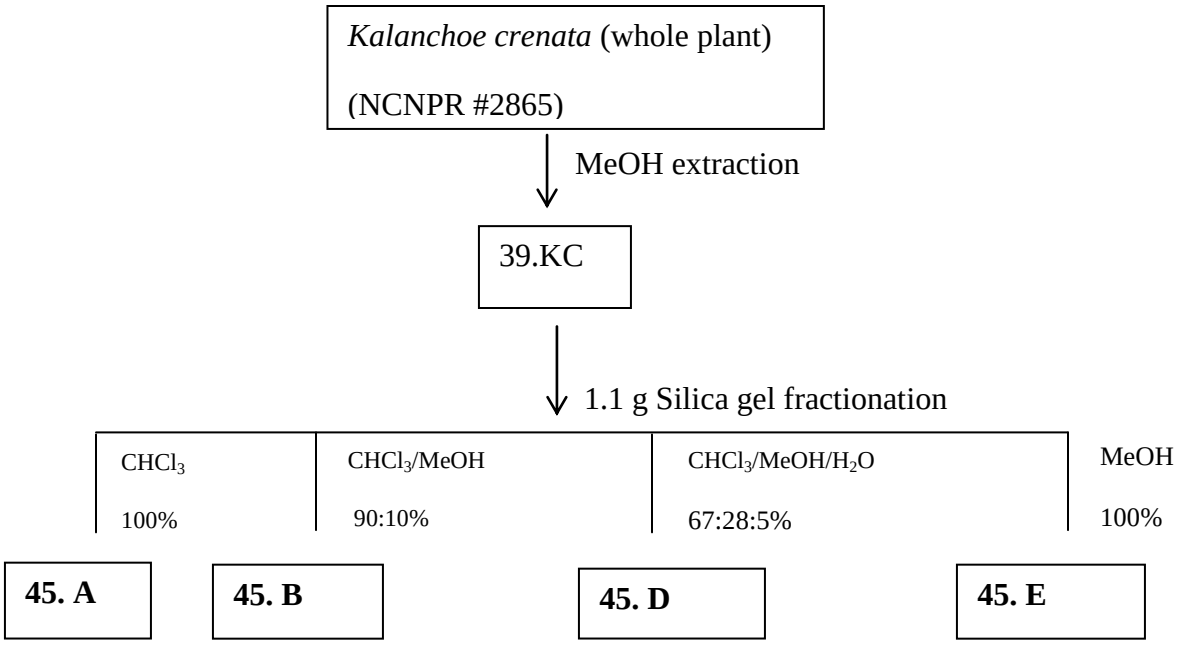


Figure 2.2C: Schematic representation of fractionation of methanolic crude extract of *K. crenata*

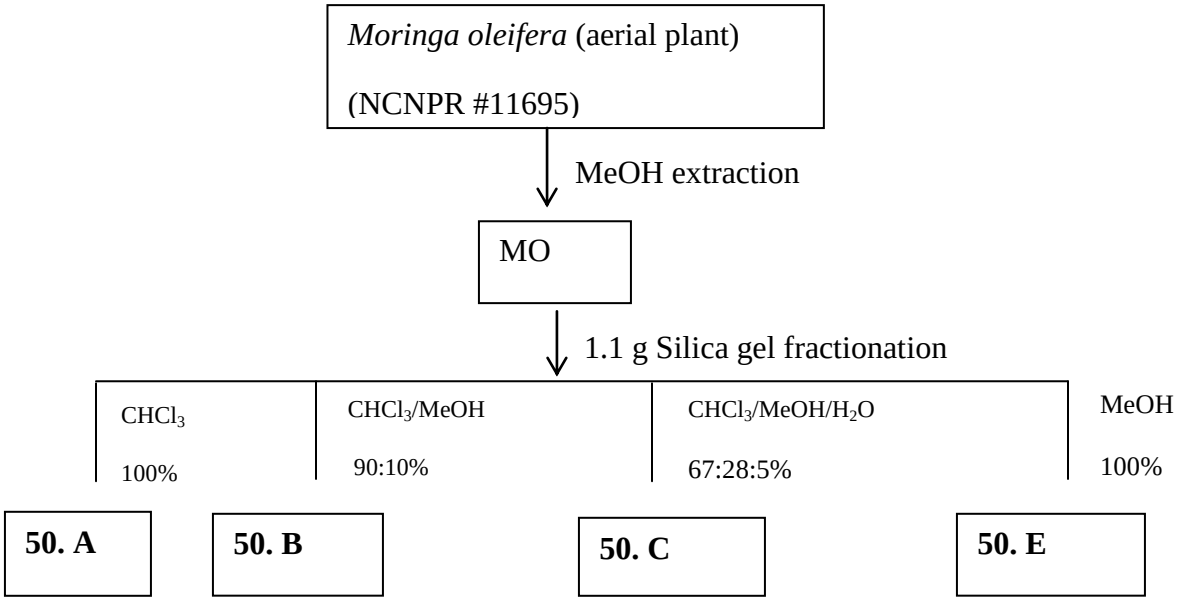


Figure 2.2D: Schematic representation of fractionation of methanolic crude extract of *M. oleifera*

2.2.1.5 Extraction of herbal tablets

Tablets of *Hypoxis* were ground in a mortar before extraction. 200 mg of pulverized tablets were transferred into Eppendorf tubes and dissolved in water or methanol, hand shaken, sonicated for 30 mins and centrifuged as described in sub-section 2.2.1.4. The final supernatant was dried, dissolved in appropriate solvent used for the extraction (10 mg/mL) and stored at -20^{o}C for further use throughout the study.

2.2.3 Inhibition of CYPs by herbal extracts and fractions

2.2.3.1 Reversible inhibition assays

2.2.3.1.1 Two point screening and IC_{50} determination

The herbal extracts were evaluated for CYP inhibition in an assay set up based on recombinant CYPs metabolizing substrates which produce fluorescent metabolites. The assays for the 5 major CYPs were set up and run according to the method described by Crespi *et al*., (1997) with slight modifications as described by Bapiro *et al*., (2000). A two stage evaluation procedure was followed, the first one being a two concentration of herbal extract inhibition screening against each of the 5 CYPs. The second stage involves the determination of IC_{50} for each herbal extract for only CYPs in which over 20% enzyme inhibition was observed during the two point concentration screening.

Each herbal extract was screened in black Costar 96-well plates (Corning Incorporated, Corning, NY) according to the protocol published by Crespi *et al*., (1997) under experimental conditions shown in Table 2.2. Each reaction mixture consisted of the appropriate concentration of enzyme, NADPH, substrate concentration equivalent to its K_m, aqueous herbal extracts (0.2 and 2.0 mg/mL) and methanol extracts (1.1 and 100 µg/mL) and potassium phosphate buffer (pH 7.4) as described by GENTEST (www.gentest.com). Phytochemical constituents of herbal medicines have the tendency to behave as fluorogenic compounds, hence auto-fluorescent readings were taken to avoid such errors. The mixtures of cofactors, NADPH, herbal extracts or positive control and buffer in the 96-well plates were pre-incubated for 10 min. An appropriate concentration of enzyme/substrate mixture was added to each well and incubated for 30 mins. The reaction was terminated by addition of ice-cold 20% Tris base/80% acetonitrile (ACN) after

incubation. Activity of the enzymes was monitored by measuring the formation of fluorescent metabolites at specific emission and excitation wavelengths as shown in Table 2.2. The experimental conditions in Table 2.2 were based on the protocol provided by the manufacturer. Fluorescence was measured on a Victor Wallace (1420 multi-plate reader). The per cent residual activity was determined using the equation:

$$\%\ \text{residual activity} = \frac{\text{Test} - \text{Test blank}}{\text{Control} - \text{Control blank}} \times 100$$

For IC_{50} determination, herbal extracts, fractions and pure compounds of final concentration 4 mg/mL (aqueous preparations), 100 µg/mL (methanol preparations), 100 µM (pure compounds from *L. frutescens*) were serially diluted (1:3) in a 100 µL of cofactor/phosphate buffer/NADPH reaction mixture in each 96-well plates. The plates were pre-incubated for 10 mins. 100 µL of enzyme/substrate reaction mixture was added to each well and incubated for 30 mins. The reaction was terminated by addition of ice-cold 20% Tris base/80% acetonitrile (ACN) after incubation. Activity of the enzymes was monitored by measuring the formation of fluorescent metabolites at specific emission and excitation wavelengths for each enzyme.

Table 2.2 Experimental conditions for fluorescent CYPs assays

CYP	1A2	2C9	2C19	2D6	3A4
Enzyme concentration	2.5 pmol/mL	25.0 pmol/mL	25.0 pmol/mL	30.0 pmol/mL	25.0 pmol/mL
NADPH concentration	1.0 mM	1.0 mM	1.0 mM	0.4 mM	1.0 mM
K-Phosphate -Buffer concentration	0.1M	0.1 M	0.1 M	0.1 M	0.1M
Substrate concentration	CEC (3 μM)	MFC (85 μM)	MFC (85 μM)	MAMC (15 μM)	BFC (10 μM)
Metabolite	CHC	HFC	HFC	AHMC	HFC
Excitation/Emission wavelength (nm)	405/460	405/535	405/535	390/460	405/535

Substrates: 7-ethoxy-3-cyanocoumarin (CEC), 7-methoxy-4-trifluoromethylcoumarin (MFC), 7-methoxy-4-(aminomethyl)-coumarin (MAMC), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), Cyano-hydroxycoumarin (CHC), 7-hydroxy-4-trifluoromethylcoumarin (HFC), 3-[2-(diethylamino)ethyl]-7-hydroxy-4-methylcoumarin (AHMC).

2.2.3.1.2 *In vivo* prediction of HDI from *in vitro* data

One of the objectives of this study is to rank the potential risk of HDI according to the US FDA guidelines based on the $[I]/K_i$ ratio where [I] is the concentration of the inhibitor (drug or herb) an individual is exposed to and K_i is the inhibition constant. The predicted percentage of inhibition of a specific CYP *in vivo* was calculated using the estimated K_i values and the estimated [I] as explained in sub-section 1.3.2.

With both the estimated K_i and [I] values, the I/K_i inhibitory ratio was used to rank the herbs with respect to risk for HDI based on FDA guidelines where, I/K_i >1.0 is associated with high risk for DDI, I/K_i = 0.1-1 is associated with intermediate risk for DDI and I/K_i<0.1 is unlikely to result in DDI (Huang *et al*., 2008; Prueksaritanont *et al*., 2013).

2.2.3.1.3 Comparative inhibition of CYP3A4 in recombinant CYP and HLM

The methanol extracts showed stronger inhibitory effect on human recombinant CYP3A4 than the aqueous extracts. To examine whether the inhibition profile of the methanol extracts observed in recombinant CYP3A4 is devoid of overestimated or underestimated, comparative studies in both recombinant CYP3A4 and HLM were done to re-evaluate the inhibition profile of the methanol extracts and fractions. The inclusion of fractions prepared from different solvents serves as a guide to determine the appropriate solvent for isolation of the active phytochemicals in future experiments.

6β-hydroxytestosterone formation was used as a marker reaction for the activity of CYP3A4. Based on the protocol provided by the manufacturer, the incubation mixture contained human liver microsomes (HLMs; 0.6 mg/mL protein) or recombinant CYP3A4 (with protein concentration of 0.04 mg/mL leading to activity identical to that of HLMs incubations) in 0.1M potassium phosphate buffer (pH 7.4). The methanol herbal extracts and fractions (25 – 100 µg/mL) were added to the enzyme mixture and pre-warmed for 10 mins. NADPH regeneration solution B (1.0 mM) was added to the mixture and incubated for 30 mins at 37^{o}C. The reaction was terminated by addition of ice-cold acetonitrile containing 5µL corticosterone as an internal standard. The mixture was centrifuged at 13,000 x g for 5 mins and the supernatant transferred into vials for HPLC analysis.

2.2.3.1.4 HPLC analysis

Testosterone samples were analyzed using a Waters 2695 separation module C_{18} column (3.9 x 150 mm, 5µm), with an isocratic elution and UV detection (254 nm) according to the methods described by Sonderfan *et al*. (1987). The HPLC mobile phase consisted of 30% water / 69.9% ACN (A) and 99.9% ACN (B) both containing 0.1% formic acid at a flow rate of 1.0 mL/min. Each run was followed by 5 min wash with 100% ACN and equilibration period of 10 min. The peak area ratio between 6β-hydorxytestosterone and the internal standard were used to determine the percent remaining activity. The chrotograms indicated retention times of 6.976 mins, 8.322 mins and 10.842 mins for 6β-hydorxytestosterone, corticosterone and testosterone, respectively. Figure 2.3 demonstrates a typical chromatogram obtained from the HPLC analysis.

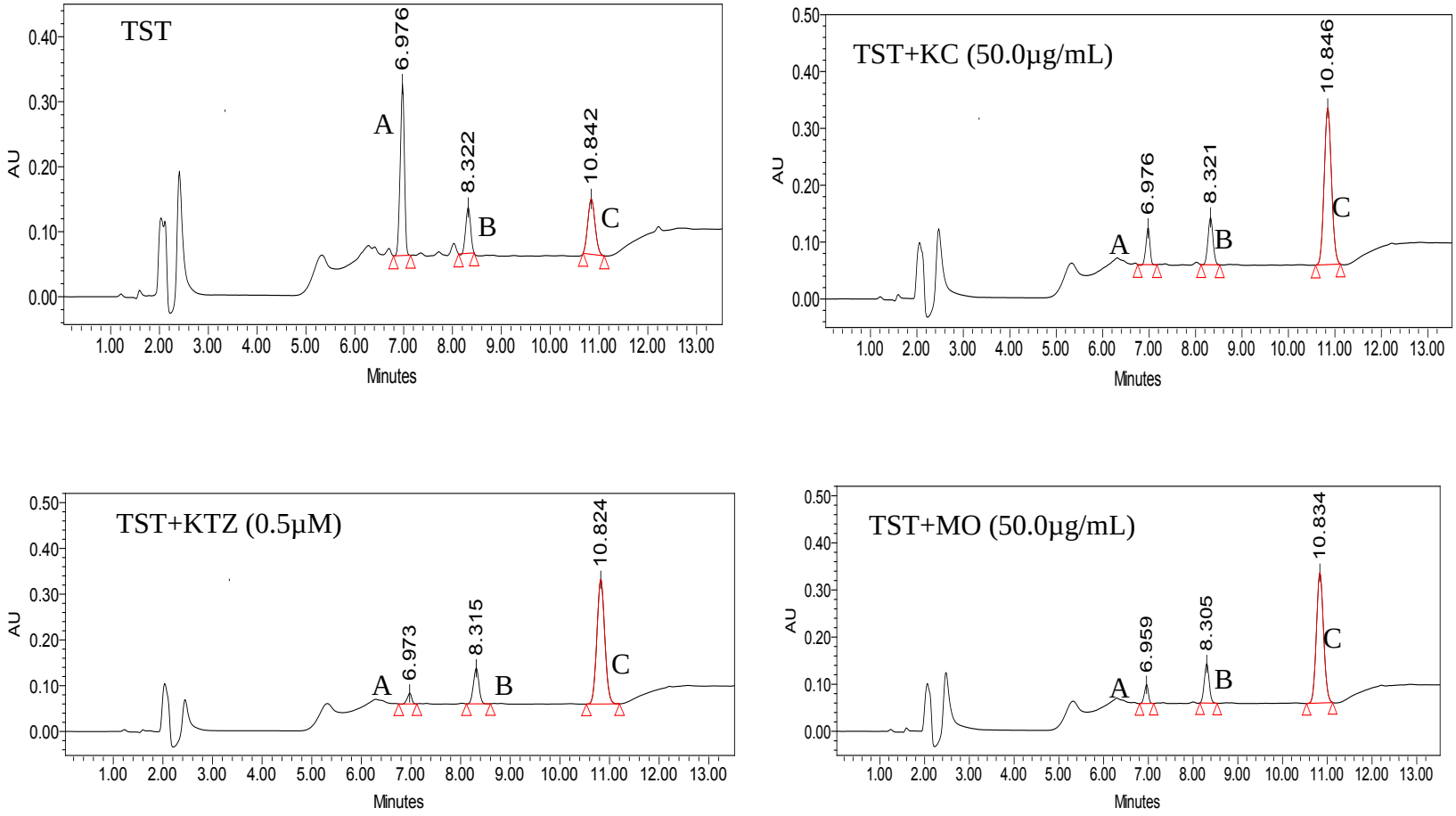


Figure 2.3 Typical chromatograms of testosterone (TST) in the absence and presence of ketoconazole (KTZ), *Kalanchoe crenata* (KC) and *Moringa oleifera* (MO). The three peaks represent 6β-hydroxytestosterone (A), corticosterone (B) and testosterone (C). Details of the chromatographic conditions are provided in the text.

2.2.3.1.5 Assessment of reversible IC_{50}s using a cocktail substrate approach with recombinant human CYPs

As described in the conventional IC_{50} determination (sub-section 2.2.3.1.1), 3 µL of methanol crude extract and fractions of final concentration 100 µg/mL in 150 µL reaction mixture consisting of phosphate buffer (0.1 M), glucose 6-phosphate dehydrogenase (0.6 units/mL in 5 mM sodium citrate buffer 7.5), control protein (0.15 mg/mL) and cofactors (0.14 mM $NADP^+$, 7.2 mM $MgCl_2$, 7.2 mM glucose 6-phosphate) was serially diluted in Costar 96-well black plates. The plates were pre-warmed for 10 mins. 100 µL of enzymes/substrates reaction mixture of 25 pmol CYP3A4/10 µM BFC, 25 pmol CYP2C19/3 µM CEC, 30 pmol CYP2D6/ 15 µM AMMC and 2.5 pmol CYP1A2/ 3 µM CEC mixture in phosphate buffer (0.1 M) was added to each well except for the blank wells and incubated for 30 mins at 37^{o}C. The reaction was terminated by addition of ice-cold 20% Tris base/80% ACN. Activity of the enzymes was monitored by measuring the formation of fluorescent metabolites at specific emission and excitation wavelengths.

2.2.3.1.6 Inhibition of CYP1A2 and CYP3A4 by herbs after absorption through PAMPA

The initial screening of herbs prepared using the hot aqueous extraction as practiced by the THPs implicated CYP1A2 and CYP3A4 as the most affected enzymes. Hence, the inhibitory effect of the hot aqueous extracts on CYP1A2 and CYP3A4 after permeation through PAMPA was investigated. This could give an indication of the inhibitory potential of each extracts when consumed orally if only passive transport is considered.

The pre-coated PAMPA plate system was warmed at 25 ^{o}C for 30 mins. 200 µL of phosphate-buffered saline (PBS) was added to the acceptor compartment. 300 µL of PBS containing 2 mg/mL of aqueous herbal extracts were introduced to the respective wells in the donor compartment (Awortwe *et al*., 2014b). 200 µM of caffeine and cimetidine were used as positive and negative controls, respectively. The plate was incubated at 25 ^{o}C for 5 hours. Concentrations of the controls in both acceptor and donor compartments were measured using UV spectroscopy and effective permeability (P_e) determined. 0.2 mg/mL of herbal extracts were collected from the acceptor compartment and tested on CYPs (1A2 and 3A4) as described in the screening assays (sub-section 2.2.3.1.1).

The integrity of the PAMPA plate system was assessed using the P_e of both positive and negative controls according to the equation (2.1), permeability (in unit of cm/s):

$$P_e = \frac{-\ln[1-C_A(t)/C_{equilibrium}]}{A*\left(1/V_D + 1/V_A\right)*t} \qquad \text{Equation 2.1}$$

$$C\ equilibrium = [C_D(t)*V_D + C_A(t)*V_A]/(V_D+V_A)$$

Where:

C_D (t) = compound concentration in donor well at time t, C_A (t) = compound concentration in acceptor well at time t, V_D = donor well volume (0.3 mL) and V_A = acceptor well volume (0.2 mL), A = filter area (0.3 cm^2), t = incubation time (18000 s).

2.2.3.2 Time-dependent inhibition (TDI) of CYP

2.2.3.2.1 TDI IC_{50} shift fold

Three microliters (3 µL) of crude methanol extract and fractions of herbal medicines of final concentration of 100 µg/mL in 150 µL enzyme/phosphate buffer reaction mixture consisting of phosphate buffer (0.1 M), glucose 6-phosphate dehydrogenase (0.6 units/mL in 5 mM sodium citrate buffer 7.5), control protein (0.15 mg/mL) and cofactors (0.14 mM $NADP^+$, 7.2 mM $MgCl_2$, 7.2 mM glucose 6-phosphate) was serially diluted as described in sub-section 2.2.3.1.1. The concentrations of CYPs were similar to that used in the reversible IC_{50} assay. The plates were pre-incubated for 30 mins followed by addition of 100 µL of substrate reaction mixture of 10 µM BFC or 3 µM CEC for CYP3A4 and CYP2C19, respectively, in phosphate buffer (0.1 M) to each well except for the blank wells and incubated for additional 30 mins. The reaction was terminated using ice-cold 20% Tris base/80% ACN and the activity of the enzymes was monitored by measuring the formation of fluorescent metabolites at similar condition to that of reversible IC_{50}. The fold shift in IC_{50} was determined for each herbal extracts and fractions.

2.2.3.2.2 Application of normalized ratio for TDI screening

TDI screening was conducted using a protocol described by Yamamoto *et al*., (2002) with slight modification. In brief, a two- step incubation consisting of an inactivation and activity assay were performed. In the inactivation assay, 100 µL of aqueous herbal extracts (0.2 and 2.0 mg/mL) and methanol herbal extract (50 and 100 µg/mL), positive control, troleandomycin (1

and 20 μM) and negative control, ketoconazole (0.1 and 1 μM) were incubated with recombinant CYPs and 0.1 M phosphate buffer, pH 7.4 in the absence and presence of NADPH for 30 mins at 37 ^{o}C. The activity assay consisted of aliquots (100 uL) of the inactivation assay to which an appropriate substrate was added and the reaction done for another 30 mins. The mixture was stopped with cold-ice 20% Tris base/80% ACN after incubation and the fluorescent detected immediately.

The time dependent inhibitory effect of each herbal extract on the activity of CYPs was expressed as the normalized ratio as demonstrated in the equation (2.2) below:

$$\text{Normalized ratio} = \frac{(R+1^{NADPH})/(R-1^{NADPH})}{(R+1^{noNADPH})/(R-1^{noNADPH})} \qquad \text{Equation 2.2}$$

Where $R+1^{NADPH}$ is the rate of reaction when incubation was performed in the presence of both inhibitor and NADPH; $R-1^{NADPH}$ is the rate of reaction when incubation was performed in the presence of NADPH but in the absence of inhibitor; $R+1^{noNADPH}$ is the rate of reaction when incubation was done in the presence of inhibitor without an NADPH; and $R-1^{noNADPH}$ is the rate of reaction when incubation was performed in the absence of both inhibitor and NADPH. The normalized ratio was used to classify each test compound as follows: below 0.7 (clear TDI), between 0.7 and 0.9 (intermediate zone with undefined TDI), and above 0.9 (non-TDI) as described by Atkinson *et al*., (2005).

2.2.3.2.3 Single point NADPH and TDI

Methanol extracts and fractions with potential TDI selected based on the IC_{50} fold shift were preincubated (50 μg/mL) with 250 pmol CYP3A4 or CYP2C19/well reaction mixture in the absence and presence of NADPH for 30 mins at 37^{o}C. The single point NADPH dependent inhibition was repeated for 10 and 20 mins preincubation. For TDI, the primary incubate was preincubated for 0, 5, 10 and 20 mins. At the end of each preincubation time point, 10 μL aliquot of the primary incubates was added to 90 μL of substrate mixture in appropriate wells for respective CYPs and incubated for additional 30 mins. The reaction was terminated using an ice-cold 20% Tris base/80% ACN and the activity of the enzymes monitored by measuring the formation of fluorescent metabolites.

2.2.3.2.4 Kinetics of CYP3A4 inactivation

Herbal extracts with strong TDI based on the normalized ratio, the single point NADPH- and the time dependent screening were selected for the TDI kinetics. With reference to the initial TDI screening assays, the test samples demonstrated stronger NADPH-, time- and concentration dependent inhibitory effects on CYP3A4 compared to that of CYP2C19; hence CYP3A4 was focused for the inactivation experiment. The two-step incubation method was used to characterize the time- and concentration-dependent inhibition of CYP3A4 by the active methanol extracts and fractions of *K. crenata* and *M. oleifera*. In the inactivation assay, varying concentrations of the methanol extracts and fractions (1 – 100 µg/mL) were incubated with CYP3A4 (250 pmol/well), cofactor, control protein, and 0.1 M phosphate buffer, pH 7.4. At 0, 5, 10, 20 and 30 mins preincubation time points, an aliquot of the preincubation mixture (1:10 dilution) was taken and added to the activity assay (similar to the TDI assay), and a further 30 mins incubation was carried out at 37°C. The reaction was terminated by addition of ice-cold 20% Tris base/80% ACN solution, and activity was followed by measuring the formation of HFC. To determine k_{obs} values, the decrease in natural logarithm of the remaining activity of CYP3A4 (%) against time was plotted for each inactivator concentration, and the k_{obs} values were described as negative slopes of the lines. Inactivation kinetic parameters were evaluated applying the nonlinear regression of the data to the following expression:

$$k_{obs} = \frac{k_{inact}.[I]}{K_I+[I]} \qquad \text{Equation 2.3}$$

Where [I] represents the conentrations of the inactivators in the preincubation mixture; k_{obs} or λ represents the negatives values of the slopes for the natural logarithm of the percentage remaining activity of CYP3A4; k_{inact} is the limit maximum inactivation rate constant measured in the absence of the inactivator and K_I is the inactivator concentration which yields half of k_{inact}.

2.2.3.2.5 *In vivo* prediction of CYP3A4 inactivation from *in vitro* data

Quantitative prediction of mechanism-based DDI *in vivo* based on *in vitro* data is very challenging even though several mathematical models have been presented. The US FDA draft guidelines for DDI studies published in 2012 recommended the calculation of R-value to assess the in *vivo* DDI risk of potential MBIs. The R-value is mathematically expressed as:

$$R = \frac{\lambda + k_{deg}}{k_{deg}} = 1 + \frac{\lambda}{k_{deg}}$$ Equation 2.4

Where k_{deg} is the rate constant for enzyme degradation *in vivo* and λ is equivalent to k_{obs}. Compounds with R-value >1.1 (or 11 for CYP3A4 inhibition in the gut) are considered possible CYP inhibitor *in vivo* which warrant further investigation *in vivo*. However, such approach fails to predict the magnitude of *in vivo* DDI risk. In this study, *in vivo* categorisation of DDI risk of MBIs based on λ/k_{deg} was adapted. According to this approach, the likelihood that a drug will cause *in vivo* interactions was classified as; likely (λ/k_{deg} >1), intermediate ($1>\lambda/k_{deg}$ >0.1) and remote ($0.1>\lambda/k_{deg}$) (Fujioka *et al*., 2012).

2.2.4 Effects of herbal extracts on metabolic clearance of testosterone in human hepatocytes and HLM

2.2.4.1 Incubation procedure

Cryopreserved hepatocytes were thawed and the cells were suspended in HepatoZYME media (BD Gentest). The cell viability was monitored at the beginning and the end of the incubation in the presence and absence of methanol extracts of herbal medicines with the use of Bio-Rad TC20 automated cell counter following the instructions provided by the supplier. The cell density was adjusted with HepatoZYME media to 0.5 $x10^6$ viable cells/mL. For HLMs assays, final protein concentration of 0.6 mg/mL in NADPH/$MgCl_2$/phosphate buffer mixture was used for the metabolic clearance of testosterone (TST). 100 µM of TST and 100 µg/mL of each herbal extract were co-incubated with 1 mL hepatocyte mixture at 37^{o}C under humidified atmosphere of 95% air and 5% CO_2 in Heraeus incubator. Aliquots of the hepatocytes incubation mixture were taken at 0, 0.5, 1, 2, 4, and 6 hours of incubation. Samples were stored in eppendorf tubes at -80^{o}C for 24 hours to break the cell walls. In the HLMs assay, an aliquot of the incubation mixture was taken for 0, 3, 7, 10, 20 and 30 mins. Each sample was stored at -20^{o}C for a maximum of 5 hours. Both hepocytes and HLM samples were centrifuged at 13000 x g and 200 µL of the supernatant stored in HPLC vials for analysis. The metabolic reaction was stopped by the addition of ACN containing 5 µL of corticosterone as an internal standard. Samples were kept at -80^{o}C for at least 4 hours before analysis.

2.2.4.2 Clearance calculations

The half-life ($t_{1/2}$) and the elimination rate constants of TST in hepatocytes and HLMs incubation mixture were determined using log linear regression of TST concentration profile against incubation time. The intrinsic clearance *in vitro* was calculated from the half-life and the cell density and scaling of intrinsic clearance *in vitro* to intrinsic clearance *in vivo* was made by employing the human liver mass (25.7 g/kg body mass), hepatocellularity (number of hepatocytes per gram of liver = 99.0 million cells/kg liver) and HLM protein concentration used as illustrated in the equations below (Emoto *et al*., 2006):

$$T_{1/2} = {Ln2}/{-(\text{slope of the Ln \% remanining of drug vs. time plot})} \quad \text{Equation 2.5}$$

$$\text{Microsome } CL_{int,\ app} = Ln2.\frac{1}{T_{1/2(min)}}.\frac{\text{mL incubation}}{\text{mg microsomal protein}}.\frac{1000\mu L}{mL} \quad \text{Equation 2.6}$$

$$\text{Microsome } CL_{int,\ scaled} = Ln2.\frac{1}{T_{1/2(min)}}.\frac{\text{mL incubation}}{\text{mg microsomal protein}}.\frac{45\text{ mg microsmal protein}}{\text{g liver}}.\frac{25.7\text{g liver}}{kg} \quad \text{Equation 2.7}$$

$$\text{Hepatocyte } CL_{int,\ app} = Ln2.\frac{1}{T_{1/2(min)}}.\frac{\text{mL incubation}}{0.5\text{Mcells}}.\frac{1000\mu L}{mL} \quad \text{Equation 2.8}$$

$$\text{Hepatocyte } CL_{int,\ scaled} = Ln2.\frac{1}{T_{1/2(min)}}.\frac{\text{mL incubation}}{0.5\text{Mcells}}.\frac{99\text{ Mcells}}{\text{g liver}}.\frac{25.7\text{g liver}}{kg} \quad \text{Equation 2.9}$$

Based on CL_{int} *in vivo* and the hepatic blood flow [Q (20.7 ml/min)/kg body mass], the hepatic metabolic clearance ($CL_{h,b}$) was predicted using the well-stirred model:

$$CL_{h,b} = {CL_{int,in\ vivo} \times Q}/{CL_{int,in\ vivo} + Q} \quad \text{Equation 2.10}$$

The free fractions *in vitro* or *in vivo* were assumed to be identical; hence no corrections were made as reported by Davies and Morris, (1993).

Influence of each herbal extract on plasma concentration of TST in both HLM and hepatocytes was determined applying:

$$\frac{AUC_i}{AUC} = \frac{CL}{CL_i} \quad \text{Equation 2.11}$$

AUC and AUC_i represent plasma concentration area of TST under the curve in the absence and presence of herbal extract. CL and CL_i represent intrinsic clearance of testosterone in the absence and presence of testosterone. The AUC ratios were used to classified the interaction of each extract with testosterone as; strong (AUC ratio >5), intermediate (2 ≤ AUC ratio ≤ 5), weak (1.25 ≤ AUC ratio <2) and no interaction (AUC ratio < 1.25) as recommended by FDA guidance for drug-drug interaction studies (2012).

2.2.5 Echinacea-mediated activation of PXR in co-transfected HepG2 cell lines

2.2.5.1 Cell viability

Cell viability was examined using the methyl thiazolyl tetrazolium (MTT) cell proliferation assay kit (Life technologies, Grand Island, New York, USA). HepG2 cells were seeded at 1.0 x 10^4 cell/100µL/well and cultured in DMEM/F12 medium containing 10% fetal calf serum, 2mM glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Cells were grown to 70-80% confluence and placed in serum-free media for 24 hours. The cells were treated with 25, 50 and 100 µg/mL of test samples for 24 hours. After the treatment period, the media was aspirated and the cell viability was immediately assessed by first incubating the cells in MTT solution for two hours, followed by measurement of absorbance at 570 nm after removal of untreated dye and addition of 200 µL of DMSO. Cell viability was calculated in comparison to untreated control

In prinicple, the MTT compound is bioreduced by cells into a coloured formazan product via NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (Berridge and Tan, 1993). The quantity of formazan product as measured by absorbance at 570 nm is directly proportional to the number of living cells in culture.

2.2.5.2 Transient transfection and Luciferase gene reporter assays

HepG2 cells (10 x 10^6 cells) transfected with a luciferase reporter construct (0.4 mg/well) and expression plasmid encoding pSG5-PXR (50 ng/well) by electroporation at 180V, 1 pulse for 70 msec, were seeded into 96-well plate at a density of 5 x 10^4 per well. The cells were subse-

quently maintained in a medium supplemented with 10% fetal calf serum containing 2.4 g/L sodium bicarbonate, 100 U/ml penicillin-G, and 100 µg/ml streptomycin for 24 hours. Test samples and rifampicin (positive control drug) were added to co-transfected cells at different concentrations and further incubated for 24 hrs. The media was aspirated from the cells and 40 µL of luciferase reagent (Promega Corporation, Madison, WI, USA) added to each well and luminescence was measured using Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA).

For time dependent induction, the above protocol was repeated. The luminescence activity was determined after 24 and 48 hours incubation with different concentrations of test samples. Luminescence of test samples was determined as background that was subtracted in the final calculation from luminescence of cell samples transiently transfected with reporter plasmids containing response elements of tested genes. The fold induction of each sample was calculated as a ratio between the luminescence of test samples to that of the vehicle treated cells.

2.2.5.3 Real-Time -PCR analysis of CYP3A4, CYP1A2 and P-gp mRNA

HepG2 cells (1.5 x10^5 per well) transfected with PXR expression plasmid (400 ng/well) were seeded into 96-well plate and cultivated for 24 h and appropriate transfected cells were exposed to different concentrations of test samples for 48 h. The cells were washed with PBS and total RNA was extracted using the Quick-start protocol (Qiagen® Texas, USA). The following primers were used for:

CYP3A4, forward (5'-TTCAGCAAGAAGAACAAGGACAA-3') and, reverse (5'-GGTTGAAGAAGTCCTCCTAAGC-3') primers; CYP1A2, forward (5-AAC-AAG-GGA-CAC-AAC-GCT-GAA-T-3) and reverse (5'-GGA-AGA-GAA-ACA-AGG-GCT-GAG-T-3') primers; *MDR1*, forward (5'-TGCTCAGACAGGATGTGAGTTG-3') and reverse (5'-AATTACAGCAAGCCTGGAACC-3) primers; and housekeeping genes *HPRT* (hypoxanthine-guanine phosphoribosyl transferase), forward (5'-CTGGAAAGAATGTCTTGATTGTGG-3'), reverse (5'-TTTGGATTATACTGCCTGACCAAG-3') primers.

cDNA was prepared from 1 µg of total RNA with iScript MMLV-RT ($RNaseH^+$) from Bio rad using oligo (dT) random primers and porcine RNase inhibitor. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed in a 96-well plate employing an iCycler iQ™ Real time PCR detector system (Bio-Rad). cDNA (40 ng of reverse transcribed RNA) was amplified with hot-start i*Taq* DNA polymerase (QIAGEN, Valencia, CA) under the following conditions: 3 mM $MgCl_2$, 0.2 mM deoxynucleoside-5-triphosphate, 0.3 µM each primer, 0.025 U/l polymerase, SYBR Green I in 1:100,000 dilution, and fluorescein (10 nM). The thermal cycler was programmed on the real-time PCR instrument using the following experimental conditions: 3 min of polymerase activation and denaturation at 95^{o}C, DNA amplification consisting of 10-15 sec denaturation at 95^{o}C and 30-60 sec anneling/extension with plate reading at optimized temperature (55-60^{o}C), cycles at 35-40 min and melt curve analysis between 2-5 sec at 55-95^{o}C. Test samples were run in triplicate with negative controls. The processing of real-time amplification curves was performed on iCycler software version 4.6 (Bio-Rad). Pfaffl's method was applied for relative quantification of gene expression normalized to endogenous control (housekeeping) gene (Pfaffl, 2001). Results were presented as the means of at least three experiments. Fold induction was expressed as the ratio between the mRNA expression of treated samples and that of the untreated sample.

2.2.5.4 Measurement of CYP3A4 enzymatic activity

Transfected HepG2 cells as described in subsection 2.2.5.2 were plated in 96-well plates, incubated overnight and and treated with capsules and fractions of *Echinacea* (1 - 100 µg/mL) for 48 h. CYP3A4 and CYP1A2 enzymatic activities were measured using fluorescent probe substrates kits (BD Gentest) according to the instructions provided by the manufacturer. In brief, 100 µL of cofactors mixture, control protein (0.05 mg of protein/mL), and glucose-6-phosphate dehydrogenase were added to each well. Initial readings were taken to record any autofluorescence and the plates were incubated at 37◦C for 30 min after addition of 3 µM 7-ethoxy-3-cyanocoumarin (CYP1A2 substrates) or 10 µM 7-benzyloxy-4-trifluoromethylcoumarin (CYP3A4 substrate) to respective wells. The reaction was terminated by the addition of 50 µL of ice cold ACN/0.5M Trisbase (80:20). Fluorescence was measured on Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA) at specified excitation

and emission wavelengths for CYP1A2 (405/460 nm) and CYP3A4 (405/535 nm). The concentration of crude herbal extracts that gives 50% of maximum response of the enzyme/transporter activity (EC_{50}) for each fraction was determined by plotting the fold induction against log concentrations of the test samples and the curve fitted by employing non-linear regression using GraphPad Prism.

2.2.6 P-glycoprotein assay

2.2.6.1 Calcein-AM uptake

A previously described protocol was followed with some modifications to assess the effect of *E. purpurea*, *M. oleifera*, *K. crenata* and *K. integra* on P-glycoprotein (Shiraki *et al*., 2002). Briefly, MDCKII/MDR1 and MDCKII/WT cells at 80–90% confluency were trypsinized, seeded into 96-well plates at a cell density of 7.0 x 10^4 cells/well. The medium was changed 24 h after seeding, and the assay was performed 48 h later. On the day of the study, the medium was aspirated, and monolayers were washed twice with transport buffer. Herbal extracts were added to monolayers in 50 µL of transport buffer containing 1% MeOH. Graded concentrations of each herbal extract (0.1–100 µg/mL, *n* =5) were used. The concentration ranges of 0.1–100 µM were used for verapamil as positive control. MeOH concentration (1%) was constant in test and control wells (each *n* = 2). Plates were pre-incubated at 37°C for 10 min. Calcein-AM was added, and the plates were immediately placed in a SpectraMax Gemini cytofluorimeter (Molecular Devices) for 60 min and read at 15 min intervals at excitation and emission wavelengths of 485 and 530 nm, respectively. Calcein-AM uptake and Pgp inhibition were immediately quantified.

2.2.6.2 P-gp inhibition by ^{3}H-digoxin uptake in hMDR1-MDCK-II cells

The experimental protocol described earlier by Rautio *et al*., (2006) was adopted with slight modifications. In brief, the cells were seeded at a density of 120,000 cells/well in 12-well transwell plates and cultured for 3 days. The TEER values were in the range of 500–800 Ω cm^2. Cells were washed with warm HBSS buffer (composition: 8.0 g NaCl, 0.4 g KCl, 0.14 g $CaCl_2$, 0.1 g $MgSO_4$, 0.06 g $Na_2HPO_4.2H_2O$, 0.06 g $MgCl_2$.6H2O, 0.06 g KH_2PO4, 1.0 g glucose and 0.35 g $NAHCO_3$ to 1000 mL of H_2O) supplemented with 10mM HEPES (pH7.4) and pre-incubated with 0.5 mL of buffer containing test samples on the apical side and 1.5mL of buffer

on the basolateral side for 30 min (37^{o}C, 5%CO_2, and 95% relative humidity). After incubation, buffer was removed from the basolateral side and replaced with 1.5mL of buffer containing ^{3}H-digoxin (40 nM), test samples or standard drugs (25 μM), and incubated further for 2h. Aliquots of 25 μL were taken out from the apical side, mixed with100 μL of scintillant (MicroscintTM-40, PerkinElmer) and radioactive counts were measured on a Top Count microplate scintillation counter(PerkinElmer,Waltham, MA, USA) in CPM mode.The monolayer integrity was monitored by measuring the permeability of Ly (afluorescent marker of passive paracellular diffusion) as described earlier (Manda *et al*., 2013). The inhibition of the basolateral to apical (B−A) transport of digoxin by test samples was calculated and compared to that of the vehicle control. The IC_{50} value, defined as the concentration that caused an inhibition of 50% in digoxin transport, was obtained from dose curves generated by plotting % inhibition versus log concentration using GraphPadPrism.

2.2.7 Statistical analysis

All values in the inhibition assays have been expressed as Mean ± SEM from two independent experiments. The percent activity remaining of TST in HLM and recombinant human CYPs were compared using unpaired t test with $P<0.05$ considered as significance. The unpaired t test was also used to determine the significance of enzyme activity in the presence and absence of NADPH.

For the induction experiment, one-way analysis of variance (ANOVA) was used to determine the significance of difference between untreated group and the treated groups. The Benferroni multi-comparison test was used for group comparisons. Statistical significance was accepted for p-values <0.05.

CHAPTER THREE

3. RESULTS AND DISCUSSIONS

PART ONE

3.1 Inhibition of CYPs by herbal extracts and fractions

The selected herbal medicines used by HIV/AIDS patients in sub-Saharan Africa were investigated for their tendency to inhibit the five major drug metabolizing enzymes namely, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Initially, reversible and irreversible screening of inhibition potency of each herbal medicine on specific isoform of CYP was conducted in recombinant human CYPs. Due to the importance of CYP3A4 in the metabolism of most drugs, extensive studies on metabolic clearance of testosterone, substrate for CYP3A4 in HLM and human hepatocytes were conducted.

3.1.1 Reversible inhibition assays

3.1.1.1 Two point screening of herbal extracts

In the preliminarily screening, two different concentrations of the aqueous (0.2 and 2.0 mg/mL) and methanol (1.10 µg/mL and 100µg/mL) herbal extracts were tested. The residual activities were expressed as percentages of the negative controls for respective CYPs. Aqueous herbal extracts of H. *hemerocallidea* powdered leaves (T30) and capsules (T41); *E. purpurea* capsules (T60) and *L. frutescens* tablets (T50) generally inhibited the five CYPs more than 50% at the highest concentration. The results indicated that, the aqueous herbal extracts of *M. oleifera* (T80) inhibited the CYP1A2, CYP2C9 and CYP3A4 in a concentration dependent manner (Figure 3.1A). Some herbal extracts showed an apparent activation on the activity of CYPs (2D6 and 3A4). The methanol extracts inhibited CYPs in the order of CYP3A4>CYP2C9>CYP2C19 which were also concentration dependent (Figure 3.1B). With the exception of *E. purpurea* capsule (2660), none of the methanol extracts inhibited CYP1A2 and CYP2D6 activities. The IC_{50} values of herbal extracts which inhibited more than 20% of the residual activity indicated for the negative control samples were determined.

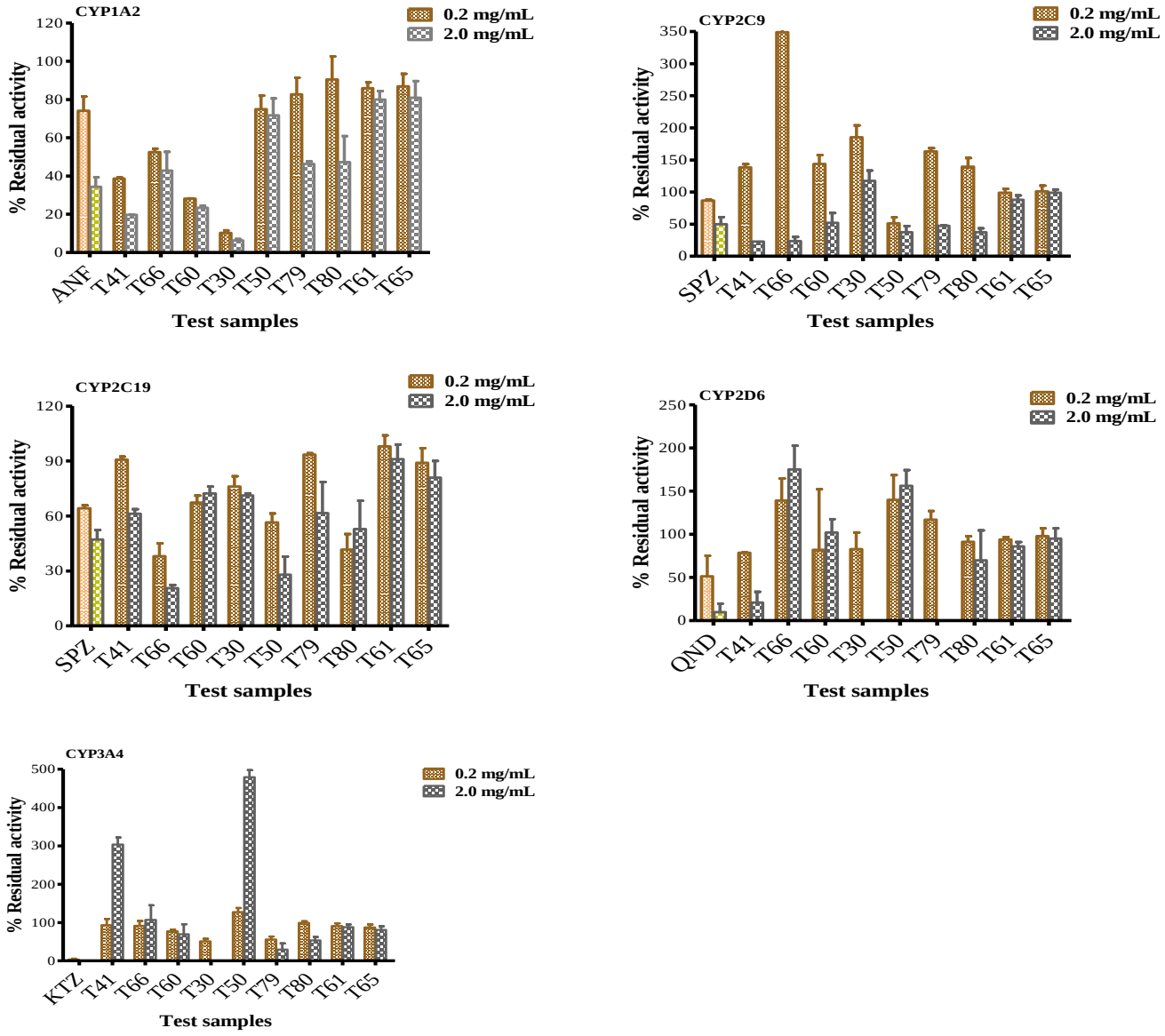


Figure 3.1A Two point screening of aqueous herbal medicines where 0.2 and 2.0 mg/ml of each herbal extract was evaluated for inhibitory effects on each of the five CYPs, 1A2, 2C9, 2C19, 2D6 and 3A4. For each of these CYPs, a positive control diagnostic inhibitor was used, alpha-nathoflavone (ANF, 0.01 and 0.10 µM) for CYP1A2, sulfaphenazole (SPZ, 1.0 and 10.0 µM) for CYPs 2C9 and 2C19, quinidine (QND, 0.01 and 0.1 µM) for CYP2D6 and ketoconazole (KTZ, 0.01 and 0.1 µM) for CYP3A4. *H. hemerocallidea* capsule (T41) and powdered leaves (T30), *L. frutescens* tablets (T50) and tea cut (T79), *T. officinale* root (T66), *E. purpurea* capsules (T60), *K. crenata* (T61), *K. integra* (T65) and *M. oleifera* powdered leaves (T80).

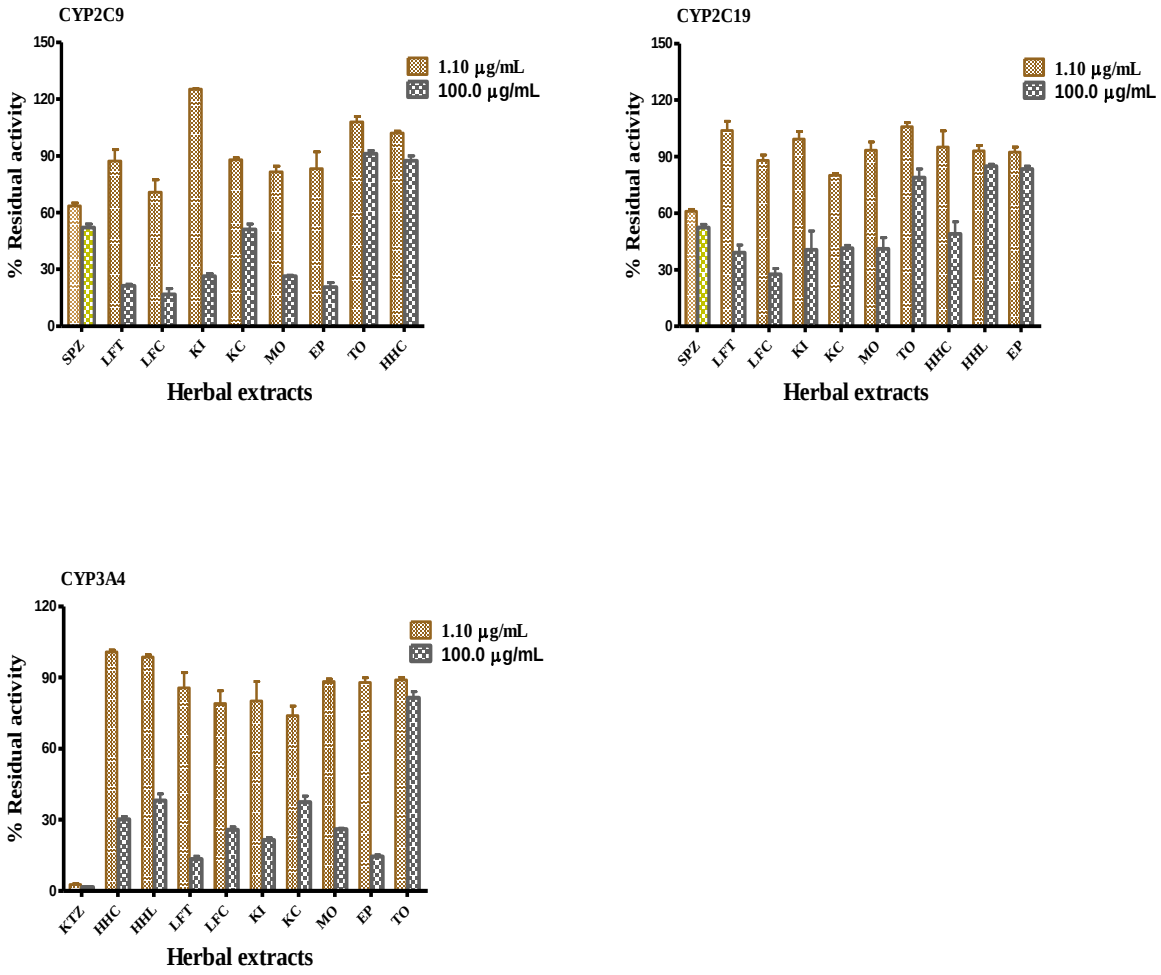


Figure 3.1B: Two point screening of methanol herbal extracts where 1.1 and 100.0 µg/mL of each herbal extract was evaluated for inhibitory effects on each of the CYPs; 2C9, 2C19 and 3A4. *H. hemerocallidea* capsule (HHC) and powdered leaves (HHL), *L. frutescens* tablets (LFT) and tea cut (LFC), *T. officinale* root (TO), *E. purpurea* capsules (2660), *M. oleifera* powdered leaves (MO), *Kalanchoe crenata* (KC) and *Kalanchoe integra* whole plant (KI).

3.1.1.2 The IC_{50} determination

The IC_{50} values were determined for only herbal extracts which indicated inhibitory effect greater than 20% on specific CYPs when compared to the negative controls. *H. hemerocallidea* (T30) and *E. purpurea* (T60) inhibited CYP1A2 with IC_{50} values of 0.63 µg/mL and 20 µg/mL, respectively. *L. frutescens* inhibited CYP2C9 with an IC_{50} value of 174 µg/mL. The result also showed *T. officinale* as an inhibitor of CYP2C19 with an IC_{50} value of 43.52 µg/mL. Like CYP1A2, *H. hemerocallidea* (T30) and *T. officinale* (T60) inhibited CYP3A4 with an IC_{50} values of 58 µg/mL and 12 µg/mL, respectively as demonstrated in Figure 3.2A.

The methanol extracts showed an inhibitory effect less than 20% on CYP1A2 and CYP2D6 compared to the negative controls; hence the IC_{50} values for the herbs on these two CYPs were not determined. All the methanol extracts indicated strong inhibition on CYP2C9, CYP2C19 and CYP3A4 with IC_{50} values within the concentrations of 1.04 – 50.87 µg/mL. However, only the methanol extract of *E. purpurea* capsule (2660) exhibited mild inhibition on CYP1A2 with IC_{50} value of 56.02 µg/mL. The sigmoidal dose response curves from which the IC_{50} plots for each herbal extract was determined are shown in Figure 3.2A (aqueous extracts) and Figure 3.2B (methanol extracts). The inhibition reaction was performed at a substrate concentration equivalent to the K_m. The IC_{50} values for the diagnostic inhibitors; 0.008 µM (CYP1A2: α-naphtoflavone), 1.351 µM and 0.191 µM (CYP2C9 and CYP2C19, respectively: sulfaphenazole), 0.011 µM (CYP2D6: quindine) and 0.021 µM (CYP3A4: ketoconazole) were in agreement with the values reported in literature (Helsby *et al*., 1998; Sai *et al*., 2000). Table 3.1 shows the IC_{50} values of the herbal extracts on various recombinant CYPs.

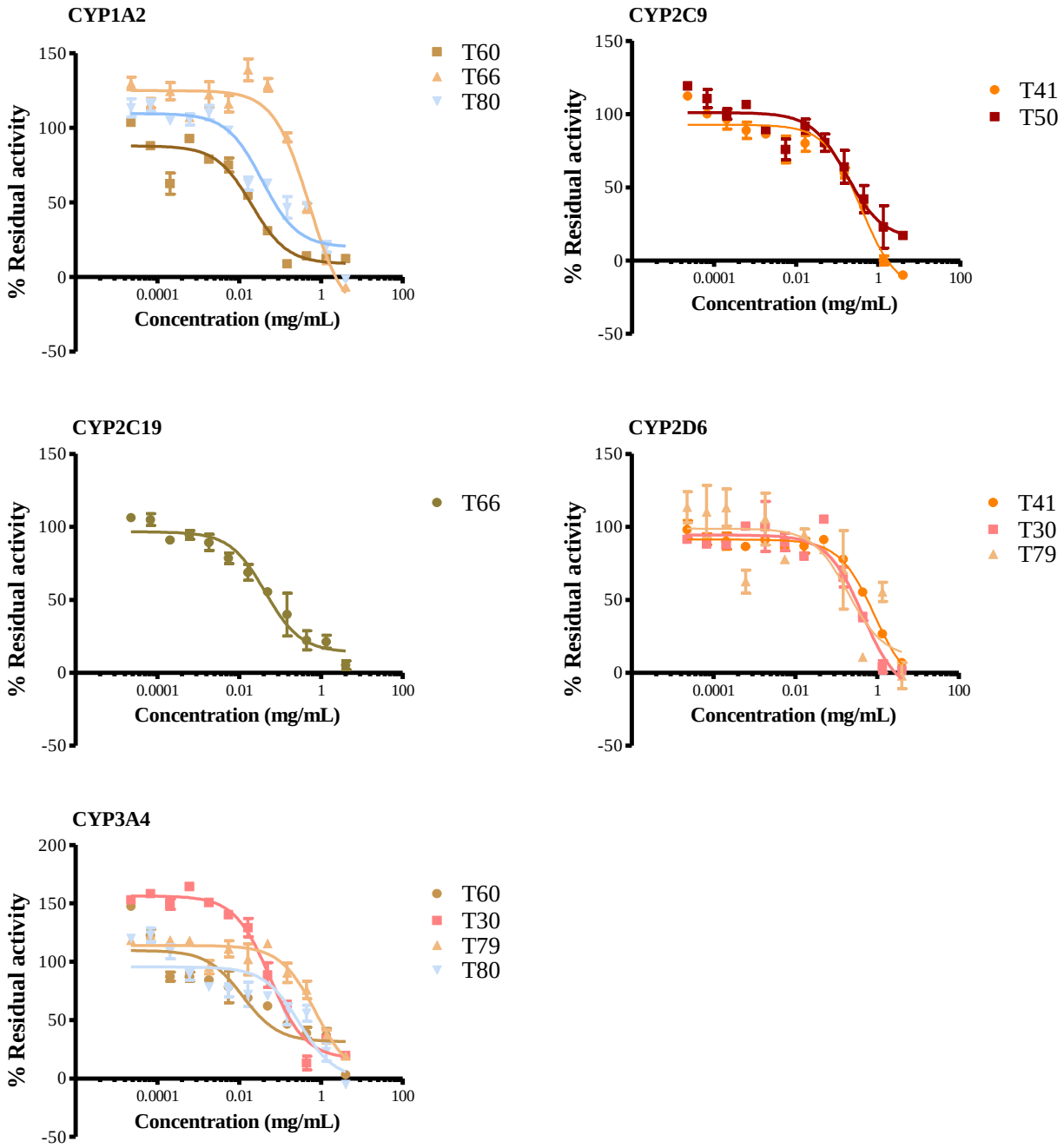


Figure 3.2A Dose-response curves from which the IC_{50} values were determined. Data are expressed as Mean ± SEM, n=2. The IC_{50} value is the concentration of inhibitor bringing about a 50 % reduction in enzyme activity.

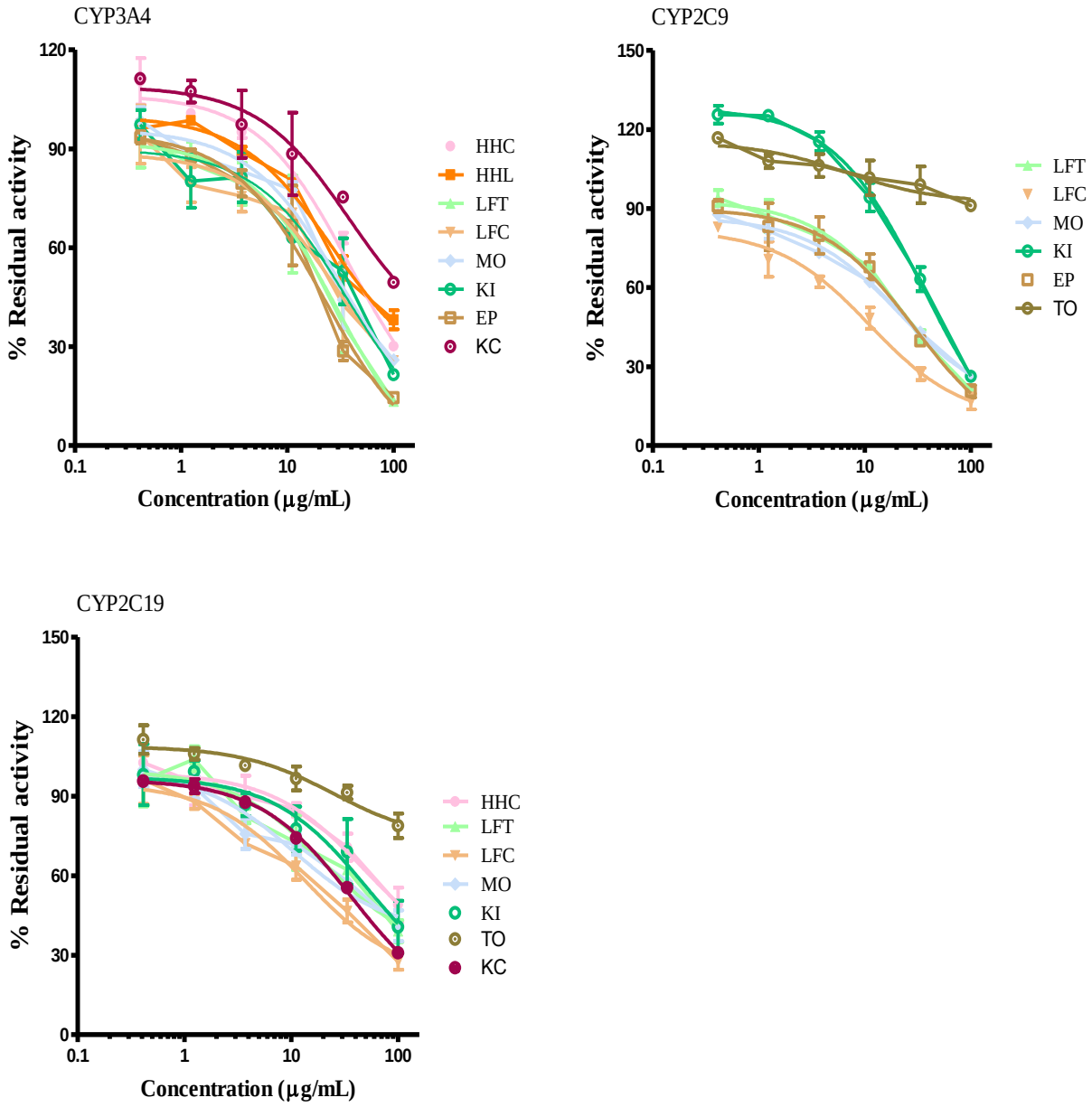


Figure 3.2B: Dose-response curves from which the IC_{50} values were determined for the methanol extracts. Data are expressed as Mean ± SEM, n=2. The IC_{50} value is the concentration of inhibitor bringing about a 50 % reduction in enzyme activity.

Table 3.1: IC_{50} values of herbal extracts on various recombinant CYPs

Herbal medicines	**Aqueous extract**	**Methanol extract**
	IC_{50} µg/mL	IC_{50} µg/mL
Hypoxis capsule		
CYP1A2	162.00 ± 3.87	-[a]
CYP2C9	383.36 ± 11.06	17.56 ± 1.24
CYP2C19	-[a]	48.96 ± 3.21
CY2D6	870.60 ± 23.54	-[a]
CYP3A4	-[a]	38.88 ± 4.61
Hypoxis leaves		
CYP1A2	0.63 ± 0.02	-[a]
CYP2C9	-[a]	-[a]
CYP2C19	-[a]	-[a]
CYP2D6	446.00 ± 10.89	-[a]
CYP3A4	58.00 ± 5.65	30.42 ± 2.54
Lesertia tablet		
CYP1A2	-[a]	-[a]
CYP2C9	174.00 ± 9.54	25.48 ± 4.16
CYP2C19	-[a]	20.10 ± 1.89
CYP2D6	-[a]	30.62 ± 0.78
CYP3A4	-[a]	-[a]
Lessertia tea cut		
CYP1A2	-[a]	-[a]
CYP2C9	-[a]	10.68 ± 2.10
CYP2C19	-[a]	13.04 ± 1.04

CYP2D6	198.00 ± 6.24	-[a]
CYP3A4	740 ± 5.20	28.41 ± 2.10
Moringa leaves		
CYP1A2	37.00 ± 1.89	-[a]
CYP2C9	-[a]	20.60 ± 1.45
CYP2C19	-[a]	10.60 ± 0.60
CYP2D6	-[a]	-[a]
CYP3A4	300.00 ± 18.21	31.48 ± 1.70
Taraxacum root		
CYP1A2	520.00 ± 21.70	-[a]
CYP2C9	-[a]	-[a]
CYP2C19	43.52 ± 4.24	-[a]
CYP2D6	-[a]	-[a]
CYP3A4	-[a]	-[a]
Echinacea capsule		
CYP1A2	20.20 ± 0.90	52.20 ± 2.10
CYP2C9	-[a]	29.60 ± 0.79
CYP2D6	-[a]	-[a]
CYP2C19	-[a]	1.04 ± 0.04
CYP3A4	12.00 ± 0.53	22.32 ± 1.60
Kalanchoe crenata		
CYP1A2	-[a]	-[a]
CYP2C9	-[a]	-[a]
CYP2C19	-[a]	38.21 ± 4.10
CYP2D6	-[a]	-[a]

CYP3A4	-[a]	36.78 ± 1.51
Kalanchoe integra		
CYP1A2	-[a]	62.37 ± 6.72
CYP2C9	-[a]	36.44 ± 2.57
CYP2C19	-[a]	50.87 ± 8.34
CYP2D6	-[a]	-[a]
CYP3A4	-[a]	36.48 ± 5.10

- [a] No inhibition at the concentration tested

3.1.1.3 *In vivo* prediction of HDI from *in vitro* data

Based on FDA guidelines for *in vivo* prediction of DDI from *in vitro* data, certain assumptions were made for both inhibitor concentration individual is exposed to in the GIT [I] or the maximum hepatic input concentration $[I]_{in}$. and the inhibitor constant (K_i). These parameters are difficult to estimate for herbal medicines. The commonly used working concentration is therefore based on the highly unlikely assumption that the whole soluble extract prepared employing the 250 mL GIT fluid is available to interact with intestinal CYPs and P-glycoprotiens. The concentrations of herbal extracts in the GIT were therefore estimated as extract per dose used in human divided by the GIT fluid volume (250 mL) as shown in Appendix 4. For soluble extract that interact with liver CYPs and drug transporters, the maximum hepatic input concentration $[I]_{in}$ was determined based on the assumption that there was complete absorption of herbal extracts from the GIT into the portal vein (Fa = 1.0). The values of absorption rate constant (k_a), hepatic blood flow rate (Qh) were also assumed to be 0.1 min^{-1} and 1610 ml/min (Kanamitsu *et al*., 2000) based on the equation 3.1:

$$[I]_{in} = [I]_{gut} + k_a F_a D/Qh \qquad \text{Equation 3.1}$$

Secondly, the K_i which is an inhibitory constant characteristic of the interaction of a pure compound with an enzyme is difficult to determine for herbal extracts for which we do not know the chemical constituents. For the use of the inhibition index proposed by FDA, a number of

assumptions are necessary, including that the IC_{50} observed is due to unique chemical constituent in the herbal extract. Furthermore, based on the design of the *in vitro* assay for the determination of IC_{50} with substrate concentration at K_m, M-M kinetic derivations allow one to estimate K_i from IC_{50}, where for competitive inhibition, $K_i = IC_{50}/2$ and for non-competitive inhibition, $K_i = IC_{50}$. For determining the safety of herbal extracts, the assumption of competitive inhibition would result in a higher inhibition index than non-competitive inhibition. Again, most documented modes of CYP inhibition are competitive and not non-competitive inhibition. Therefore, the assumption of competitive inhibition will be employed to estimate the K_i.

Based on the above assumptions, the estimation of HDI will be based on the following FDA guidelines: $[I]/K_i > 1$ (likely), $1 > [I]/K_i > 0.1$ (possibly) and $0.1 > [I]/K_i$ (remote) as presented in Table 3.2 for each test compound on respective CYPs. The IC_{50} values for herbal extracts which inhibited CYPs less than 20% based on preliminary screening were not investigated. The predicted likelihood of *in vivo* CYP inhibition by the herbal extracts is indicated in Table 3.2.

Table 3.2 Prediction of *in vivo* herb-drug interactions from *in vitro* data

Herbal medicines	Aqueous extract					Methanol extract				
	$[I]_{gut}$ (µg/mL)	$[I]_{in}$	K_i (IC_{50}/2) (µg/mL)	GIT $[I]_{gut}/K_i$	Hepatic $[I]_{in}/K_i$	$[I]_{gut}$ (µg/mL)	$[I]_{in}$	K_i (IC_{50}/2) (µg/mL)	GIT $[I]_{gut}/K_i$	Hepatic $[I]_{in}/K_i$
***Hypoxis* capsule**	1400.00	1422.00				242.00	264.00			
CYP1A2			81.00	17.00	17.60			_a	_a	
CYP2C9			191.65	7.30	7.40			8.78	27.59	30.07
CYP2C19			_a	_a				24.48	9.89	10.78
CYP2D6			435.30	3.20	3.30			_a	_a	
CY3A4			_a	_a				19.44	12.46	13.58

***Hypoxis* leaves**	321.84	342.70				378.00	380.00			
CYP1A2			0.32	510.0	1087.90			_a	_a	
CYP2D6			223.00	1.44	1.50					
CY3A4			29.00	11.00	11.80			15.21	24.85	26.30
***Lessertia* tablets**	1200.00	1218.60				192.00	229.20			
CYP2C9			87.00	13.80	14.00			12.74	15.07	17.99
CYP2C19			_a	_a				10.05	19.10	22.8
CY3A4			_a	_a				15.31	12.54	14.97
***Lessertia* tea cuts**	131.00	149.60				146.40	165.00			
CYP1A2			_a	_a				_a	_a	
CYP2C9			_a	_a				5.34	27.42	30.90
CYP2C19			_a	_a				6.70	21.85	24.63

CYP2D6			99.00	1.30	1.50			_a	_a	
CY3A4			370.00	0.35	0.001			14.21	10.30	11.60
***Moringa* leaves**	1600.00	1615.50				224.00	239.50			
CYP1A2			18.50	86.50	87.30			_a	_a	
CYP2C9			_a	_a				10.30	21.75	23.25
CYP2C19			_a	_a				5.31	42.18	45.10
CY3A4			150.00	10.70	10.77			15.74	14.23	15.21
***Taraxacum* roots**	1192.16	1238.70								
CYP2C9			260.05	4.60	4.76			_a	_a	
CYP2C19			21.76	59.61	56.92			_a	_a	

***Echinacea* capsules**	1280.00	1298.60				238.08	256.7		
CYP1A2			10.00	128.00	129.86		28.10	8.47	9.13
CYP2C9			_a	_a			14.80	16.08	17.34
CYP2C19			_a	_a			0.52	475.8	493.6
CY3A4			6.00	213.00	216.43		11.16	21.33	23.00

[–a]No inhibition at the concentration tested, intra-hepatic concentration ($[I]_{in}$) = $C_{max} + k_a F_a D/Qh$.

3.1.1.4 Comparative inhibition of CYP3A4 in recombinant CYP and HLM

The methanol extracts exhibited high risk of HDI based on the *in vivo* prediction from the reversible IC_{50} values. Fractions of the various methanol extracts which showed high risk of HDI were prepared and comparative inhibition profile investigation in both HLMs and recombinant human CYP3A4 was conducted. HPLC was used to monitor the inhibitory effects of various crude methanol extracts and fractions of herbal medicines on CYP3A4-catalyzed 6β-hydroxylation of TST in HLMs and recombinant human CYP3A4. The concentration of TST used as a probe substrate in both HLMs and recombinant human CYP3A4 in this assay was approximately the K_m. As shown in Figure 3.3A, the crude KC (*K. crenata*) weakly inhibited CYP3A4 yielding remaining enzyme activity of 85% (HLM) and 101% (rCYP3A). The fraction 45D significantly reduced ($P = 0.006$) the activity of CYP3A4 in HLM compared to that of rCYP at 50 μg/mL. Conversely, the activity of CYP3A4 was reduced significantly ($P = 0.02$) in rCYP compared to that in HLM by fraction 45D at 100 μg/mL. The fraction 45B indicated significant reduction in CYP3A4 activity in rCYP compared to that of HLM at all the three different concentrations tested. Crude methanol extract (39KC) and fraction 45A showed no significant differences in the activity of CYP3A4 when tested in both HLM and rCYP.

Both crude and fractions of *M. oleifera* exhibited strong inhibition in both HLM and rCYP though activity in the latter was stronger than those observed in the HLM. For example, inhibition of 60% (HLM) and 84% (rCYP3A4) were observed for the crude methanol extract. The fraction 50B showed a strong inhibition of 84 % in rCYP and mild inhibition of 40% in HLM. Additionally, fraction 50A indicated strong inhibition of 45% and weak inhibition of 20% in rCYP3A4 and HLM, respectively as depicted in Figure 3.3B.

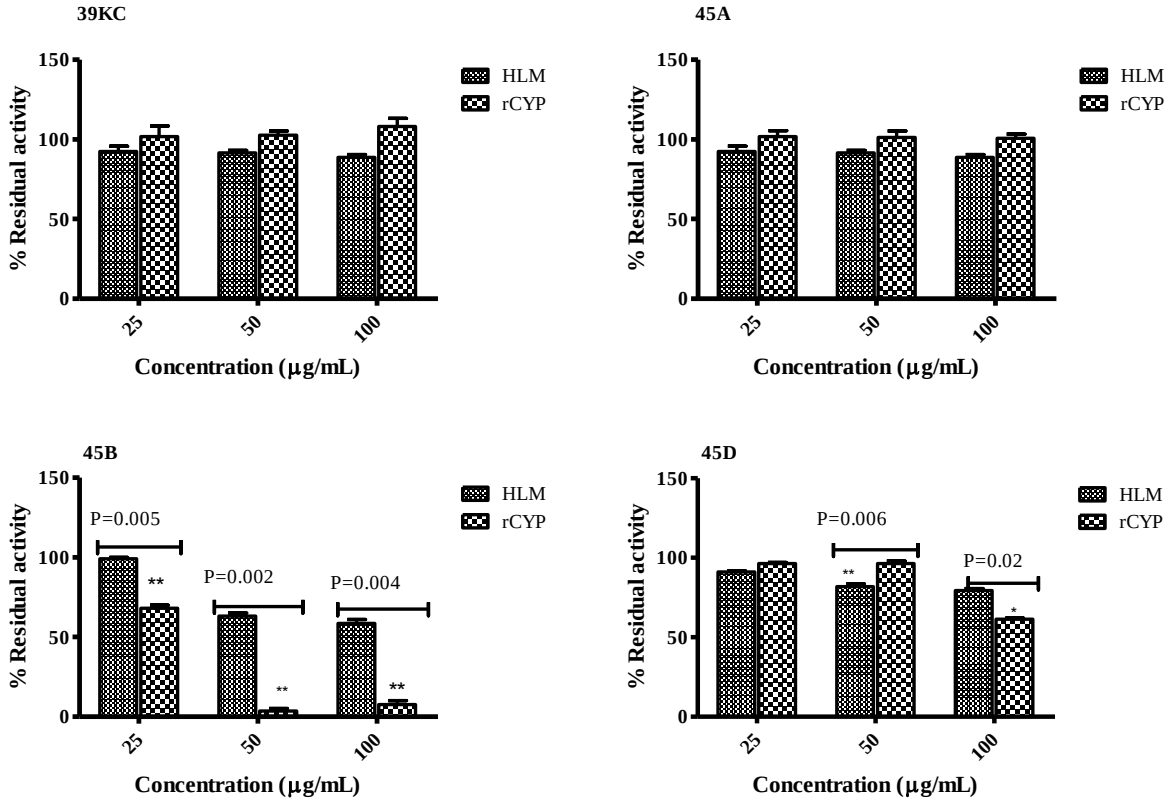


Figure 3.3A Inhibitory effect of crude *K. crenata* (39KC) and fractions 45A, 45B and 45D (25 - 100 µg/mL) on CYP3A4-catalyzed 6β-hydroxytestosterone formation in HLMs and recombinant human CYP3A4. Data expressed as Mean ± SEM of duplicate determinations.

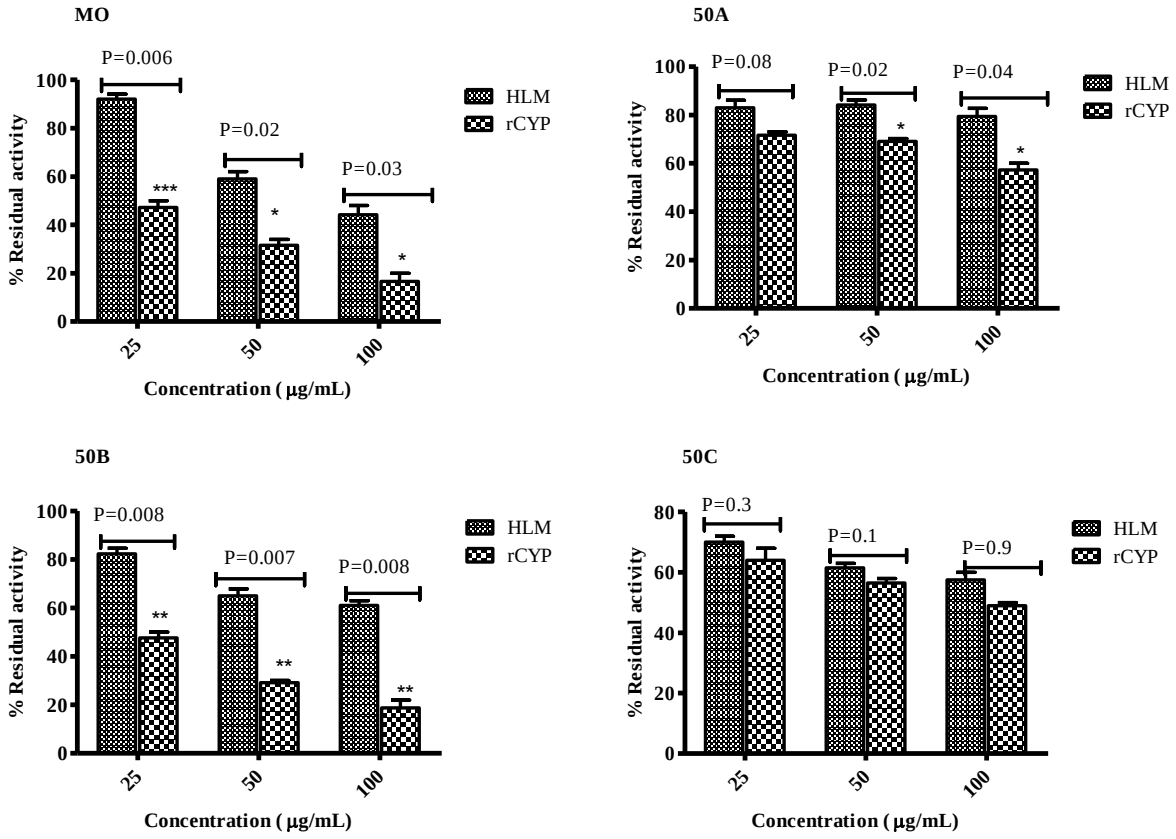


Figure 3.3B: Inhibitory effect of crude *M. oleifera* (MO) and fractions 50A, 50B and 50C (25 - 100 µg/mL) on CYP3A4-catalyzed 6β- hydroxytestosterone formation in HLMs and recombinant human CYP3A4. Data expressed as Mean ± SEM of duplicate determinations.

3.1.1.5 Comparative evaluation of conventional and cocktail IC_{50}s using fluorescent substrate in rCYPs

The IC_{50} values of crude methanol and fractions of *M. oleifera* and *K. crenata* were re-evaluated using cocktail enzyme/substrate for the five major recombinant human CYPs. A fold decrease or increase in the inhibitory potency of each extract or fraction was determined as a ratio of the cocktail to the conventional IC_{50} values as shown in Table 3.3. Crude methanol extract of *K. crenata*, fractions 45B and 45D showed a 3.32-, 2.55- and 4.8-fold decrease in inhibition potencies, respectively on CYP3A4. A 1.18- and 1.63-fold decrease in inhibition potency of *M. oleifera* fractions 50A and 50B, respectively on CYP3A4 was observed. Crude methanol extract of *M. oleifera* indicated an increase in inhibition potency (0.38-fold) on CYP3A4. For

CYP2C19, there was no difference in the inhibitory effect of 39KC, 45B, 50A and 5B, whereas fraction 45D of *K. crenata* and crude methanol extract of *M. oleifera* (80) showed a reduction in inhibition potency of 4.36 and 43.55, respectively.

Table 3.3 Comparison of inhibitory potency of test samples in cocktail and single rCYPs

Fraction	Conventional IC_{50} (µg/mL)	Cocktail IC_{50} (µg/mL)	Fold decrease or increase in inhibitory potency
CYP3A4			
39 KC	38.21	127	3.32[b]
45B	5.33	13.6	2.55[b]
45D	15.76	75.72	4.80[b]
80	46.30	18.10	0.39[c]
50A	17.69	20.96	1.18[b]
50B	7.66	12.51	1.63[b]
CYP2C19			
39KC	36.78	_a	_a
45B	10.30	_a	_a
45D	32.18	140.4	4.36[b]
80	5.91	257.40	43.55[b]
50A	7.90	_a	_a
50B	5.91	_a	_a

–[a] No inhibition at the concentration tested, [b] fold decrease in inhibition potency and [c] fold increase in inhibition potency.

3.1.1.6 Integration of PAMPA screening into rCYP assays

To monitor the inhibitory effect of various herbal medicines used in this study after permeation through absorption membrane, the PAMPA model was used. The hot aqueous herbal extract prepared similar to the method used by the THPs was employed for the PAMPA screening. The integrity of the PAMPA was intact as the effective permeability (P_e) values for the positive control (caffeine = 27.8 x 10^{-6} cm/s) and the negative control (cimetidine = 1.3 x 10^{-6} cm/s) were within the ranges reported for high and low permeability compound, respectively by BD Gentest. The inhibitory effect of most herbal extracts on CYPs (1A2 and 3A4) before and after PAMPA was similar especially for *L. frutescens* tablets (T50) and tea cut (T79), *H. hemerocallidea* capsule (T41) and *M. oleifera* powdered leaves (T80). However, the inhibitory effect of *E. purpurea* capsules (T60) and *H. hemerocallidea* powdered leaves (T30) on CYP1A2 was reduced by almost three fold after PAMPA compared to that of before PAMPA as depicted in Figure 3.4.

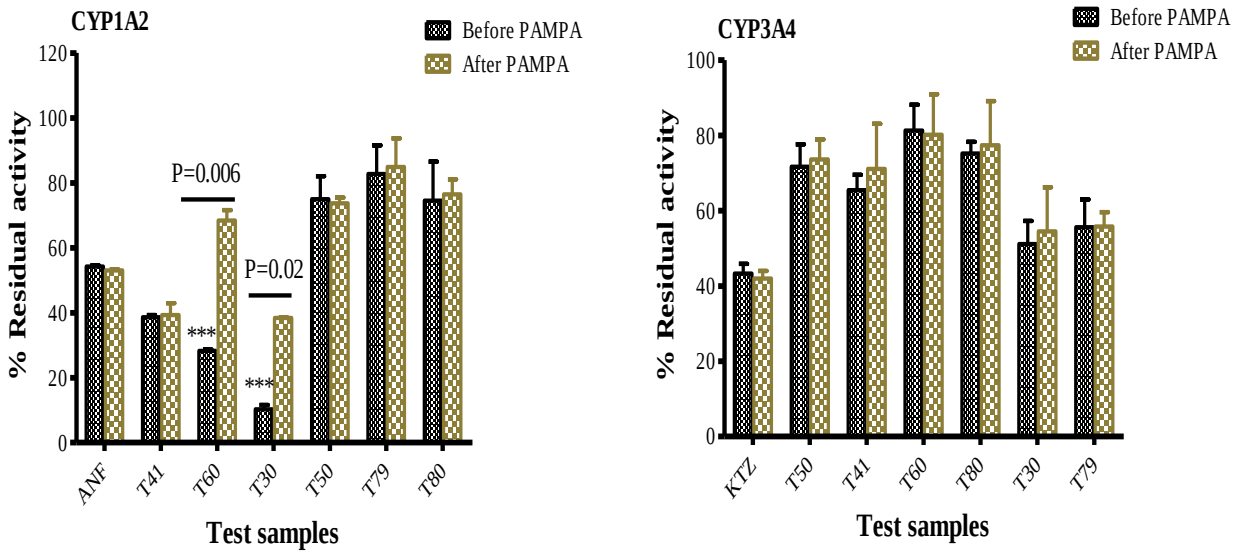


Figure 3.4 Inhibitory effects of herbal medicines on CYPs (1A2 and 3A4) after and before PAMPA. Positive control of: CYP1A2 – ANF (0.01µM), CYP3A4 – KTZ (0.01 µM) were used. Data are expressed as Mean ± SEM, n=2.

DISCUSSION

Reversible inhibition of CYPs

The screening of new chemical entities for metabolism based DDI is standard practice in medicines development. Scientists from the pharmaceutical industry and FDA have published opinion documents and guidelines on how such studies should be done (Zhang *et al*., 2009; Prueksaritanont *et al*., 2013). Knowledge and systems for evaluating DDI involving CYP inhibition and induction are well established compared to other enzymes and drug transporters (Crespi *et al*., 1997). As the use of herbal medicines either as nutritional supplements or complementary and alternative medicines is on the increase and well established in developing countries, concerns about the risk of HDIs have resulted to an increase number of studies on inhibitory effects on CYPs based on published guidelines. Such studies have indeed resulted to drug label revisions such as indinavir and postmarket withdrawal of mibefradil (Riley *et al*., 2002; Clauson *et al*., 2008). In this study, we evaluated the inhibitory effects of 7 herbal medicines used by HIV/AIDS patients to treat HIV or related co-morbidities and control disease symptoms. Many of the herbs were shown to potently inhibit the 5 major CYPs (1A2, 2C9, 2C19, 2D6 and 3A4) at levels predicted to result to significant *in vivo* HDI if co-administered with drugs which are substrates of these enzymes. These herbal medicines are being used in populations which are also receiving ARVs under national treatment roll out programs, thus present a real healthcare risk for HDIs with ARVs which are metabolized by CYPs such as the NNRTI (nevirapine and efavirenz, metabolized by CYP3A4 and 2B6) and protease inhibitors (metabolized by CYP3A4).

The initial screening assay using two concentrations showed that generally the aqueous herbal extracts inhibited CYPs in the order CYP1A2 > CYP3A4 > CYP2D6 > CYP2C9 > CYP2C19 in a concentration dependent manner. Aqueous extracts of T30 (*H. hemerocallidea* powdered leaves), T41 (*H. hemerocallidea* capsule), T60 (*E. purpurea* capsules) and T50 (*L. frutescens* capsules) at a concentration of 2.0 mg/mL inhibited the five major CYPs more than 50%. The methanol extracts of *Lessertia* tablets and tea cuts, *K. integra* and *E. purpurea* capsule at a concentration of 100 µg/mL exhibited inhibition on CYP3A4, CYP2C9 and CYP2C19 activities by at least 50%. *Taraxacum officinale* methanol extracts weakly (20%) inhibited CYP2C9.

Comparing the inhibition profile of aqueous extracts to that of methanol indicated a stronger effect by phytochemicals in the latter. This can be attributed to the recovering higher amounts of phytochemicals from the plant matrix using methanol as solvent for the extraction process. The herbal extracts prepared employing the hot aqueous decoction showed weak inhibition on CYPs; CYP3A4, CYP2C9 and CYP2D6. Thus extraction procedures which do not take into account the indigenous mode of preparation (hot aqueous decoction) of these herbal medicines can yield different phytoconstituents with varying inhibitory effects on CYPs and other drug metabolizing enzymes. Nevertheless, as a form of scientific investigation, several solvents of different polarity were employed in this study to improve the recovery of phytochemicals and to flag the potential of herbal medicines likely to cause HDI.

The aqueous herbal extract of *T. officinale* showed an apparent activation on the activity of CYPs (2D6 and 3A4). An increase in activity of more than 20% compared with the control activity was considered to be an apparent activation. The apparent activation needs to be interpreted with caution as this has been shown to be a typical *in vitro* artifact of the fluorimetric assays especially when dealing with compound (herbal extracts in this case) with high florescent activity. To further study such cases, different assay platforms using HLM and other standard CYP marker reactions with HPLC-UV or LC-MS/MS analysis need to be used (Masimirembwa *et al*., 1999).

Although the recombinant human CYPs are used as an HTS model, the HLM are preferred as an ideal *in vitro* tool for evaluation of the inhibitory activity of new drug candidates by employing specific substrates. In comparing the inhibitory effects of crude methanol extracts and fractions of the two potent inhibitors of CYP3A4; *M. oleifera* and *K. crenata*, CYP3A4 activity in HLMs and recombinant CYP3A4 was examined using TST as probe substrate. The results showed a general weak inhibition profile of the test samples in HLMs. This could be attributed to some phytochemical compounds of the test samples acting as substrates, therefore metabolized via other pathways than CYP3A4. A study by Patki *et al*, (2003) comparing inhibitory potency of known CYP3A4 inhibitors employing both HLMs and recombinant CYPs indicated higher IC_{50} values in the former. The weak potencies of inhibitors in HLMs were attributed to compounds acting as substrates for other isozymes, and therefore metabolized to less active forms which weakly inhibit CYP3A4. However, in a situation where metabolites with strong inhibitory effect

are generated due to the activities of other isozymes in HLMs, lower IC_{50} values may be observed (Nomeir *et al*., 2001). In this study, although weaker inhibition activity was observed in HLMs assay, the difference was marginal when compared with that of recombinant human CYP. The results of this study agree with the findings by Bapiro *et al*., (2001) and Bell *et al*., (2008) which demonstrated positive correlation between compounds screened with both HLMs and recombinant human CYP for their inhibition effects. However, strong inhibition was observed in recombinant human CYP compared to HLMs for fraction 45B. This indicates the possibility of underestimation of potential inhibitor when HLMs are used as the sole *in vitro* model for the prediction of drug candidates' potential for DDI or HDI. Conversely, the use of recombinant human CYP might also lead to an overestimation of inhibitory effect of such compounds on CYPs; hence a combination of these models should be employed in screening herbs purported to have potential risk for HDI to avoid loss of important information. Crude *K. crenata* exhibited a strong inhibition profile in HLMs compared to rCYP3A4. Such activity could be due to production of metabolites by other enzymes present in HLMs and these metabolites later acting as inactivators of CYP3A4.

The IC_{50} profile screening indicated CYP1A2 and CYP3A4 as the most sensitive to the aqueous herbal extracts whilst the methanol extracts strongly inhibited CYP3A4, CYP2C9 and CYP2C19. The aqueous herbal extract of *H. hemerocallidea* (T30) inhibited CYP1A2 the strongest, with an IC_{50} value of 0.64 ± 0.02 µg/mL, although the methanol did not show any effect. The methanol extracts of *H. hemerocallidea* capsules (HHC) and leaves (HHL) indicated strong inhibition on CYP2C9 with IC_{50} of 17.56 µg/mL and 10.68 µg/mL, respectively. Such finding contrasts those of Fasinu *et al*., (2013) who reported the crude methanol as weak inhibitor of CYP2C9 (IC_{50} = 156 µg/mL) in HLMs. This could be due to the differences in phytoconstituents of *H. hemerocallidea* used for the two independent studies. Additionally, whilst recombinant human CYPs contain a single isoform of enzyme, HLMs have several enzymes. The phytochemicals in *H. hemerocallidea* might serve as substrates to other enzymes which could account for the weak inhibitory activity reported earlier. Thus for mechanistic purposes, the methanol extract of *H. hemerocallidea* is considered a strong inhibitor of CYP2C9 in rCYPs even though the same preparation is weak in HLMs and could therefore cause HDI upon concurrent administration with substrates of CYP2C9 such as NSAIDS, S-warfarin and tolbutamide. The aqueous extract of *H. hemerocallidea* capsule indicated no effect on CYP3A4.

This finding agrees with an *in vivo* study conducted by Gwaza *et al*., (2013) in health volunteers to evaluate the influence of another subspecies of *Hypoxis* (*H. obtusa*) on pharmacokinetics of lopinavir/ritonavir.

The hot aqueous extract of *L. frutescens* tea cut exhibited weak inhibition on CYP3A4 with IC_{50} of 740 ± 5.2 µg/mL whilst both the methanol extracts of the tea cut and tablets inhibited the same enzyme with IC_{50} values of 28.42 ± 2.1 µg/mL and 30.62 ± 0.78 µg/mL, respectively. The strong inhibitory effects of methanol extracts of *L. frutescens* observed in this study agree with the findings by Fasinu *et al*., (2012). However, the weak inhibitory effects of the hot aqueous extracts of *L. frutescens* show that the degree of HDI depends on the nature of solvent used for the extraction of the herbal medicine (Pan *et al*., 2012).

Both aqueous and methanol extracts of *E. purpurea* indicated strong inhibition on CYP3A4, CYP2C9 and CYP2C19. The aqueous extracts of EP exhibited strongest effects on CYP1A2 (IC_{50} = 20.20 ± 0.9 µg/mL) and CYP3A4 (IC_{50} = 12.0 ± 0.53 µg/mL). A similar trend was observed for the methanol extract yielding IC_{50} values of 15.6 ± 0.79 µg/mL, 1.04 ± 0.01 µg/mL and 22.32 ± 1.6 µg/mL for CYP2C9, CYP2C19 and CYP3A4, respectively. Studies conducted by Modarai *et al*., (2010) and Hansen and Nilsen (2008) reported *E. purpurea* as a weak inhibitor of CYP3A4 supersomes (IC_{50} = 364 - 1812 µg/mL) which contradicts with the findings of this study. Such discrepancies between the current results and that of the Modarai *et al*., (2010) and Hansen and Nilsen (2008) can be attributed to the differences in developmental stages, season of collection, postharvest preservation and extraction procedures (Bolling *et al*., 2010). Also the type of substrate and the source of enzyme (HLM or rCYP) used could be responsible for the differences in the inhibition profile observed especially for CYP3A4. In this study, BFC was used as substrate for CYP3A4 which has been shown to have high affinity for the enzyme compared to other substrates such as TST, midazolam and nifedipine (Fowler *et al*., 2002).

Moringa oleifera, a popular herbal medicine consumed for treatments and management of cardiovascular and gastrointestinal complications in HIV/AIDS patients on ARVs also indicated strong inhibition of CYPs. The methanol extracts exhibited strong inhibition with IC_{50} values of 20.60 ± 1.45 µg/mL, 10.62 ± 0.60 µg/mL and 31.48 ± 1.7 µg/mL for CYP2C9, CYP2C19 and CYP3A4, respectively. The strong inhibition of *M. oleifera* on CYP3A4, agrees with the previous findings by Monera *et al*., (2008). Although the methanol extracts was ineffective on

CYP1A2, strong inhibition was observed for the aqueous extracts of *M. oleifera* with IC_{50} value of 37.00 ± 1.89 µg/mL while weak effect was indicated for CYP3A4 (IC_{50} = 300.00 ± 18.21 µg/mL).

As a method of confirming the inhibition effects of the crude methanol extract and fractions of *M. oleifera* and *K. crenata* in the presence of other isozymes, the cocktail approach was adopted. A general decrease in inhibition profile was observed as the IC_{50} values for the cocktail were higher compared to the single CYPs. In an attempt to establish differences between potencies of known inhibitors using conventional HLMs or recombinant human CYPs against the cocktail approach, Dierks *et al*., (2001) reported a strong correlation between the two models. For example, comparable IC_{50} values of ketoconazole, quinidine, sulfaphenazole and tranylcypromine were observed for their respective CYPs in both conventional single CYP and the cocktail approach. However, for herbal extracts or fractions with multi-phytochemicals, there is a high probability of at least a constituent acting as a substrate to other isozymes or binding to other CYP protein(s). Such effects might be responsible for the general weak inhibitory effects observed in the cocktail approach. Surprisingly, the crude methanol extract of *M. oleifera* indicated strong inhibition of CYP3A4 using the cocktail approach compared to the conventional assay. Generally, the outcome of the cocktail approach confirms the presence of other isozymes in HLMs contributing to the reduction of inhibition activity of test samples as opposed to the single CYPs.

Prediction of *in vivo* HDI

Prediction of HDI based on IC_{50} values without inclusion of the concentration of herbal extracts reaching the liver and interacting with the enzyme could lead to false positive or negative estimations. Unlike pure compounds or new candidate drugs, no guidelines for the conduct of HDI studies are available. Most research laboratories have adopted the protocol for the conduct of DDI by US FDA and/or PhARMA (Bjorsonn *et al*., 2003). The adoption of current industry and FDA guidelines for prediction of DDI to estimate the degree of HDIs comes with a number of important caveats. Firstly, in industry, pure compounds are evaluated whereas for herbs mixtures of unknown chemical composition are used making it impossible to know the identity

or the concentration of the inhibiting components. The use of the total GIT concentration of the herbal extract (µg/mL) as the assumed soluble extract available to interacts with the intestinal CYPs and P-glycoprotein is therefore a gross exaggeration. Secondly, the IC_{50} and estimations of K_i from this value using specific M-M kinetic conditions is also difficult to do with herbal extracts for the same reason indicated above. The IC_{50} and K_i used for herbal extracts are therefore based on the assumption that at least one pure constituent of the herbal extract is responsible for the inhibitory effect observed on the CYPs. Additionally, the substrate concentration used for the *in vitro* assay is equivalent to the K_m, hence the speculated phytoconstituent is expected to demonstrate competitive inhibition. The K_i value is therefore half of the IC_{50} based on competitive inhibition. Thirdly, industry uses the metabolism-based DDI studies to both guide the molecular design of NCE devoid of potential DDI risks during the early hit and lead identification and lead optimization stages. At candidate drug selection stage, the DDI data is used to select compounds devoid of such DDI risk or the design of *in vivo* DDI studies to determine the extent of the risk of DDI in candidate drugs which still bear the CYP inhibitory effects. For herbal extracts already in use, the HDI studies are therefore mainly to assess risk, guide the design of *in vivo* HDI studies and possibly revise product labels to highlight the risk of co-administering some herbs with certain conventional drugs. The mechanistic approach of using *in vitro* systems adopted in this study allows for rapid evaluation of many herbal medicines and for general or class label recommendations with respect to HDI which can guide patients and clinicians in avoiding drug-herb combinations likely to result in HDI.

Despite the study design and results limitations such evaluations allow to identify herbal extracts with likely risk for HDI for which more detailed biochemical studies (identification of possible components in the extract causing the observed inhibition) and clinical studies (identifying herbs for which *in vivo* evaluation can be conducted) might be required. So for the 7 herbal extracts evaluated in this study, a number of outcomes can be highlighted with respect to their possible clinical risk to cause HDI.

A preliminary risk evaluation from *in vitro* data to *in vivo* using the FDA guidelines predicted that most of the herbal medicines are likely to pose high risk for CYP metabolism-based HDIs. The K_i values for aqueous extracts; T41 (*H. hemerocallidea* capsules), T30 (*H. hemerocallidea*

powdered leaves), T60 (*E. purpurea* capsules), and T80 (*M. oleifera* powdered leaves) obtained with recombinant CYP1A2, CYP2D6 and CYP3A4 were far below the estimated gut and intra-hepatic concentrations of the respective herbal medicines. These herbal medicines were therefore predicted to inhibit the three CYPs *in vivo* to a major extent with potential high risk for HDI as the estimated C_{max}/K_i is greater than 1.0. For most methanol extracts, the K_i values were almost 10-fold greater than the estimated gut and intra-hepatic concentrations for CYP2C9, CYP2C19 and CYP3A4. Based on these findings, it is speculated that inadequate concentration of the methanol extract is likely to reach the liver to interact with CYPs to cause inhibition and possible HDI. Thus, although the methanol extracts were estimated to have high risk to cause HDI based on C_{max}/K_i, such prediction can be false positive. Incorporation of maximum hepatic input into the prediction of HDI indicated severe consequences compared to that of GIT estimation. However, due to the several assumptions considered in such estimation, it is inappropriate to conclude that hepatic estimation superceeds gut prediction since herbal products consist of polyphytochemical with some components unlikely to be completely absorbed. To confirm the inhibitory effects of these extracts, *in vivo* studies in animal models or human is recommended. Other models for characterization of absorption parameters can be employed to evaluate the probability of specific phytochemicals in herbal extracts to actively or passively traverse the intestinal barrier. In this study, a specific absorption model, PAMPA, was introduced to monitor the inhibitory effect of the aqueous herbal extracts which showed high risk of HDI based on the *in vivo* prediction.

Inhibition of CYP1A2 and CYP3A4 by herbs after absorption through PAMPA

The intensity of HDI of orally administered medications depends on transport of the phytochemical constituents via the intestinal transmembrane barrier to the enterocytes and through the portal vein to the hepatocytes. Herbal medicines consist of multi-phytochemical constituents with different physicochemical properties. Certain constituents in herbs are impermeable to the intestinal transmembrane barrier. Influx drug transporters can also concentrate herbal constituents in hepatocytes with the result that the actual concentration in the liver far exceeds that in plasma. It is therefore important to incorporate models that mimic

intestinal absorption of drugs in the conduct of *in vitro* HDI. This will reduce the overestimation and underestimation of HDI. The PAMPA plate system which is frequently used for screening permeability of new chemical entities was employed in this assay to assess the transcellular permeability of the investigated herbal preparations. The plate system employed has triple lipid-oil-lipid layers which mimic the exterior and interior biological membrane of the intestinal barrier (Faller, 2008). The membrane is attached to the basal component of the acceptor plate, acting as a barrier between the acceptor (AC) and donor (DC) compartments. Initially, once test sample is introduced to the donor compartment in phosphate buffer, passive transport occurs unilaterally from the DC to AC. After 5 hours of incubation, the system equilibrates; bidirectional transport of test sample from AC to DC and DC to AC occurs, thus parallel movement is observed.

In this study, the inhibitory effects of most herbal medicines on CYPs (1A2 and 3A4) before and after PAMPA were similar, indicating that significant amount of phytochemical constituents in the herbal medicines evaluated in this study permeated through the lipid- oil- lipid tri-layer. The inhibitory effect of T30 (*H. hemerocallidea* powdered leaves) and T60 (*E. purpurea* capsules) on CYP1A2 was significantly reduced after passive transport through PAMPA compared to the observation for CYP3A4. The active site of CYP1A2 has a planar shape which favours chemicals with aromatic rings such as polyphenols predominantly found in herbal extracts. CYP3A4 however, has different binding pocket and the degree of inhibition depends on the affinity of a specific inhibitor for a given active site (Ohkura *et al*., 2009).

First-line treatment of most HIV-infected patients in both developing and developed countries includes NNRTIs (nevirapine and efavirenz) and PIs (ritonavir and atazanavir) as a therapy (Shubber *et al*., 2013). These NNRTIs are metabolized by intestinal and hepatic CYP3A4. Consequently, co-administration of nevirapine and efavirenz with the investigated herbal preparations, especially T30 (*H. hemerocallidea* powdered leaves) and T79 (*L. frutescens* tea cut), has the potential to cause HDI. The inhibitory effect of these herbal preparations on CYP3A4 may increase plasma concentrations of NNRTIs and PIs generating possible systemic toxicity.

PART TWO

3.2 Time-dependent inhibition of CYP

3.2.1 TDI IC_{50} shift fold

The IC_{50}-shift assay with single substrate for CYP2C19 and CYP3A4 was used to evaluate the TDI potencies of the crude methanol extracts and fractions. An inhibition curve (shift IC_{50} value for TDI) obtained in the presence of NADPH was compared with inhibition curve (reversible IC_{50} value) obtained in the absence of NADPH to determine the TDI potency of individual fractions as shown in Figures 3.6, 3.7 and 3.8, respectively. Fold shift in IC_{50} as a ratio of the reversible and TDI IC_{50}s was employed to determine the potencies of each fraction by categorizing samples as positive TDI or negative TDI (Figure 3.5). Herbal extracts and fractions prepared based on the Figure 2.2A to 2.2D were used for TDI screening assays. Test samples with fold shift in IC_{50} $\geq$1.5 were classified as positive TDI. The fractions 45A and 45D of *K. crenata* showed TDI potencies with fold shift in IC_{50} of 2.26 and 1.50 for CYP3A4 and, 2.36 and 5.07 for CYP2C19, respectively. The crude methanol extract of *K. crenata* (39KC) and the fraction 45B were observed as positive TDI of CYP3A4 with fold shift of 3.29 and 1.91, respectively. However, both 39KC and 45B indicated a non-TDI effect on CYP2C19 with fold shift of 1.30 and 1.20. The crude methanol extract of *L. frutescens* and fraction 43.10 exhibited TDI potential with IC_{50} shift fold of 3.0 and 2.12, respectively on CYP3A4, whilst the pure compounds were non-TDI. However, Sutherlandioside D and, fractions 43D and 43C exhibited TDI potential with IC_{50} shift fold of 1.85, 4.73 and 2.06, respectively on CYP2C19. Fractions 50A, 50B, and 50C of *M. oleifera* demonstrated TDI activity with IC_{50} shift fold of 8.31, 7.16 and 3.92, respectively on CYP3A4. However, the crude methanol extract and fractions of *M. oleifera* showed non-TDI activity on CYP2C19. Troleandomycin and ketoconazole produced a fold shift of 3.29 and 0.67, respectively which were in agreement with previous study by Berry *et al*., (2008). The positive and negative controls of CYP2C19, ticlopidine (TPD) and tranylcypromine (TCP) yielded a fold shift of 2.10 and 0.55, respectively.

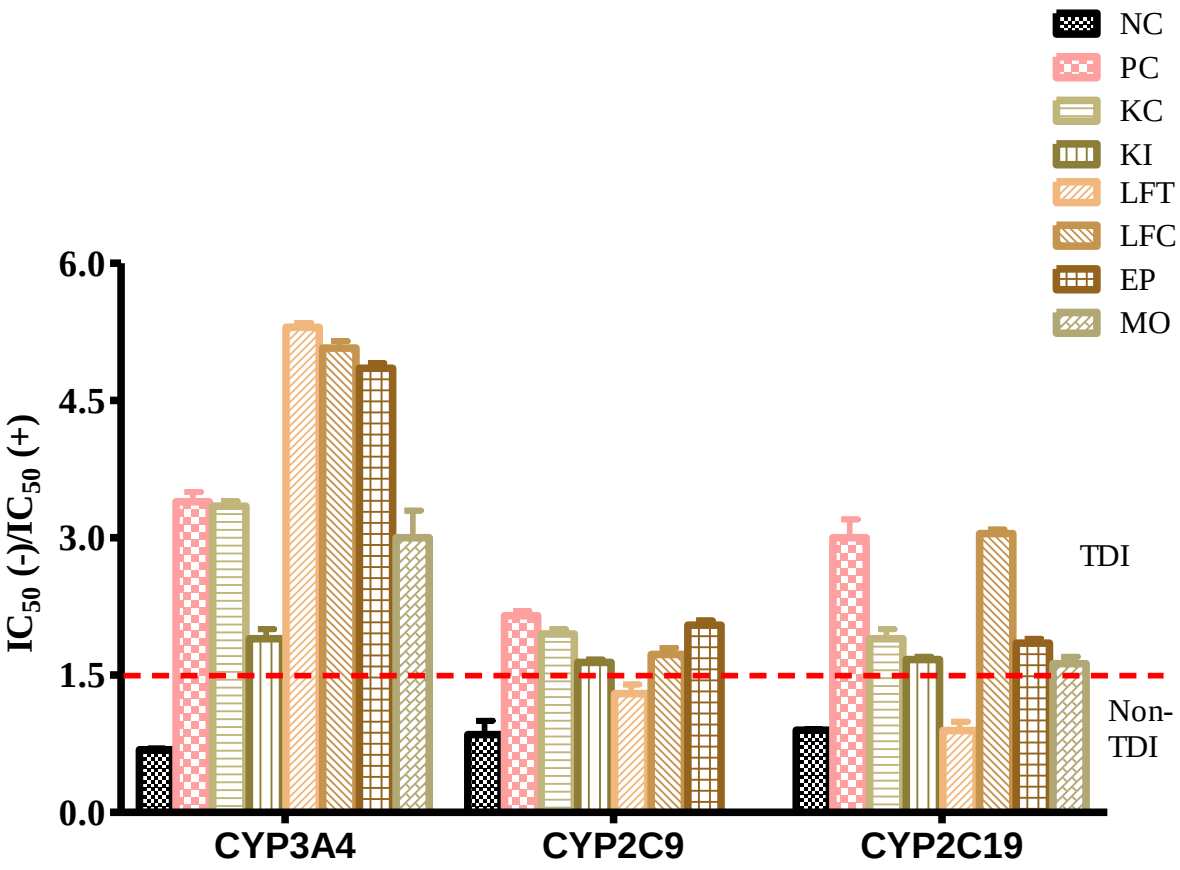


Figure 3.5 Determination of TDI based on IC_{50} shift fold. Bars represent the ratio of IC_{50} values from the no preincubation (-) assays to the 30 min preincubation (+) assays for the test samples listed. Data are the mean ± SEM (n=2). Positive control (PC) and negative control (NC) used for the various CYPs were; troleandomycine and ketoconazole (CYP3A4), ticlopidine and tranylcypromine (CYP2C19) and Tienilic acid and sulphenazole (CYP2C9).

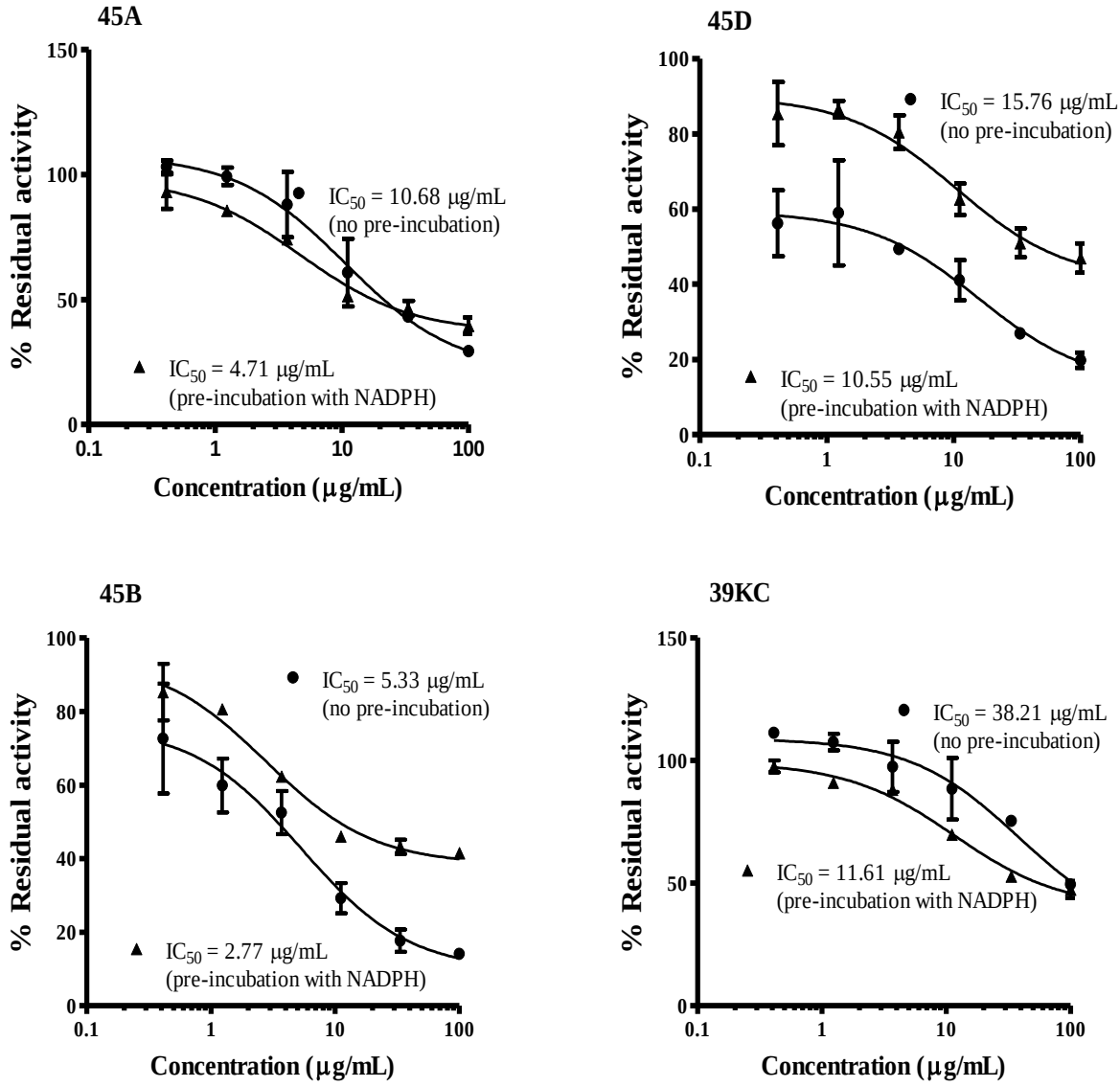


Figure 3.6A IC_{50} shift curves for rCYP3A4 in the presence of Crude (39KC) and fractions 45A, 45B and 45D of *K. crenata* in recombinant CYP3A4.

Values represent the average of duplicate experiments. Inhibition percentages for no preincubation (closed circles) and 30 min preincubation (close triangle) are shown. 39KC (crude methanol extract), 45A ($CHCl_3$: 100%), 45B ($CHCl_3$/MeOH: 90/10%), 45D ($CHCl_3$/MeOH/H_2O: 67/28/5%) and 45E (MeOH: 100%).

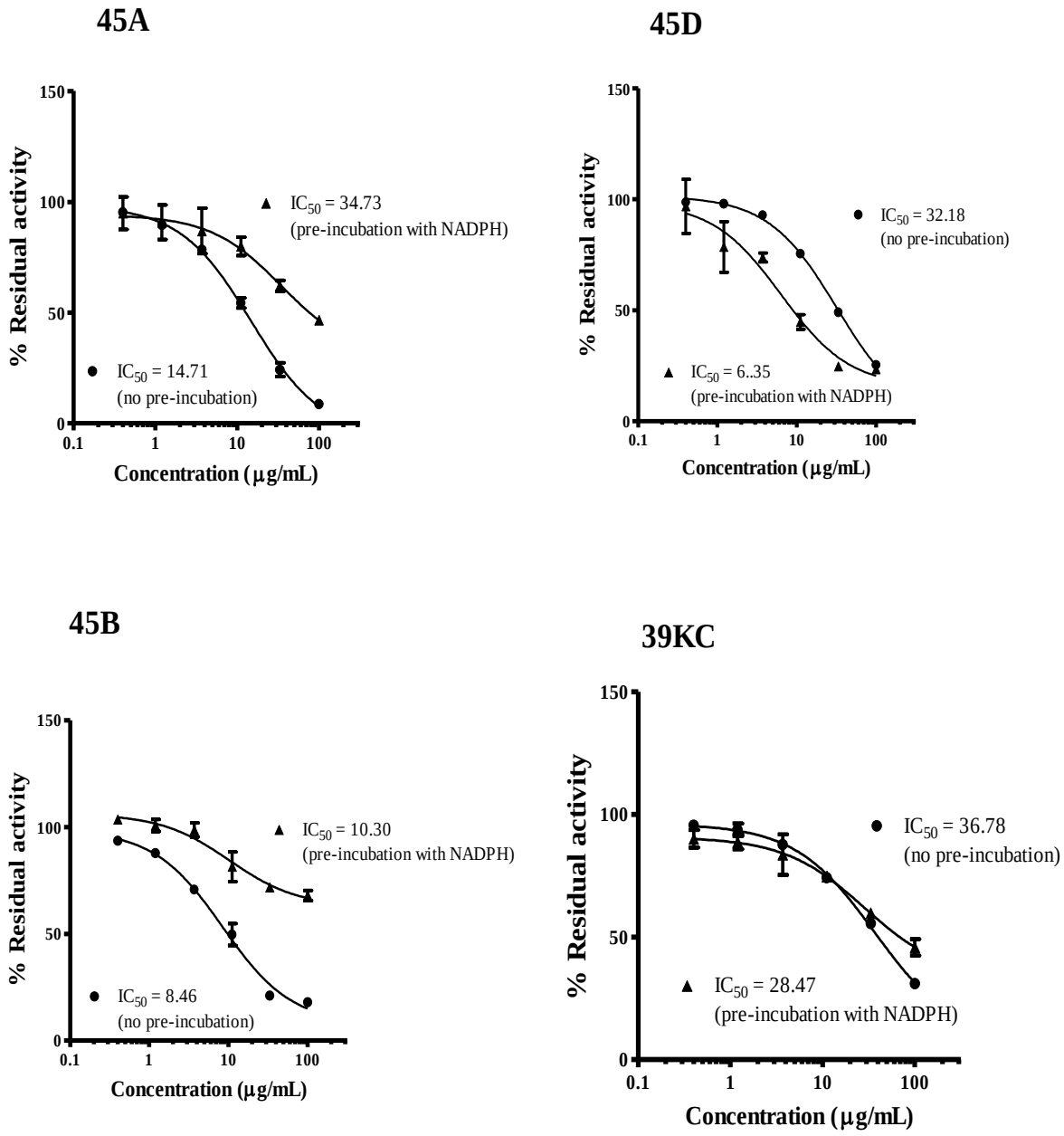


Figure 3.6B: IC_{50} shift curves for rCYP2C19 in the presence of Crude (39KC) and fractions 45A, 45B and 45D of *K. crenata*.

Values represent the average of duplicate experiments. Inhibition percentages for no preincubation (closed circles) and 30 min preincubation (close triangle) are shown. 39KC (crude methanol extract), 45A ($CHCl_3$: 100%), 45B ($CHCl_3$/MeOH: 90/10%), 45D ($CHCl_3$/MeOH/H_2O: 67/28/5%) and 45E (MeOH: 100%).

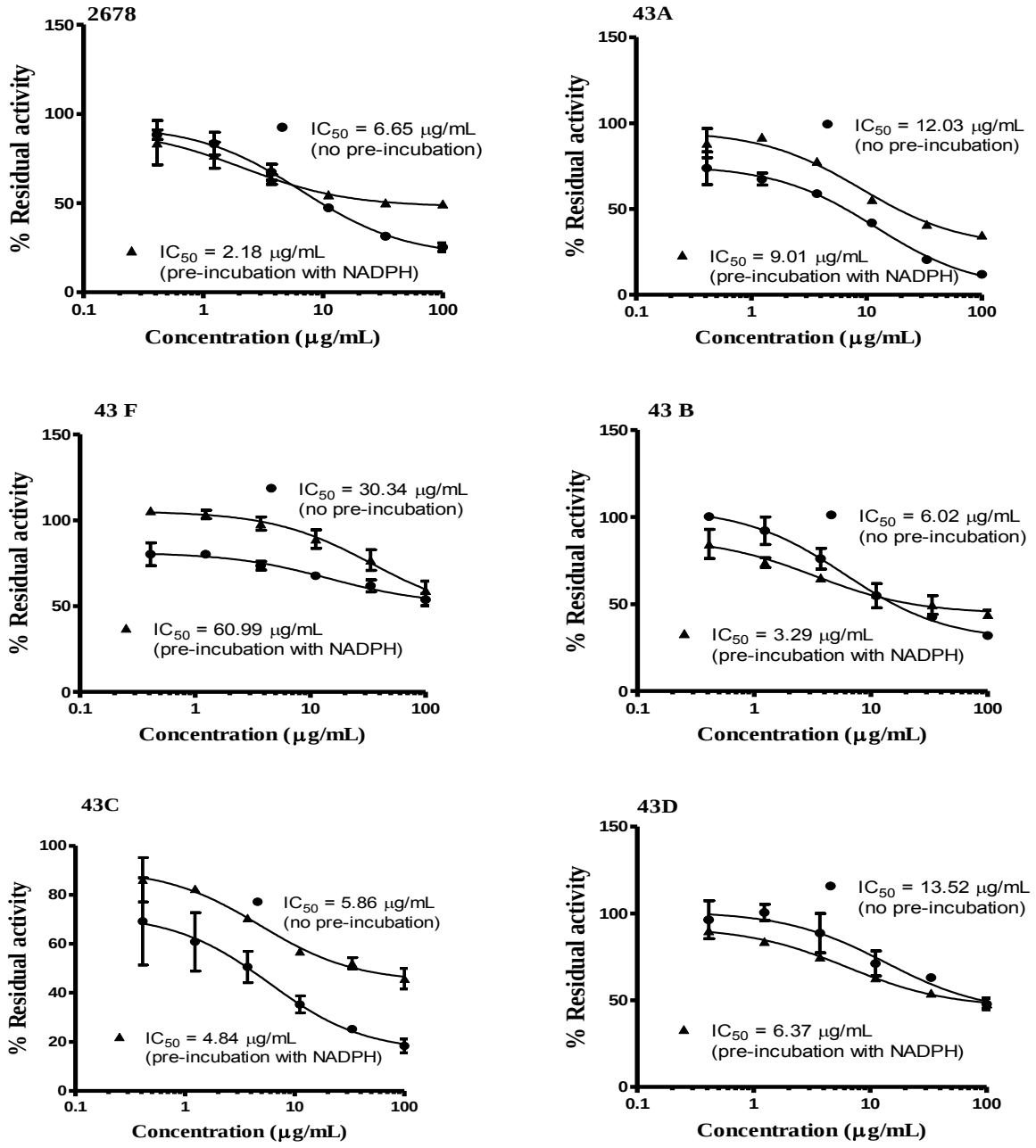


Figure 3.7A IC_{50} shift curves for rCYP3A4 in the presence of *L. frutescens* crude (2678), fractions 43A, 43B, 43C, 43D and 43F and pure compounds (sutherlandioside A-D and sutherlandin A-D). The values represent the average of duplicate experiments. Inhibition percentages for no preincubation (closed circles) and 30 min preincubation (close triangle) are shown. 2678 (crude methanol extract), 43A ($CHCl_3$: 100%), 43B ($CHCl_3$/MeOH: 90/10%), 43C-E ($CHCl_3$/MeOH/H_2O: 67/28/5%) and 43F (MeOH: 100%).

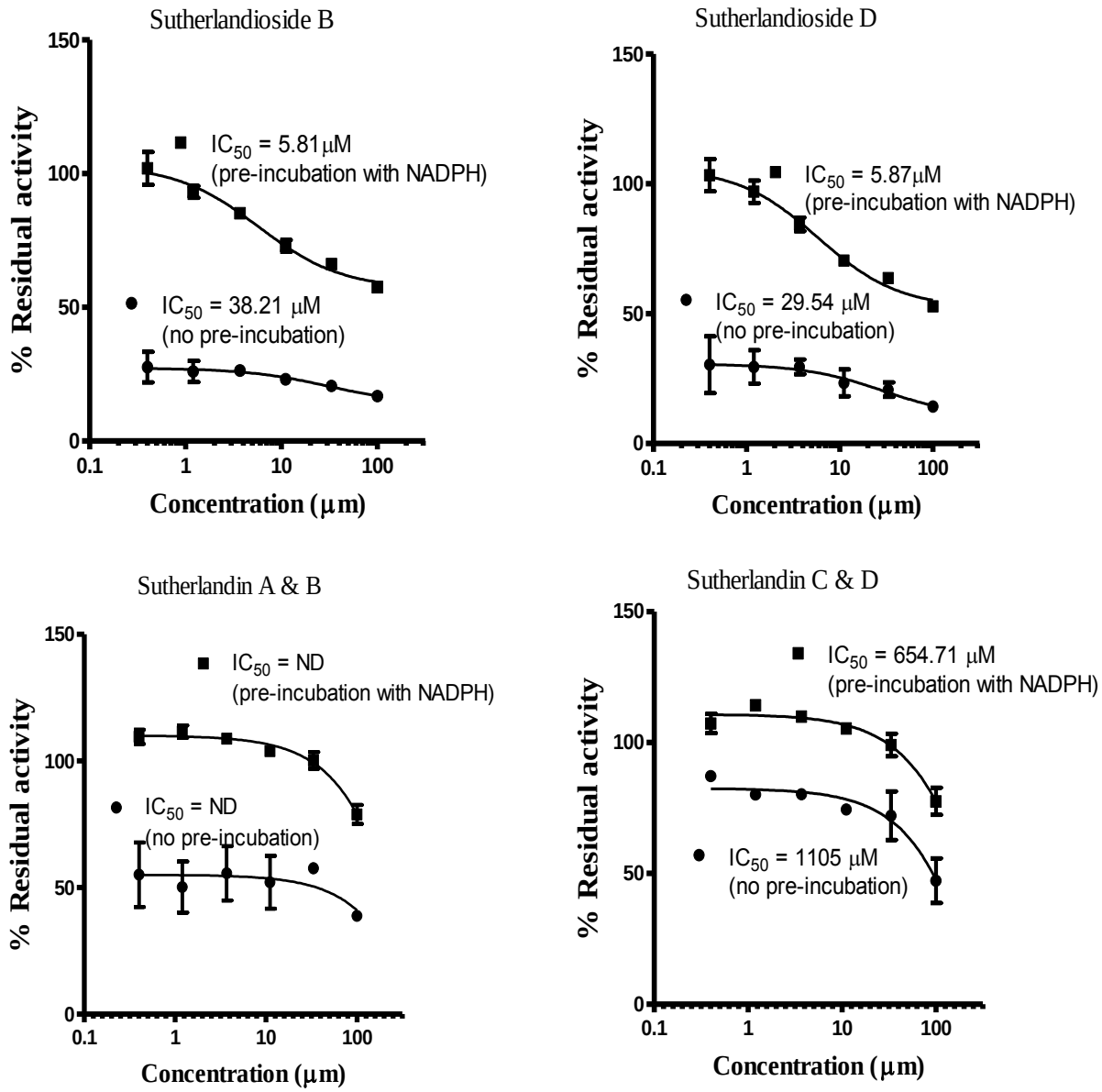


Figure 3.7A continued:

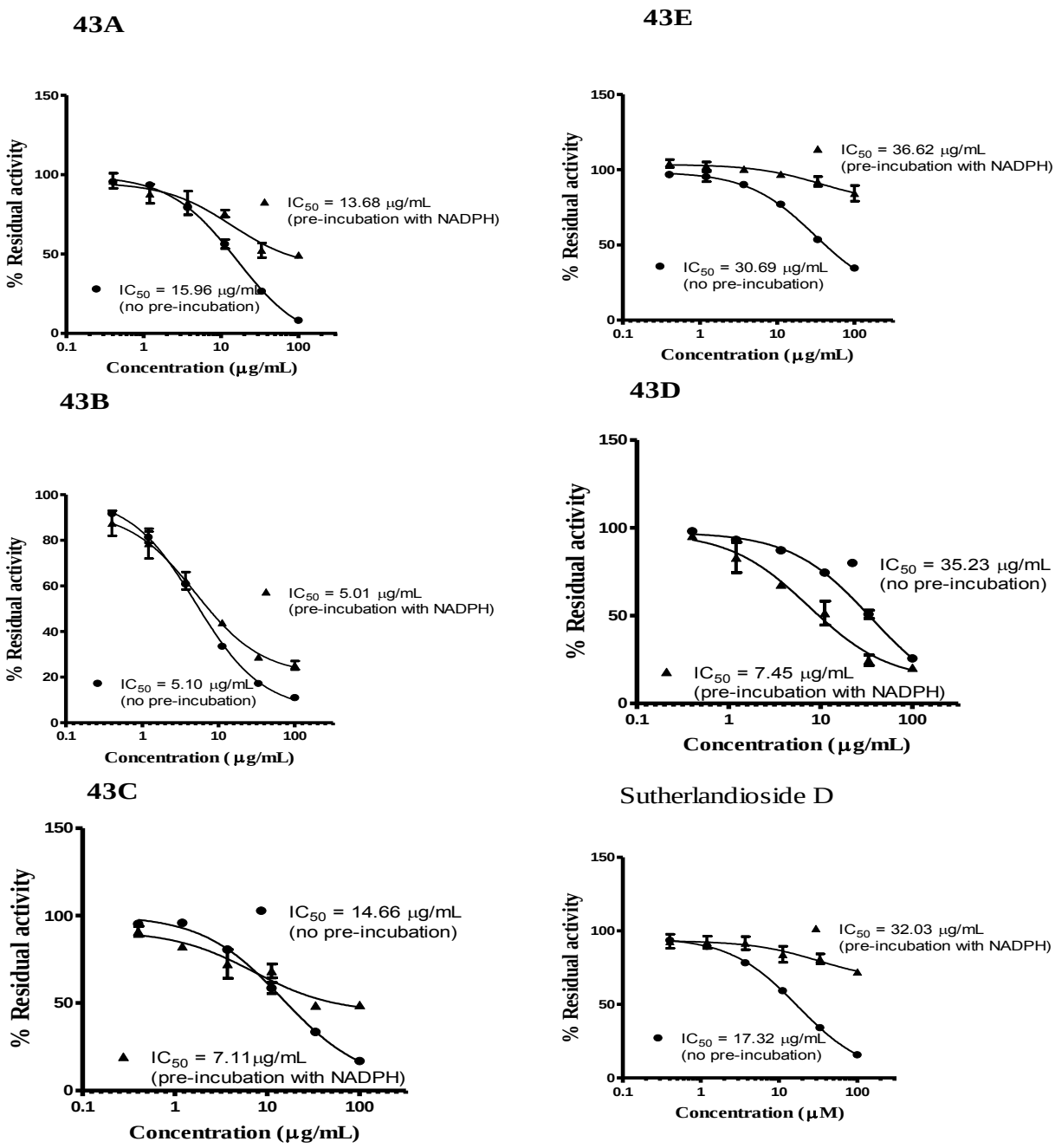


Figure 3.7B: IC_{50} shift curves for rCYP2C19 in the presence of *L. frutescens* crude (2678), fractions 43A, 43B, 43C, 43D and 43F and pure compounds (sutherlandioside D).

Values represent the average of duplicate experiments. Inhibition percentages for no preincubation (closed circles) and 30 min preincubation (close triangle) are shown.

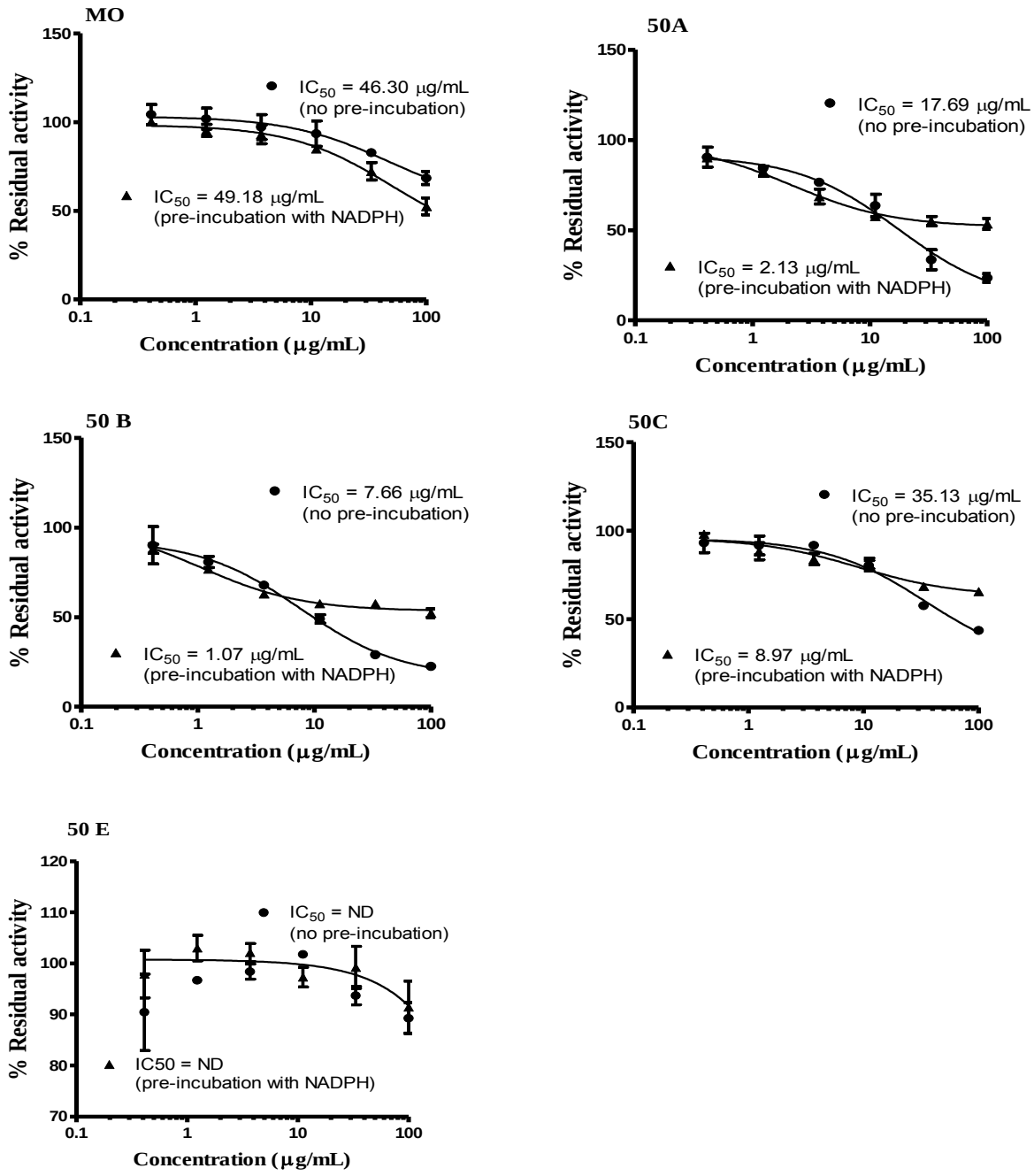


Figure 3.8A IC_{50} shift curves for rCYP3A4 in the presence of crude (MO) and fractions 50A, 50B, 50C and 50E of *M. oleifera*.

Values represent the average of duplicate experiments. Inhibition percentages for no preincubation (closed circles) and 30 min preincubation (close triangle) are shown. MO (crude methanol extract), 50A ($CHCl_3$: 100%), 50B ($CHCl_3$/MeOH: 90/10%), 50C ($CHCl_3$/MeOH/H_2O: 64/16/20%) and 50E (MeOH: 100%).

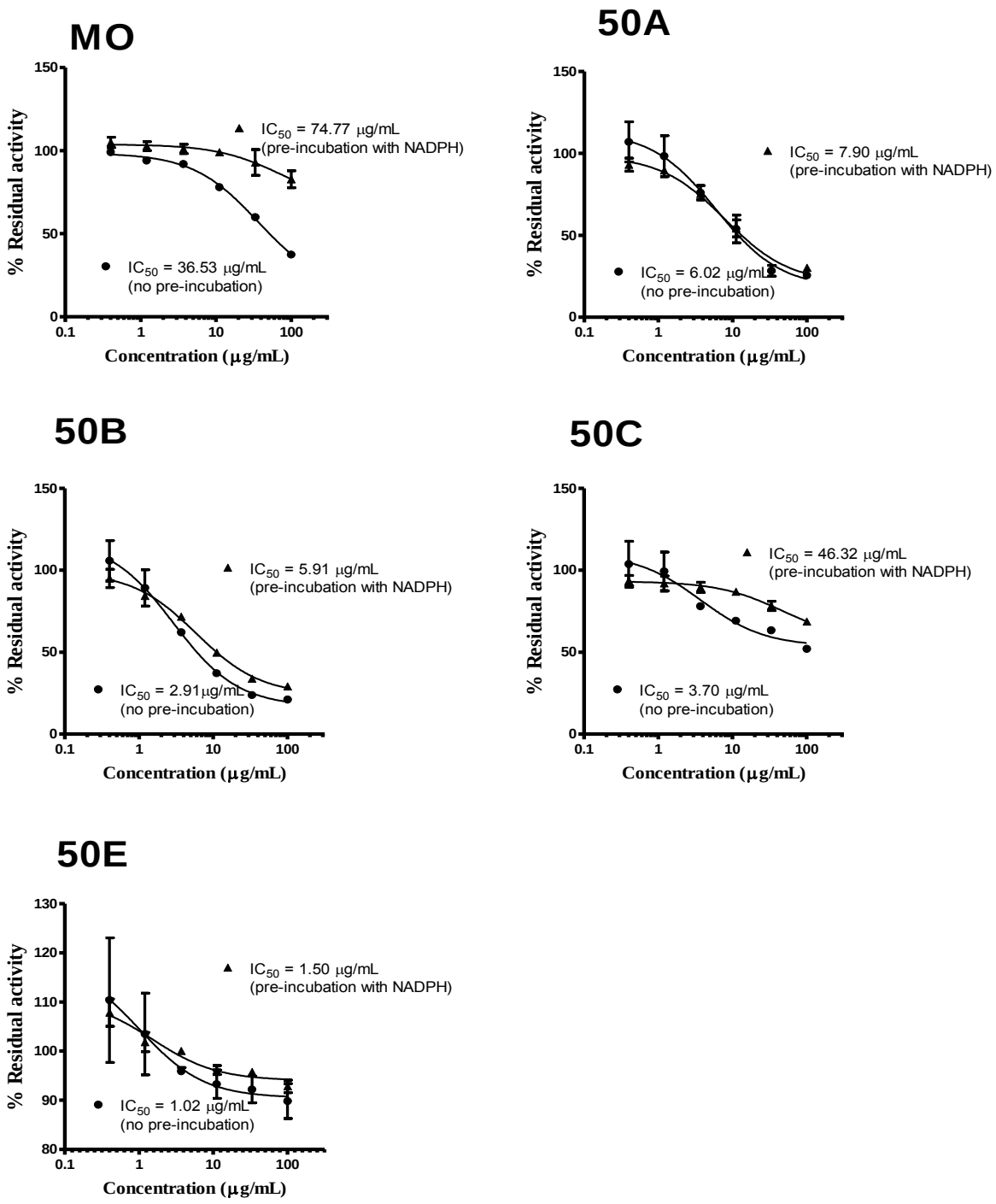


Figure 3.8B: IC_{50} shift curves for rCYP2C19 in the presence of crude (MO) and fractions 50A, 50B, 50C and 50E of *M. oleifera*.

Values represent the average of duplicate experiments. Inhibition percentages for no preincubation (closed circles) and 30 min preincubation (close triangle) are shown.

3.2.2 Application of normalized ratio for TDI screening

The aqueous and methanol extracts of the various herbal medicines were categorized into clear (≤0.70), intermediate TDI (0.7 – 0.9) or non-TDI (>0.9) applying the normalized ratio. Aqueous herbal extracts of *L. frutescens* tablets (T50) and *H. hemerocallidea* capsule (T41) showed concentration dependent TDI on CYP3A4. Although *H. hemerocallidea* powdered leaves (T30) showed a clear TDI, the effect was a non-concentration dependent. The aqueous extracts of *T. officinale* and *M. oleifera* exhibited non-TDI effect (Figure 3.9A). All methanol extracts of herbal medicines tested except *M. oleifera* indicated clear TDI at 100 µg/mL. *M. oleifera* showed an intermediate TDI at 100 µg/mL and as a non-TDI at 25 µg/mL (Figure 3.9B). The troleandomycin (TAO) and KTZ demonstrated clear TDI (<0.7) and non-TDI (>0.9), respectively with values which agreed with those reported by Atkinson *et al*., (2005).

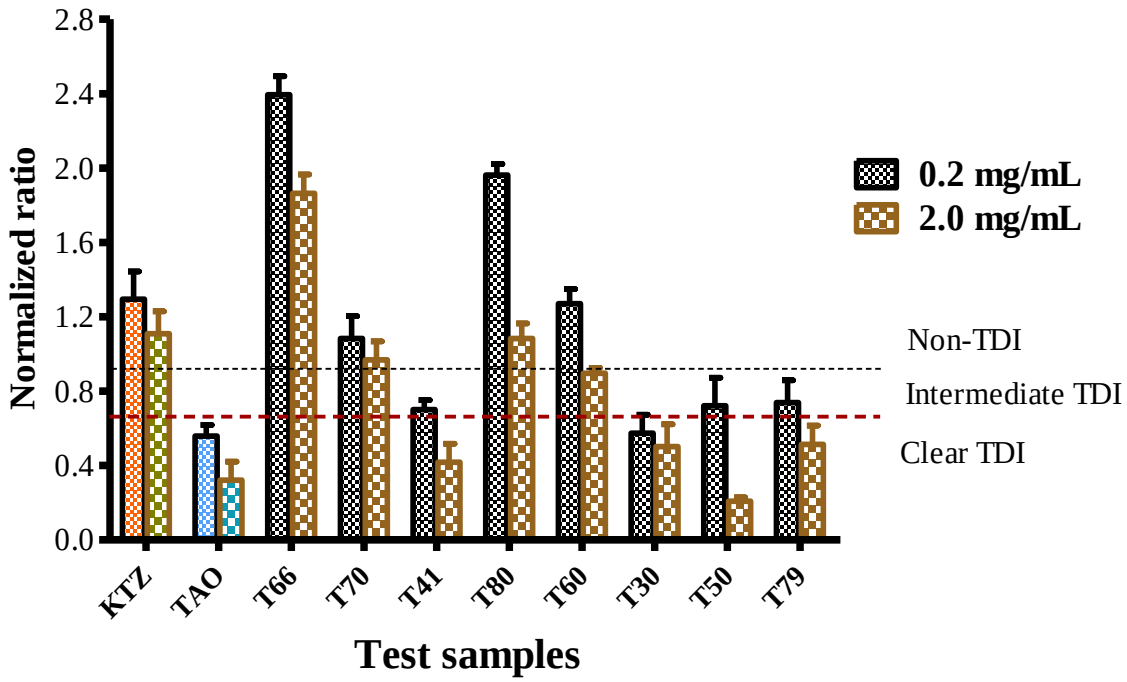


Figure 3.9A TDI classification of aqueous herbal extracts based on normalized ratio. Known TDI compound, troleandomycin (TAO, 1 and 20 µM) and non-TDI compound, ketoconazole (KTZ, 0.1 and 1 µM) were used as positive and negative controls. High and low concentrations of herbal extracts 0.2 and 2.0 mg/mL were used. The normalized ratio was calculated as described under *Materials and Methods*. Data expressed as Mean ± SEM, n= 2.

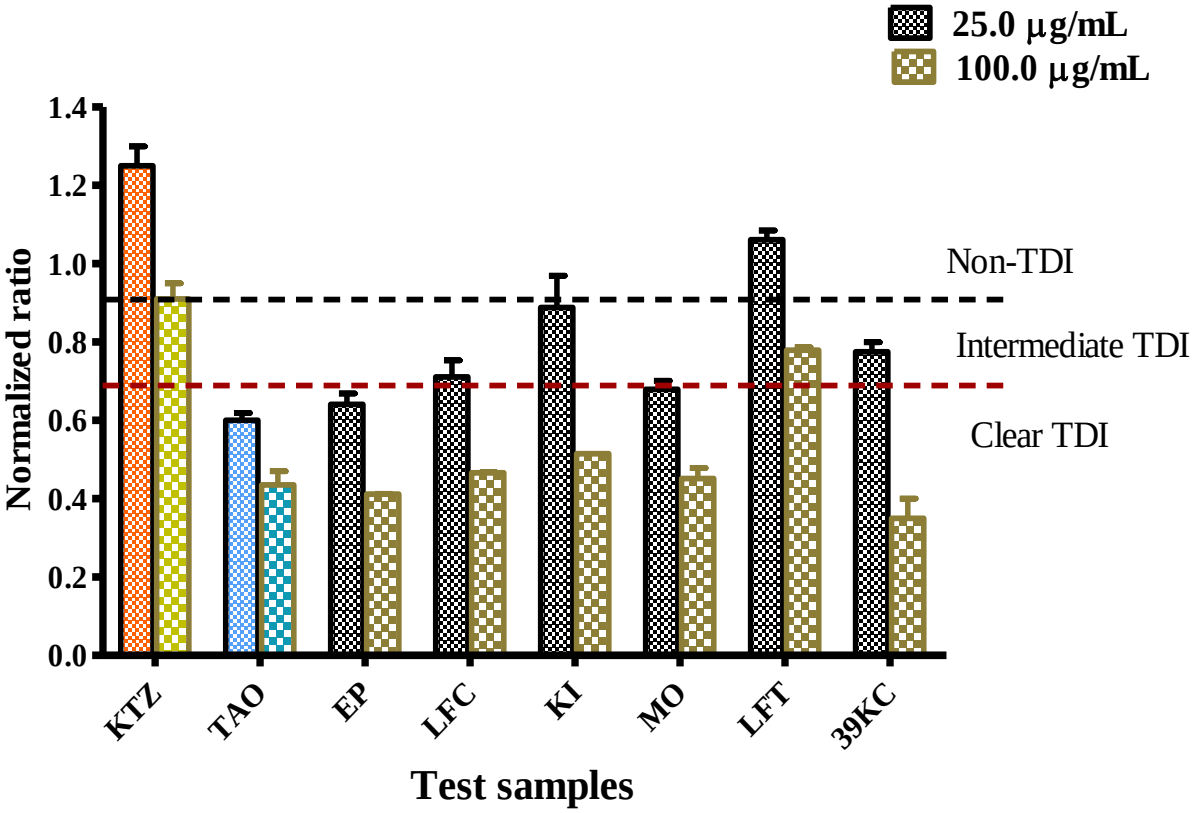


Figure 3.9B: TDI classification of methanol herbal extracts based on normalized ratio. Known TDI compound, troleandomycin (TAO, 1 and 20 µM) and non-TDI compound, ketoconazole (KTZ, 0.1 and 1 µM) were used as positive and negative controls. High and low concentrations of herbal extracts 25.0 and 100.0 µg/mL were used. The normalized ratio was calculated as described under *Materials and Methods*. Data expressed as Mean ± SEM, n = 2.

3.2.3 Time-, concentration- and NADPH- dependent inhibition

The crude extract and fractions of *K. crenata* indicated varying degree of inhibitory effect on CYP2C19 and CYP3A4 in the presence and absence of NAPDH. The fractions 45A, 45B and 45D showed an NADPH dependent inhibitory effect on both CYP2C19 and CYP3A4. However, the crude extract indicated NADPH dependent inhibition on CYP3A4 and non-NADPH dependent inhibition potential on CYP2C19 (Figure 3.10A). *M. oleifera* crude extract and the fractions indicated non-NADPH dependent inhibitory effect on CYP3A4. The fractions 50B,

50E and crude methanol of *M. oleifera* inhibited CYP2C19 significantly in NADPH-dependent manner as shown in Figure 3.10B.

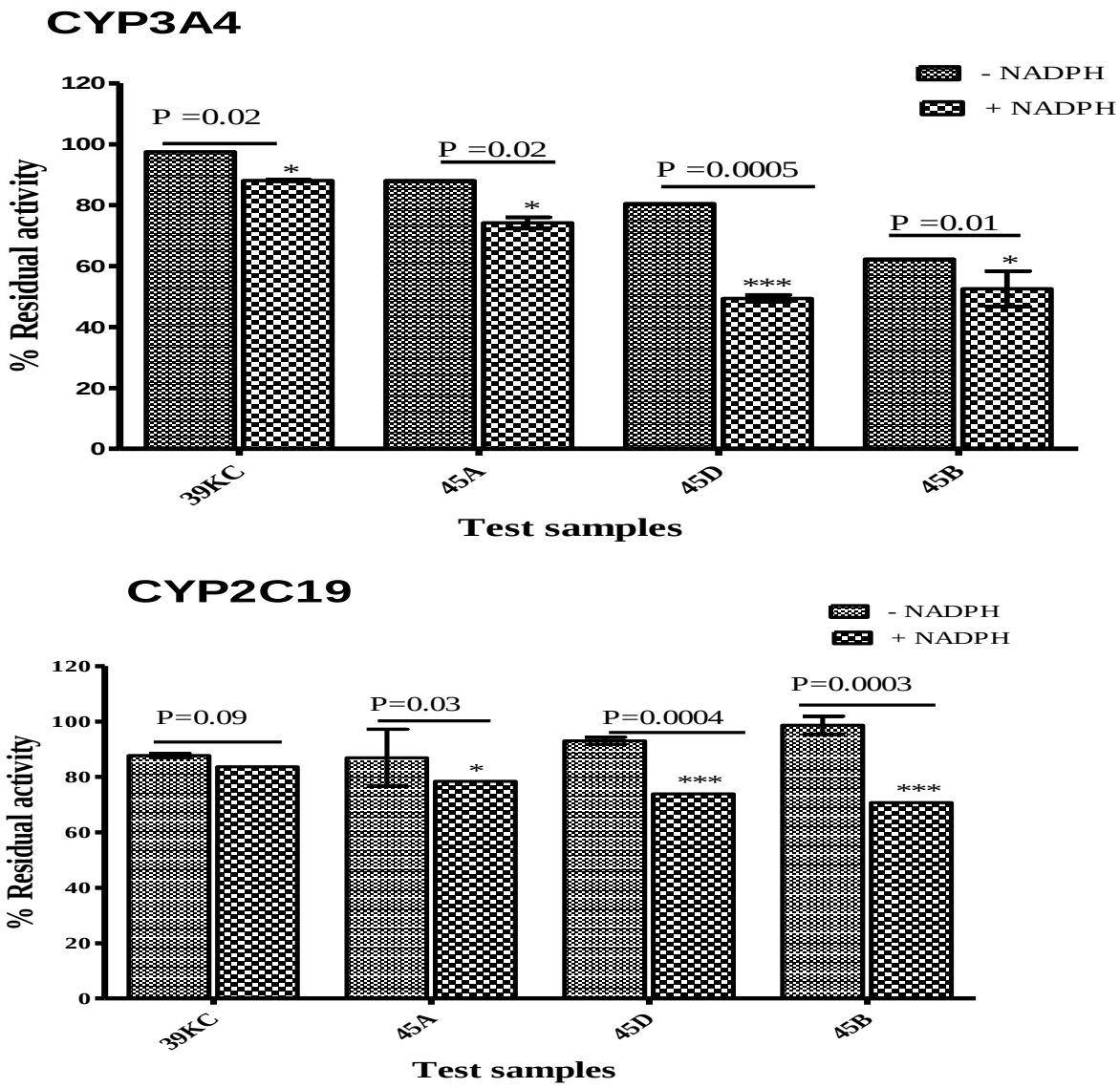


Figure 3.10A Inhibitory effects of crude and fractions of *K. crenata* (50 µg/mL) on CYP3A4 and CY2C19 preincubated for 30 mins in the presence or absence of NADPH. The percent residual activities were plotted against respective fractions. Two tailed unpaired t-test were used to compare the percent activity remaining for each fraction with $P < 0.05$ considered significant.

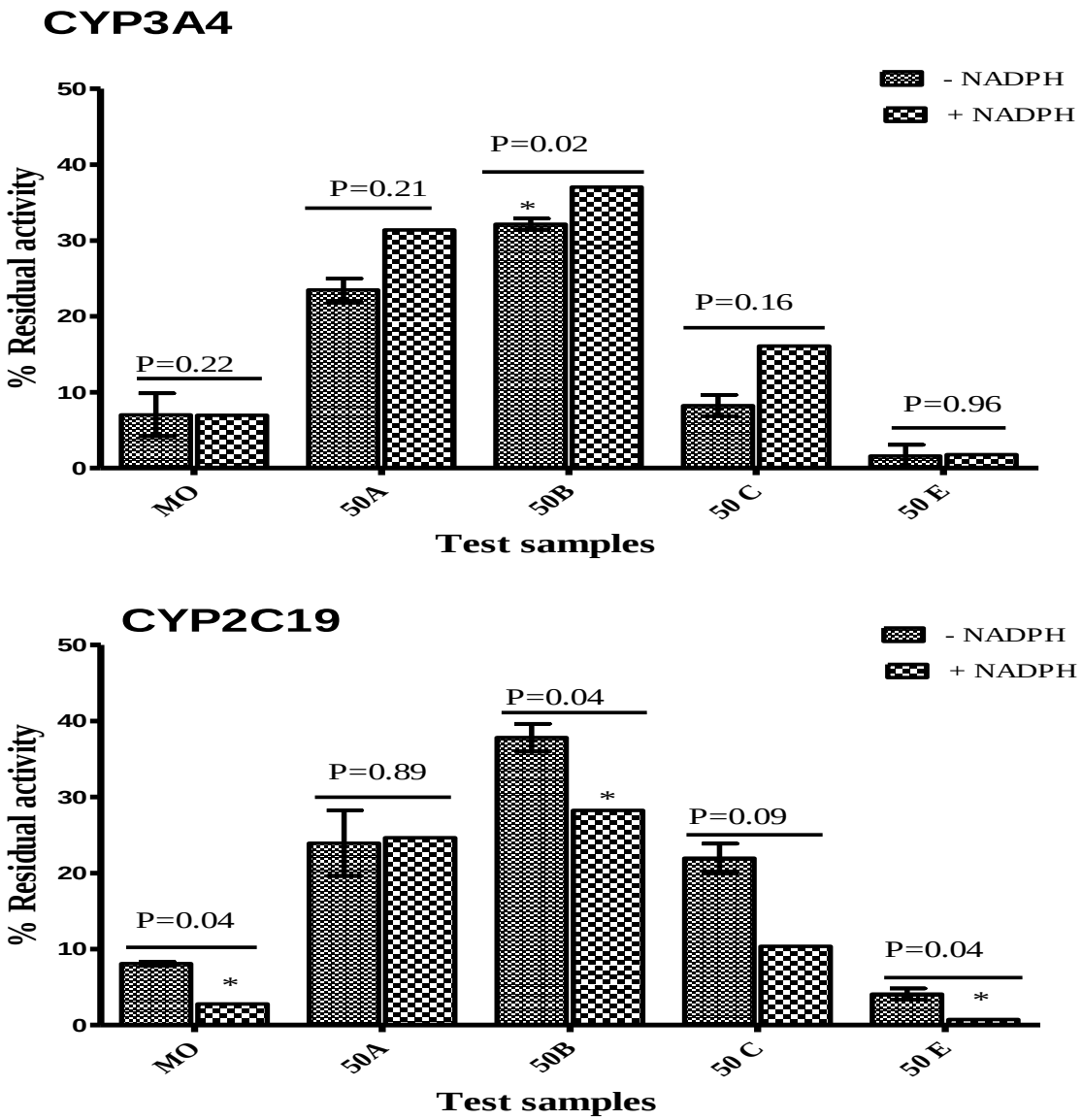


Figure 3.10B: Inhibitory effects of crude and fractions of *M. oleifera* (50 µg/mL) on CYP3A4 and CY2C19 preincubated for 30 mins in the presence or absence of NADPH. The percent residual activities were plotted against respective fractions. A two-tailed unpaired t-test was used to compare the percent activity remaining for each fraction, with $P < 0.05$ considered significant.

Preincubation of crude methanol and fractions of *K. crenata* and *M. oleifera* for 0, 10, 20 and 30 mins indicated time-dependent inactivation of CYP3A4 as the degree of inhibition increased with time (Figures 3.11A-B). However, 39KC did not show TDI following preincubation with

CYP2C19 in the presence of NADPH. The fractions 45A, 45B and 45D however indicated TDI on CYP2C19 as shown in Figure 3.11A. The large SEM indicated on residual activity of fraction 45A in the absence of NADPH could be due to artifact. Crude *M. oleifera* and fractions were inactive on CYP2C19 after preincubation at specific durations.

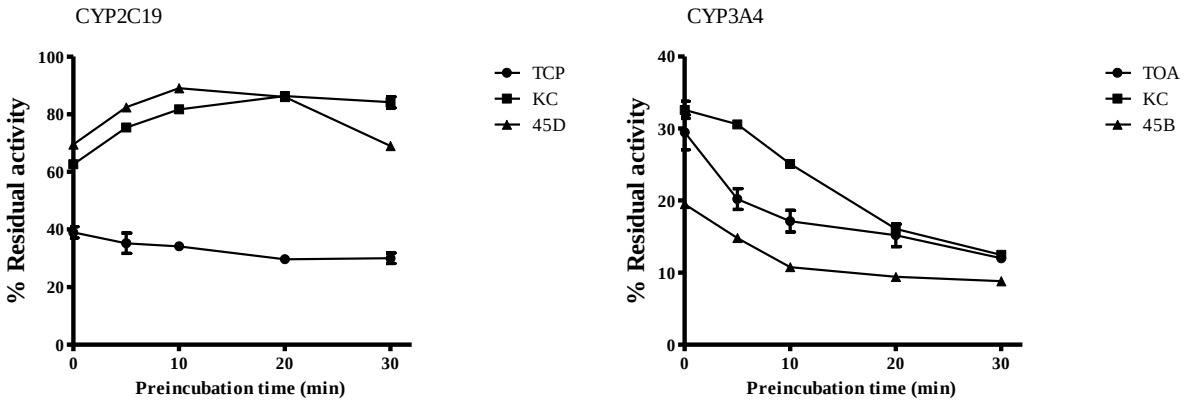


Figure 3.11A Time-dependent inhibition of *K. crenata* on CYP3A4 and CYP2C19. 50 µg/mL of 39KC, 45B or 45D were preincubated for 0, 10, 20 and 30 min. The percent remaining activity was plotted against respective time point through connecting lines. Each point represents the Mean ± SEM of duplicate determinations.

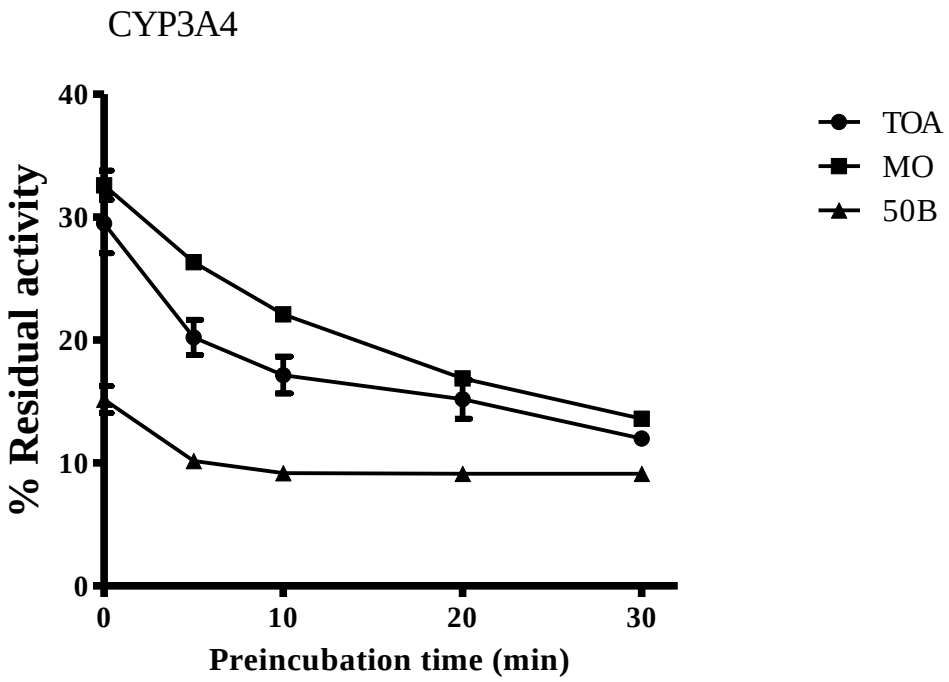
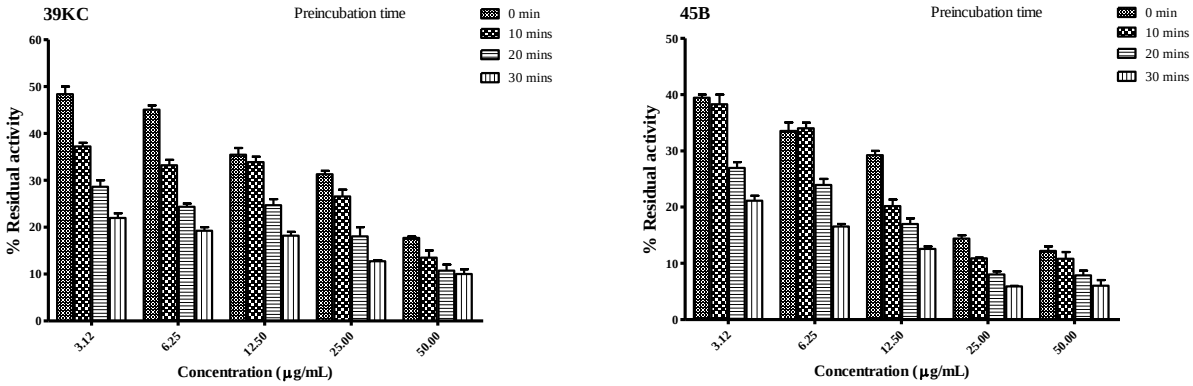


Figure 3.11B: Time-dependent inhibition of *M. oleifera* on CYP3A4. 50 µg/mL of MO and 50B were preincubated for 0, 10, 20 and 30 min. The percent remaining activity was plotted against respective time point through connecting lines. Each point represents the Mean ± SEM of duplicate determinations.

Inactivation of CYP3A4 by 39KC, 45B and 50B followed a time- and concentration-dependent pattern. Crude methanol extract of *M. oleifera* had a concentration-dependent and not a time-dependent effect on CYP3A4. However, inactivation of CYP2C19 by 39KC and 45D did not indicate time- and concentration dependency, as the inhibition profile became weaker with increase in both time and concentration of the respective extract or fractions applied to the incubation mixture (Figure 3.12A).

CYP3A4



CYP2C19

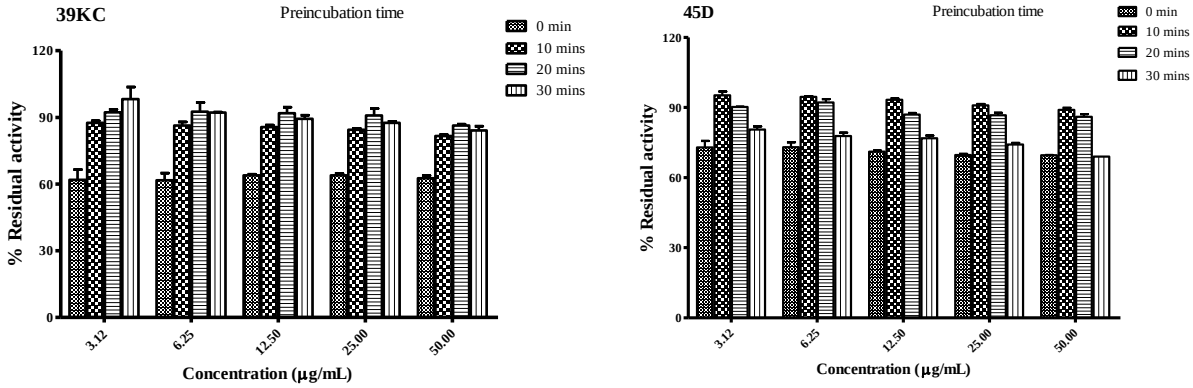


Figure 3.12A Concentration- and time-dependent inhibition of CYP3A4 and CYP2C19 by 39KC, 45B and 45D preincubation for 0, 10, 20 and 30 min. The percent remaining activity was plotted against respective time point through connecting lines. Each point represents the Mean ± SEM of duplicate determinations.

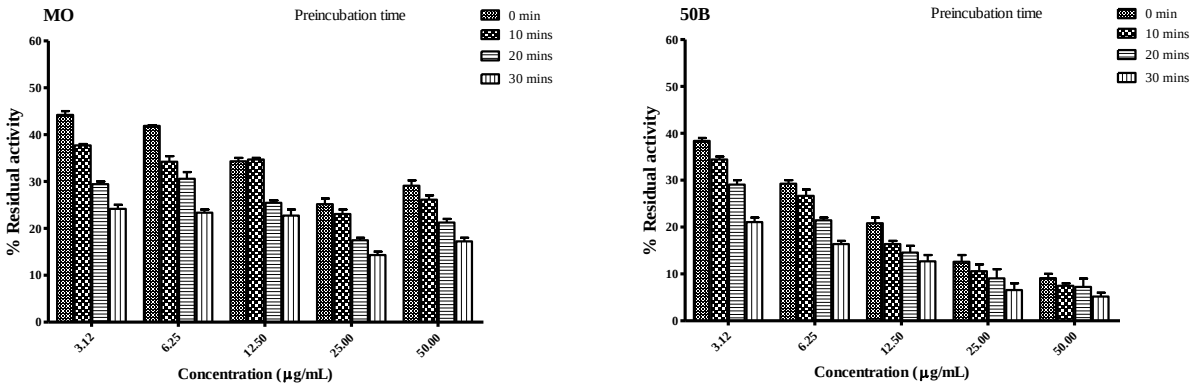


Figure 3.12B: Concentration- and time-dependent inhibition of CYP3A4 by MO and 50B preincubation for 0, 10, 20 and 30 min. The percent remaining activity was plotted against respective time point through connecting lines. Each point represents the Mean ± SEM of duplicate determinations.

3.2.4 Kinetics of CYP3A4 inactivation

CYP3A4 HFC activity was inactivated by *K. crenata* preparations (39KC and 45B) and *M. oleifera* preparations (MO and 50B) in a time- and concentration- dependent manner in the presence of NADPH regeneration system. The loss of activity followed pseudo-first-order kinetics. Linear regression analysis of the time course data was used to determine the k_{obs} values at various concentrations (Figure 3.13A-B). The K_I and k_{inact} values were determined from double reciprocal plots of k_{obs} values and inactivator concentrations. The K_I and k_{inact} values for 39KC were 1.77µg/mL and 0.091 min^{-1} while that of 45B were 6.45 µg/mL and 0.024 min^{-1}, respectively. The crude *M. oleifera* and fraction 50B yielded K_I of 25.3 and 47.85 µg/mL, respectively while their individual K_{inact} values were 0.018 and 0.044 min^{-1}.

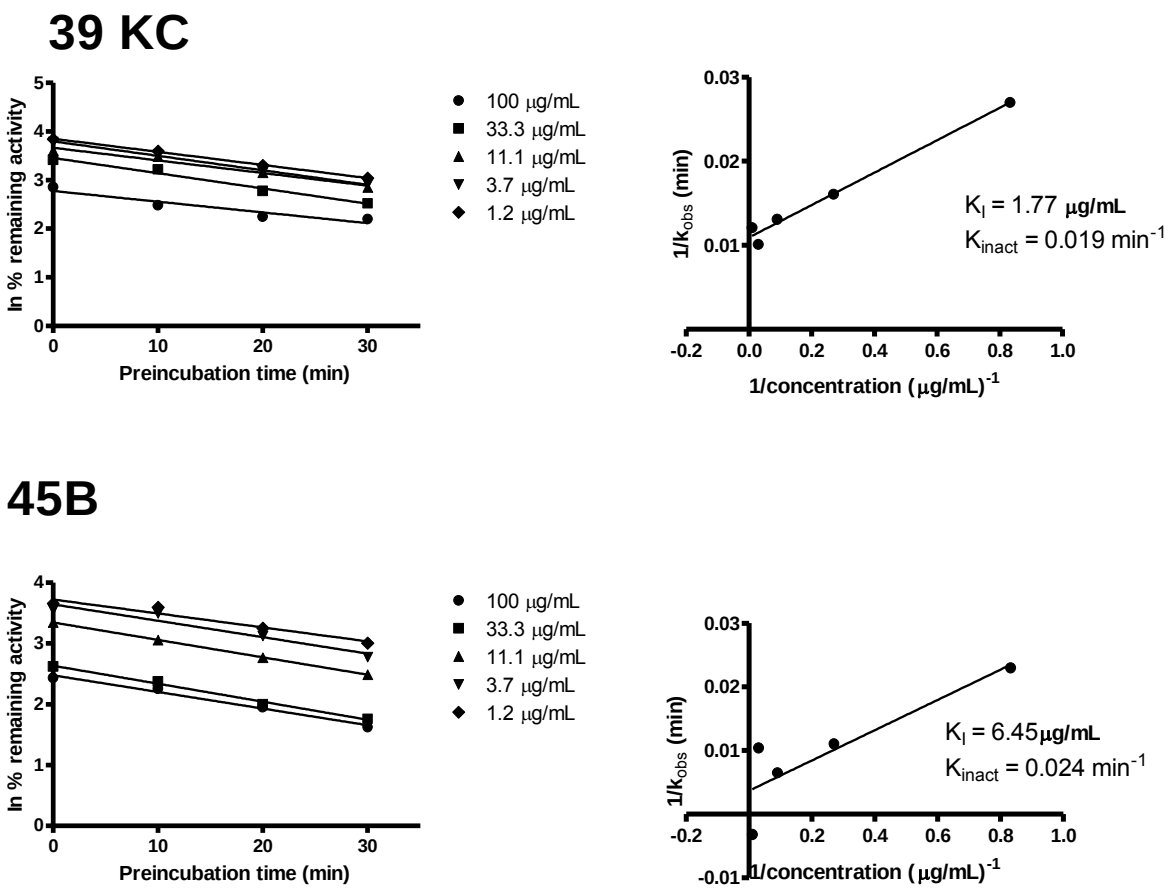


Figure 3.13A Inactivation kinetics of CYP3A4 by 39KC and 45B. Concentrations of fractions are shown on the right of the plots (A). Double-reciprocal plots of k_{obs} and inhibitor concentrations were plotted on linear graph to determine inactivation parameters K_I and K_{inact} in plot (B). Each point represents the mean of duplicate determinations.

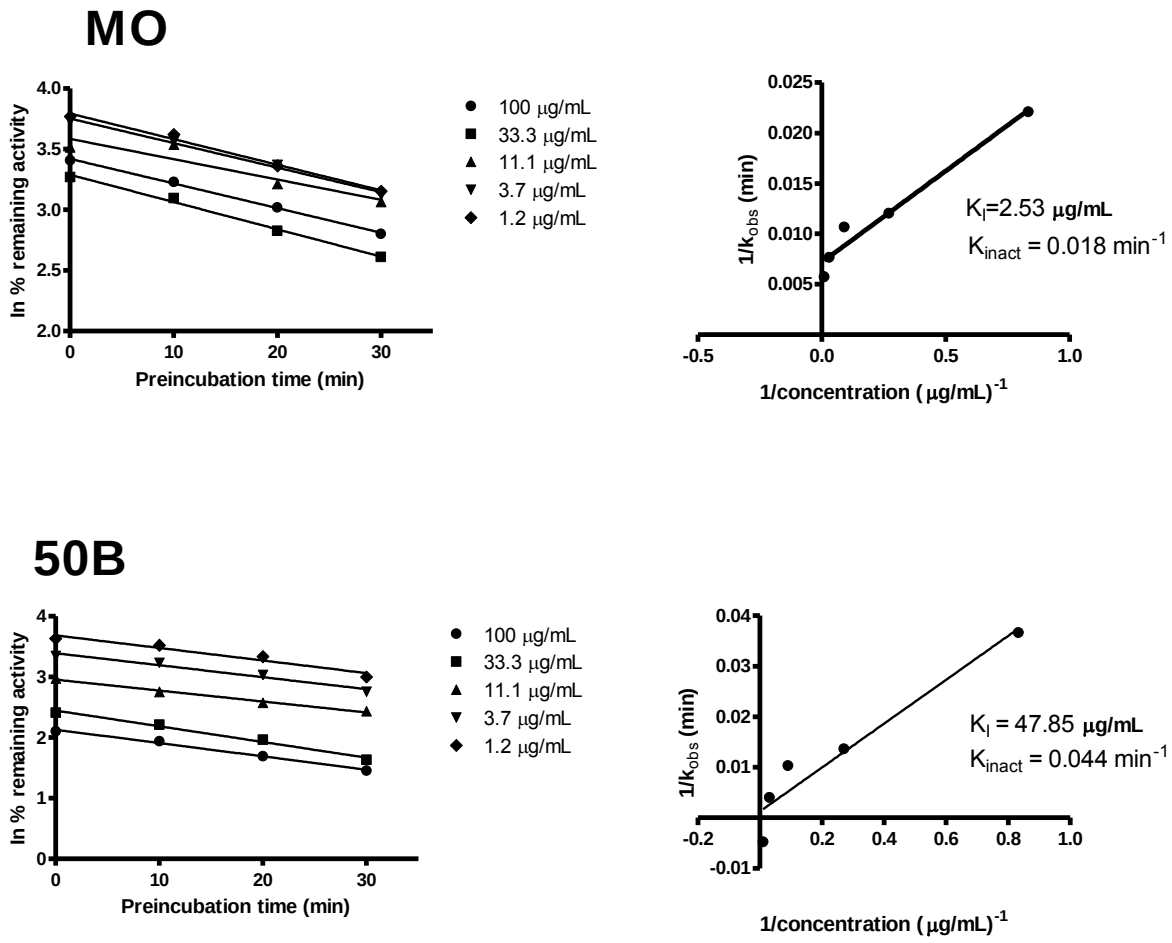


Figure 3.13B: Inactivation kinetics of CYP3A4 by MO and 50B. Concentrations of fractions are shown on the right of the plots (A). Double-reciprocal plots of k_{obs} and inhibitor concentrations were plotted on linear graph to determine inactivation parameters K_I and K_{inact} in plot (B). Each point represents the mean of duplicate determinations.

3.2.5 *In vivo* prediction of CYP3A4 inactivation

The likely *in vivo* effects of crude extracts of *K. crenata* and *M. oleifera* on both enteric and hepatic CYP3A4 were evaluated by applying the λ/k_{deg} approach of classification. The [I] of each herbal extracts was derived from the daily dosage for normal adult. Also, the rate constants of enzyme degradation (k_{deg}) 0.000321/min and 0.000481/min were used for enteric and hepatic

CYP3A4 (Obach *et al*., 2006), respectively. Crude extracts of *M. oleifera* and *K. crenata* demonstrated the potential to inactivate both enteric and hepatic CYP3A4 as shown in Table 3.4.

Table 3.4 *in vivo* prediction of CYP3A4 inactivation

Parameters	*K. crenata*	*M. oleifera*
Concentration (µg/mL)	60	244
K_{inact} (min^{-1})	0.024	0.018
K_I (µg/mL)	6.45	2.53
$\lambda = \frac{K_{inact} \times [I]}{K_I + [I]}$ (min^{-1})	0.022	0.018
Gut (λ/k_{deg})	68.53	56.07
Hepatic (λ/k_{deg})	45.73	37.42
HDI risk	Likely	Likely

DISCUSSION

Usually, novel compounds are evaluated for their potential to cause CYP-mediated drug interaction based on single concentration screening while allowing for later detailed characterization involving IC_{50} and K_i determinations for reversible inhibition. Additionally, evaluation of compounds as time-dependent or mechanism-based inhibitors following reversible inhibition has become necessary, as the consequences are more detrimental. However, due to the complexity of mechanism-based inhibition, pharmaceutical industry and FDA have developed approaches to evaluate the potential of time-dependent inactivation by funneling compounds through single concentration, single time point experiments, and determining IC_{50} shift to avoid waste of time, labour and money on compounds that are weak CYP inactivators (Obach *et al*., 2007). The choice of *in vitro* models for the conduct of mechanism-based screening of compounds is important, because false positive or negative predictions can result to inappropriate compound progression or de-selection. The HLM or recombinant human CYPs are routinely applied for the conduct of such studies (Preuksaritanont *et al*., 2013). As preliminary studies to evaluate the TDI potential of *K. crenata* and *M. oleifera* to cause HDIs, several screening approaches were adopted to de-select and select the appropriate test samples in which inactivation kinetics will be required.

Limited information is currently available regarding the interactions of *K. crenata* and *M. oleifera* with clinically prescribed medications. This is an issue of potential safety concern as these herbal products are consumed by geriatrics and patients with chronic diseases such as diabetes, HIV/AIDS and asthma. Drugs commonly used by these patients include analgesics (diclofenac, ibuprofen, celecoxib - CYP2C9 substrates), protein pump inhibitors like omeprazole (CYP2C19 substrate), antiasthmatics (theophylline –CYP1A2 substrate) and nevirapine (CYP3A4); some of these have narrow therapeutic indices and high tendencies for DDI. Furthermore, such patients are prone to co-morbidities and infections, which might require the use of medications like the sulphonylureas (tolbutamide and glipizide), antituberculosis (isoniazid, rifampicin), and macrolide antibiotics (e.g., erythromycin, clarithromycin) metabolized by CYPs. Hence, there exists a high potential risk for interaction when any of these medications are consumed concurrently with *K. crenata* or *M. oleifera* which contain multiple phyto-constituents.

Recently, the US Pharmaceutical Research and Manufacturers Association recommended the use of IC_{50}-shift approach as the first step for TDI assays (Bjornsson *et al*., 2003). This method involves the application of HLMs or recombinant human CYPs to evaluate whether the potency of the NCE to inhibit CYP increases when the drug candidate is preincubated in the presence of NADPH. The IC_{50} shift approach was subsequently used by Obach *et al*., (2007) to evaluate the TDI potential and demonstrated a progressive flow strategy in which NCEs are selected for detailed TDI kinetics estimations. Therefore, this study used the IC_{50} shift approach to examine the TDI potency of crude and fractions of *K. crenata*, *L. frutescens* and *M. oleifera*. The test samples were preincubated with single recombinant human CYPs in the presence of NADPH as a primary incubate. An aliquot of the primary incubate was taken at predetermined time points and added to the secondary incubate containing the probe substrate to make a 10-fold dilution prior to 30 mins incubation. Such approach showed a leftward shift in the inhibition curves after preincubation compared to the curves without preincubation. Although many institutions are adopting the IC_{50} shift approach for screening of TDI potential compounds, there is currently no definitive criterion for classification of compounds as negative or positive TDI. Many pharmaceutical companies have been using the fold-shift criterion of 1.2 to 1.3-fold whilst Berry *et al*., (2008) also concluded that an IC_{50}-shift decrease of >1.5 indicated significant potency. In these studies, the Berry *et al* criterion was adopted whereby compounds with a fold shift ≥1.5 were tagged as potential TDI candidates. The crude methanol extract of *K. crenata* (39KC) and *L. frutescens* together with fractions 43.10, 45A, 45B and 45D tested had TDI effects. Although crude methanol extract of *M. oleifera* exhibited a non-TDI effect, the fractions 50A, 50B and 50C showed TDI potencies with IC_{50} shift of 8.31, 7.16 and 3.92, respectively on CYP3A4. TDI potencies of 3.29 and 0.67 was observed for troleandomycin and ketoconazole, known TDI and non-TDI compounds for CYP3A4 which were in agreement with the results reported by Sekiguchi *et al*., (2009). The *K. crenata* fractions 45A and 45D also exhibited positive TDI on CYP2C19 with fold shift of 2.36 and 5.07, respectively. However, the crude methanol extract (39KC) and 45B indicated non-TDI effects, with respective fold decreases of 1.30 and 1.20. Sutherlandioside D, a saponin isolated from *L. frutescens* exhibited positive TDI with shift fold of 1.85 while those of the fractions 43D and 43C were 4.73 and 2.06 on CYP2C19.

To confirm the positive TDI of fractions evaluated in this assay, the normalized ratio was used as described by Atkinson *et al*., (2005). The aqueous extracts; T41 (*H. hemerocallidea* capsule) and

T30 (*H. hemerocallidea* powdered leaves) showed clear TDI effect on CYP3A4. These herbal products could therefore reversibly and irreversibly inhibit CYP3A4; hence caution must be exercised in administering such medications to ARV-treated HIV patients. Interestingly, clear TDI was observed for T50 (*L. frutescens* tablets) which did not show significant reversible inhibition on CYP3A4 during early screening. Thus, although acute use of T50 (*L. frutescens* tablets) may not pose any danger to patients, chronic use of such products can generate enough intermediary metabolites which are deleterious to consumers concomitantly taking other conventional medications. Clear TDI was observed for all the methanol extract except *M. oleifera* which exhibited intermediate TDI at 100 µg/mL. Thus methanol extract of these herbal medicines can be fatal to the health of perpetual consumers on conventional medications such as efavirenz and nevirapine which are substrates of CYP3A4.

Inactivators of CYPs are known to be time- and concentration dependent, hence the effects of such parameters on *K. crenata* and *M. oleifera* crude and fractions were tested. The results showed that none of the fractions of *K. crenata* was a time-dependent inactivator of CYP2C19, as the inhibition profile weakened after incubation. Such a phenomenon can be due to recovery of enzyme activity, since the inhibition potency of the phytoconstituents weakens with time regardless of the concentration applied. However, crude methanol extract of *K. crenata* and fraction 45B as well as that of *M. oleifera* (MO and 50B) exhibited time- and concentration-dependent inhibition as their inhibition profile increased on CYP3A4. Thus, MO, 50B, 39KC and 45B were identified as TDIs of CYP3A4 based on the observations that their inhibitory effects on CYP3A4 activity were dependent on: 1) the preincubation, 2) concentration, and 3) NADPH. Next, the inactivation kinetics parameters of these four samples (MO, 50B, 39KC and 45B) were determined in recombinant human CYP3A4. The kinetic parameters for the inactivation of CYP3A4 by 39KC and 45B were: K_I, 1.77 and 6.45 µg/mL; K_{inact}, 0.019 and 0.02 min^{-1}, respectively. The crude *M. oleifera* and fraction 50B also demonstrated strong inhibitory kinetics on CYP3A4. Prediction of K_{inact} based on reversible and irreversible inhibition IC_{50} yielded values of 0.015 and 0.04 min^{-1} for 39KC and 45B, respectively which were closer to the results obtained from the dilution method. Thus, shift IC_{50} values can be used to predict inactivation constant of time-dependent inhibition of herbal medicines as recommended earlier by Sekiguchi *et al*., (2009). Categorization of MBIs based on λ/k_{deg} confirmed that both MO and KC are potential inactivators of both enteric and hepatic CYP3A4.

PART THREE

3.3 RESULTS

3.3.1 Metabolic clearance of testosterone

The viability of hepatocytes (percentage viable cell relative to total cells) reduced from 78.10% at time zero to 52.3% without the herbal extracts and to 50.5% with the extracts. Hence, the cell degradation is not attributable to the presence of the herbal extracts. The TST was also stable in the HepatoZYME media.

The intrinsic clearance of TST in the presence and absence of herbal extracts was monitored in both HLM and human hepatocytes (Figure 3.14). Previous study conducted in our laboratory extensively investigated the metabolic clearance of *L. frutescens* and *H. hemerocallidea* in hepatocytes. This study therefore investigated the influence of the other four herbs (*M. oleifera*, *E. purpurea*, *K. crenata* and *K. integra*) that showed strong inhibitory effects in recombinant CYPs. Estimation of hepatic blood clearance was done using the well-stirred liver model. The intrinsic clearance of TST was reduced by *K. crenata*; 8.5% (HLM) and 14.1% (hepatocytes), *M. oleifera*; 59.2% (HLM) and 52.7% (Hepatocytes), *E. purpurea*; 63.1% (HLM) and 62.3% (hepatocytes) *in vivo* (Table 3.4 and Appendix 3). Clearance of TST by respective herbal extracts taken into account the hepatic blood flow were *K. integra*; 74.38% (HLM) and 22.00% (hepatocytes), *M. oleifera*; 49.20% (HLM) and 52.89% (hepatocytes), *E. purpurea*; 58.96 (HLM) and 56.78% (hepatocytes) and *K. crenata*; 12.6% (HLM) and 6.69% (hepatocytes) as shown in Figure 3.14.

Prediction of the degree of interaction of the herbal extracts with TST indicated that *K. integra* interacts intermediately (AUC ratio = 4.30) and weakly (AUC ratio = 1.39) in HLM and hepatocytes, respectively. *Moringa oleifera* demonstrated weak (AUC ratio = 1.47) and intermediate (AUC ratio = 2.46) interactions, respectively in HLM and hepatocytes. *Echinacea purpurea* indicated intermediate interaction in both systems whilst *K. Crenata* exhibited no interaction in both HLM and hepatocytes (Table 3.5).

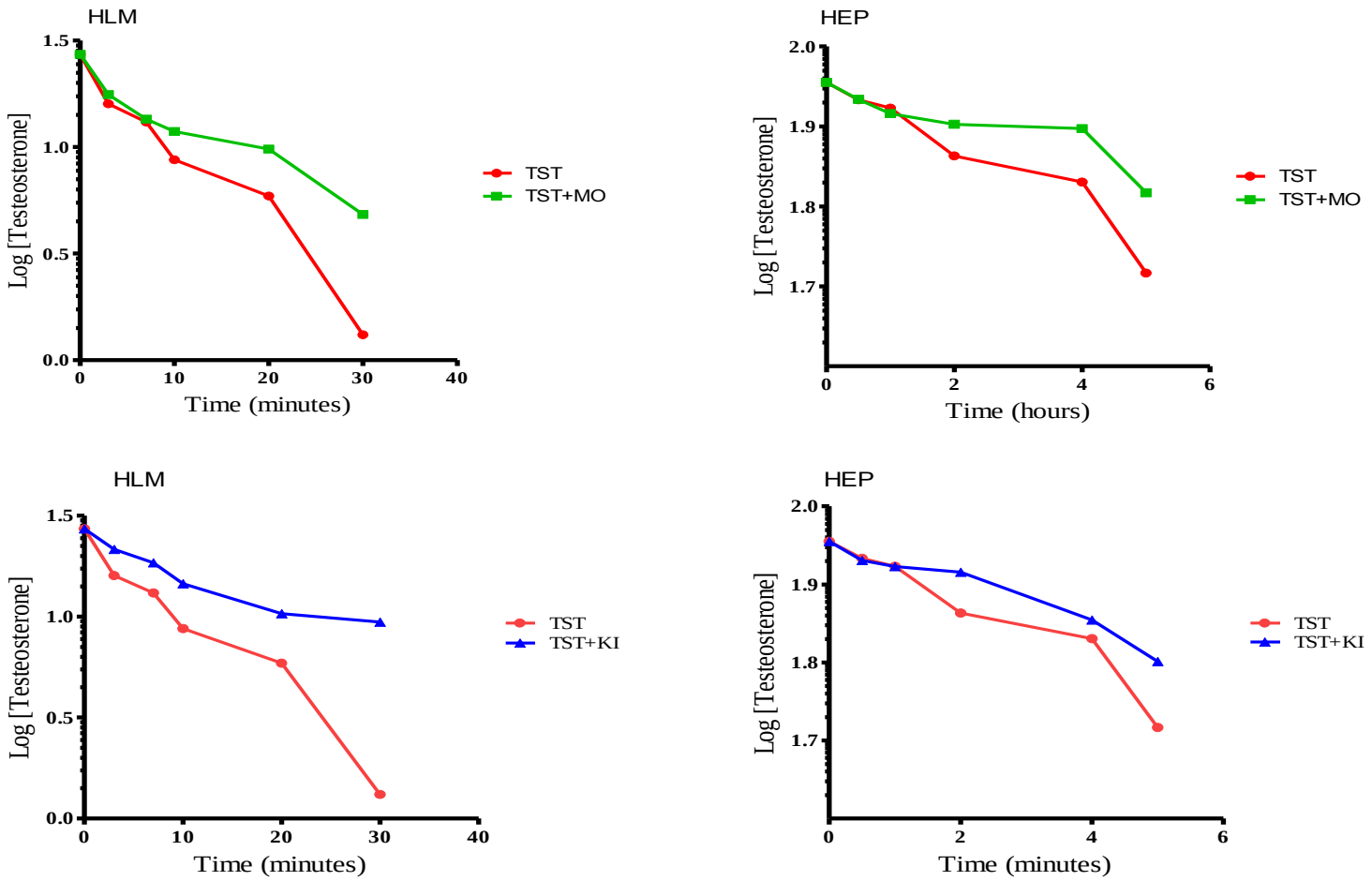


Figure 3.14A: Time course showing the influence of crude methanol extract of *M. oleifera* (MO) and *K. integra* (KI) on clearance of TST in HLM and human hepatocytes (HEP).

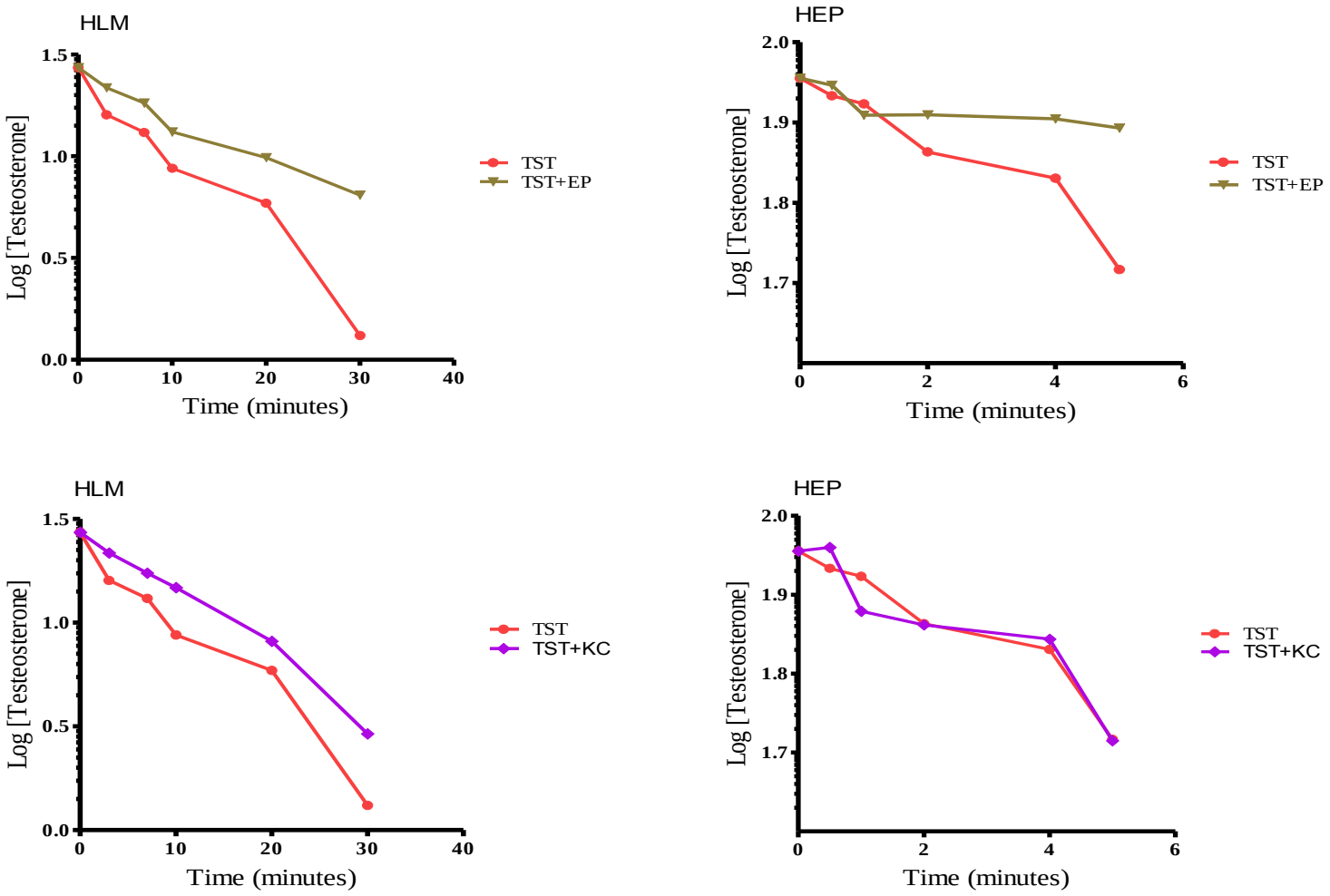


Figure 3.14B: Time course showing the influence of crude methanol extract of *E. purpurea* (EP) and *K. crenata* (KC) on clearance of TST in HLM and human hepatocytes (HEP).

Table 3.5: Comparative evaluation of metabolic clearance of TST in HLM and hepatocyte

Test samples	$CL_{int\ in\ vitro}$ (mL/min/kg)		$CL_{int\ in\ vivo}$ (mL/min/kg)		AUC_i/AUC	
	HLM	Hepatocyte	HLM	Heaptocyte	HLM	Hepatocyte
TST	2.64	2.45	3.05	6.24	-	-
TST+ KI	0.61	1.77	0.71	4.50	4.30 –b	1.39 –c
TST + MO	1.25	1.00	1.44	2.54	1.47–c	2.46–b
TST + EP	1.00	0.90	1.15	2.30	2.65–b	2.71–b
TST + KC	2.26	2.24	2.62	5.71	1.16–d	1.09–d

AUC ratio of interactions:

–a Strong (AUC ratio >5)

–b Intermediate (2 ≤ AUC ratio ≤ 5)

–c Weak (1.25 ≤ AUC ratio <2)

–dNo interaction (AUC ratio < 1.25)

DISCUSSION

During development of new candidate drugs at early drug discovery stage, the *in vitro* metabolic stability is often examined in a high-throughput manner. The conduct of *in vitro* metabolic stability studies of new drugs enhances the prediction of *in vivo* clearance in humans. The knowledge of *in vivo* clearance of a new drug is critical for the determination of plasma concentration of specific drug, allowing the prediction of the appropriate dosage regimen for specific patient populations. For herbal medicines which are mostly consumed orally, it is imperative to examine their influence on drug metabolism by using appropriate *in vitro* systems with specific probe substrates. The outcome of such investigation shows the impact of herbal extracts on intrinsic clearance of specific drug, attempting to envisage the likely magnitude of HDI to occur in humans.

Traditionally, the HLMs are commonly used as an *in vitro* system for the prediction of metabolic clearance of new drugs. However, the use of this system has resulted in an underestimation of intrinsic clearance of drugs during *in vivo* extrapolations (Hewitt *et al*., 2007). Alternatively, *in vitro* systems such as pooled human hepatocytes are envisaged as a potentially more accurate model because of the complete availability of both phase I and phase II metabolizing enzymes, drug transporters and NADPH cofactors resulting to drug concentration in the hepatocytes closer to the concentration within the liver of humans. This study used both pooled human liver microsomes and pooled human hepatocytes to predict the influence of crude methanol extracts of the herbal extracts; *M. oleifera*, *K. integra*, *K. crenata* and *E. purpurea* on the metabolic clearance of TST.

The *in vitro* prediction showed an equivalent intrinsic clearance of TST in both HLM and hepatocytes systems. Thus differences in intrinsic clearance observed in the subsequent evaluations are primarily due to the influence of herbal extracts applied. However, to avoid under- and over-estimation of intrinsic clearance of TST, the biological scaling factors such as microsomal content (HLM), hepatocellularity (hepatocytes), adult liver mass and rate of hepatic blood flow were employed to extrapolate the *in vitro* data to *in vivo* (Hallifax *et al*., 2005; Ito and Houston, 2005). The estimation of TST clearance based on the biological scaling factors showed 2-fold increase in intrinsic clearance of TST in human hepatocytes compared to that of HLM. Thus, prediction of intrinsic clearance of TST *in vitro* without application of biological scaling

factors to estimate clearance *in vivo* could lead to under-prediction in human hepatocytes and/or over-prediction in HLM.

Kalanchoe integra demonstrated intermediate interaction with TST in HLM whilst weak interaction was observed in hepatocytes. Considering hepatocytes as an intact cell with constant influx and efflux of compounds, the degree of interaction depends on the free extracellular-intracellular gradient and the concentration of herbal extract available at the enzyme active site. For herbal extract rapidly metabolized by CYPs and further conjugated by the phase II enzymes, its clearance will be facilitated to reduce interaction as observed in the case of *K. integra* in the hepatocyte. However, for HLM, the metabolism of *K. integra* is primarily mediated by the CYPs. Hence, accumulation of metabolites of *K. integra* with potential inactivation effect on CYP3A4 could be presumably attributed to the high AUC ratio observed compared to the hepatocytes. Conversely, *Moringa oleifera* demonstrated weak and intermediate interactions with TST in HLM and hepatocytes, respectively. In this case, it is speculated that the phytochemicals of *M. oleifera* are metabolized by other CYPs in addition to CYP3A4 to generate inactivators that intensify the inactivation of CYP3A4, hence the intermediate interaction between the herbal and TST observed in hepatocytes compared to HLM. The intrinsic clearance of TST in the presence of *E. purpurea* and *K. crenata* were equal in both hepatocytes and HLM. The equivalent interaction indicates non-significant influence of phase II, membrane barrier, transporters and other factors relevant to hepatocyte which might affect the disposition of the herbal drugs. Thus, the influence of herbal extract on metabolic clearance of specific drug will depend on the nature of the phytochemicals and the free concentration reaching the active site of the CYP3A4.

PART FOUR

3.4 RESULTS

3.4.1 Assesment of cytotoxic effect of herbal medicines

HepG2 cell preparations were exposed to 100, 50 and 25 µg/mL methanol extracts of *Echinacea purpurea* plant material, capsules and fractions to assess the effect on HepG2 cell mitochrondrial activity applying MTT assay. *Echinacea* demonstrated non-cytotoxic effect on HepG2 at the concentrations tested as shown in Figure 3.15. Other herbal medicines demonstrated non-toxic effect on HepG2 (data no shown).

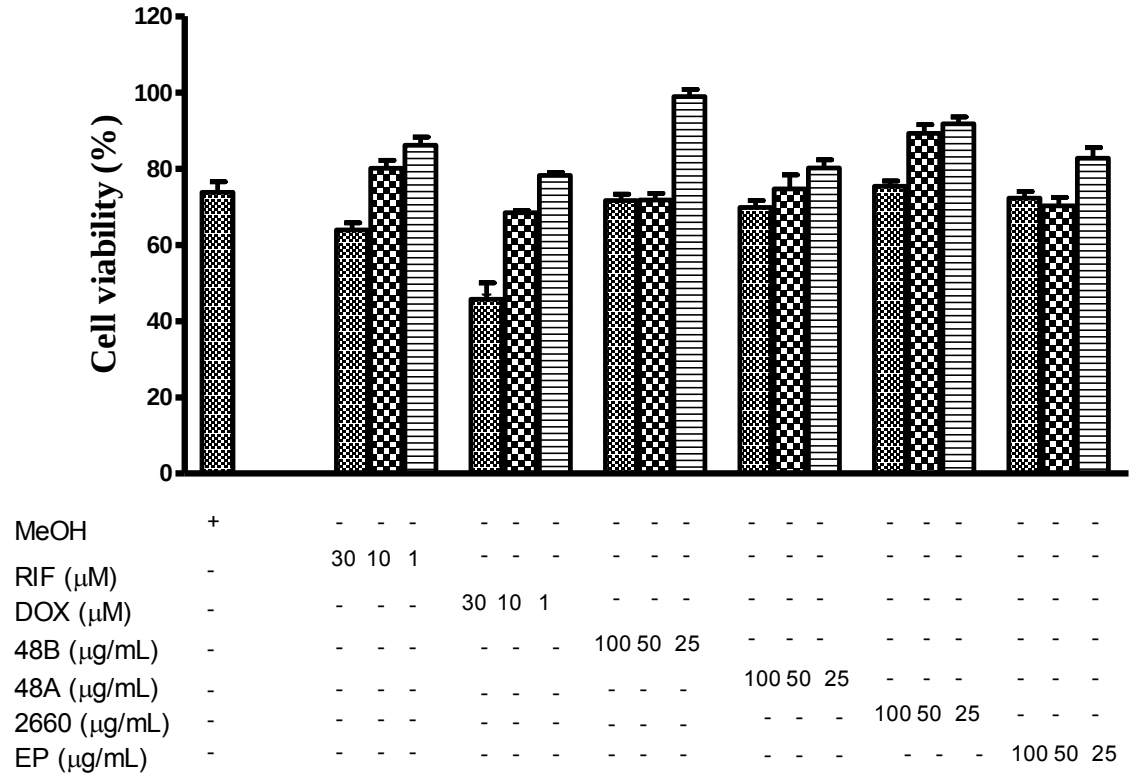


Figure 3.15: Effect of Echinacea on HepG2 cell viability.

HepG2 cells were treated for 24 h with Echinacea (100, 50, 25µg/mL) or rifampicin or doxorubicin (30, 10, 1µM). At the end of this period, cell viability was measured as described under *Materials and Methods*. The figure shows the mean of duplicate treatments expressed as a percentage of the value in MeOH-treated cells, with the SEM indicated by the vertical bars. Cytostatic agent, doxorubicin (DOX) was used as positive control, rifampicin (RIF), *Echinacea* capsule (2660), Echiancea plant material (EP) and Echinacea fractions (48A and 48B).

3.4.2 Reporter gene assay

3.4.2.1 Initial screening of herbs for PXR activation

Initial screening of herbal extracts implicated methanol extract of *E. purpurea* as significant inducer of CYP3A4 comparable to rifampicin (Figure 3.16A). Influence of the herbal extracts on rifampicin-mediated PXR activation indicated *Hypoxis* powdered leaves (HHL) and *K. integra* as non-concentration dependent competitive inhibitors. Other herbal extracts had no significant effect on rifampicin-mediated PXR activation as demonstrated in Figure 3.16B. The subsequent investigation therefore focused on *E. purpurea* crude methanol and fractions. *Echinacea* capsule (2660) activated hPXR with fold induction of 1.9 compared to the vehicle control (Figure 3.16C).

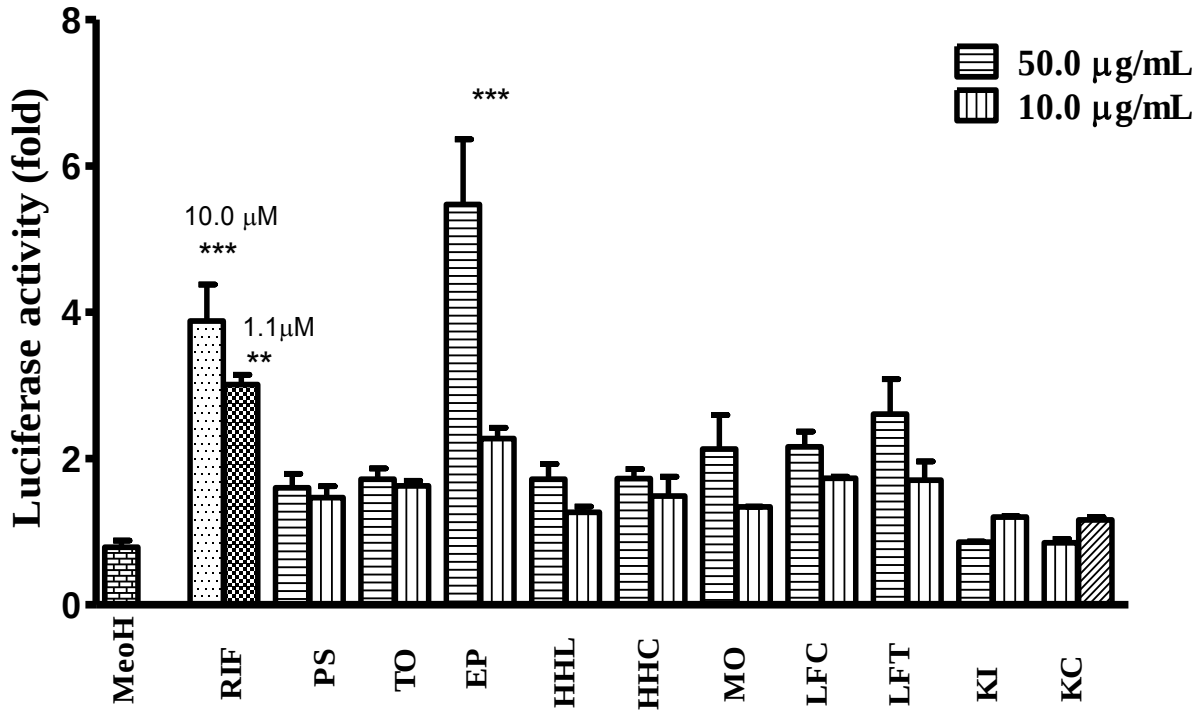


Figure 3.16A Effect of herbal medicines on activation of PXR in co-transfected HepG2 cells.

The fold induction relative to vehicle (methanol) was determined. One-way ANOVA was used to compare the mean value between the vehicle and the treatment groups and Benferroni multiple comparison was conducted for multiple comparison between the treatment groups when the $P < 0.05$. Results are expressed as Mean ± SEM. **P=0.01 and ***P = 0.001.

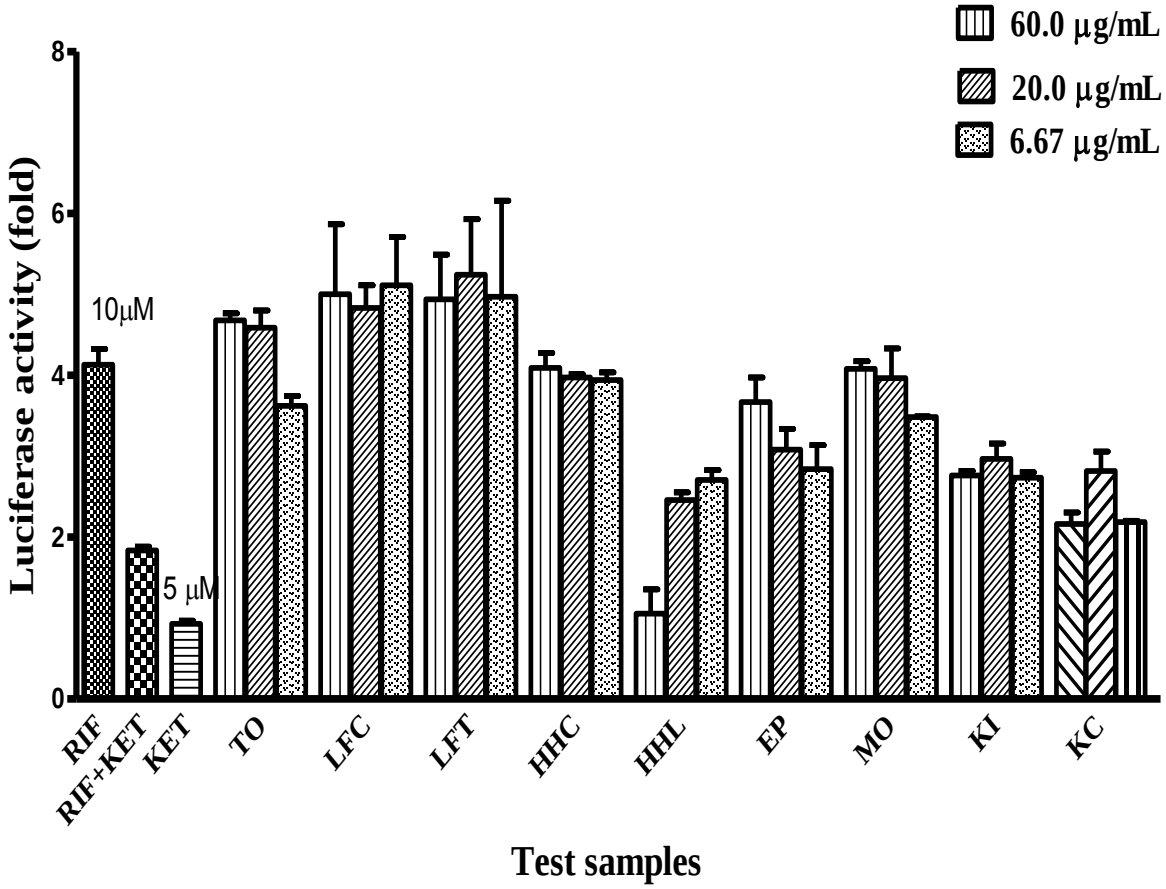


Figure 13.16B: Influence of herbal medicines on rifampicin-induced activation of PXR.

The data are represented as Mean ± SEM of triplicate measurements in two independent experiments. Ketoconazole (KET), *Lessertia* tablets (LFT), *Lessertia* tea cut (LFC), Hypoxis capsule (HHC), *Hypoxis* powder (HHL), *Echinacea* capsule (2660), *Moringa* powdered leaves (MO), *Taraxacum* root (TO), *Kalanchoe crenata* (KC) and *Kalanchoe integra* whole plant (KI).

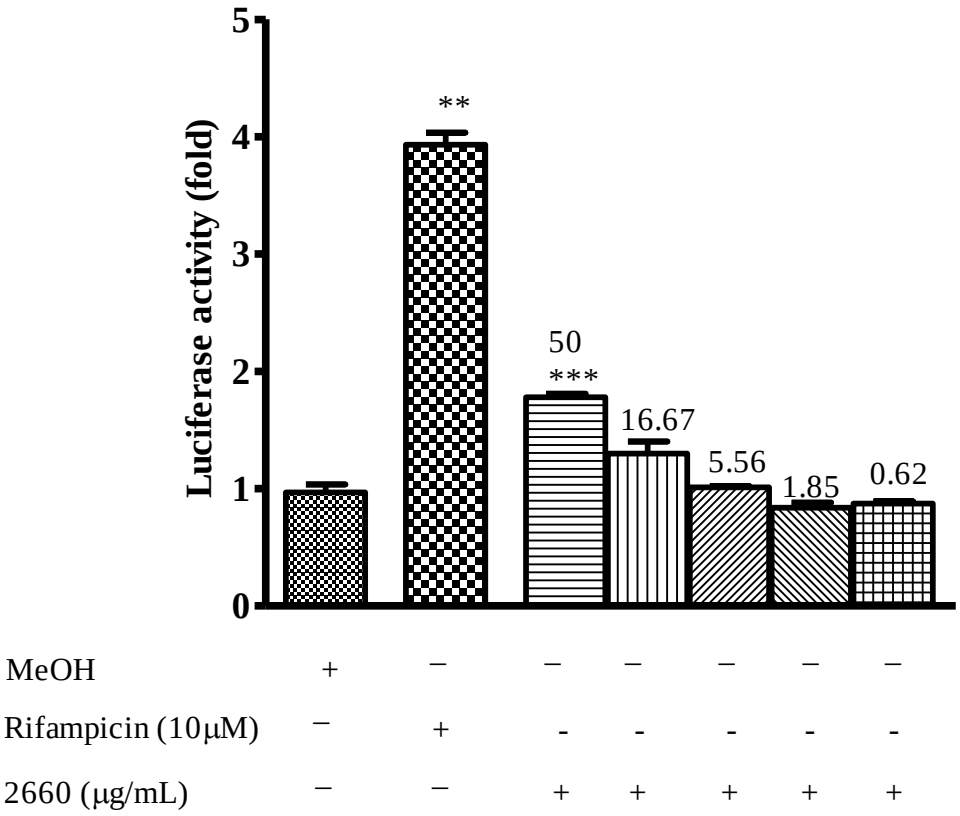


Figure 3.16C: Effect of *Echinacea purpurea* on activation of PXR.

HepG2 cells were transiently transfected with *p3A4-luc* reporter construct containing the basal promoter (-362/-53) with proximal PXR response element and the distal xenobiotic responsive enhancer module (-7836/-7208) of CYP3A4 (0.4 μg/well) and *pSG5-PXR* expression vector (50 ng) using the manufacturer's instructions. Transfected HepG2 cells were maintained in medium containing *Echinacea purpurea* at the indicated concentrations for 24 h. Luciferase activities are normalized to protein concentration and expressed as -fold activation of non-treated cells transfected with *p3A4-luc*. Results are expressed as Mean ± SEM. One-way ANOVA was used to compare the mean value between the vehicle and the treatment groups and Benferroni multiple comparison was conducted for multiple comparison between the treatment groups when the $P < 0.05$. ***$P = 0.001$.

3.4.2.2 Concentration- dependent PXR activation

To identify the appropriate solvent(s) for extraction of active constituents responsible for activation of hPXR, *Echinacea* fractions of different polarity were prepared from the methanol extract of whole *E. purpurea*. *Echinacea* plant material (EP), and fractions; 48A and 48B strongly activated hPXR with fold increase in luciferase activity of 4, 5.5 and 3.5, respectively compared to the vehicle control at 50 µg/mL. At 16.67 µg/mL, 48B caused strong activation of hPXR with luciferase activity of 3.8- fold increase while EP and 48B mildly activated hPXR with 2-fold increase in luciferase activity compare to the vehicle. However, 48C, 48D and 48E showed no activation of hPXR (Figure 3.17).

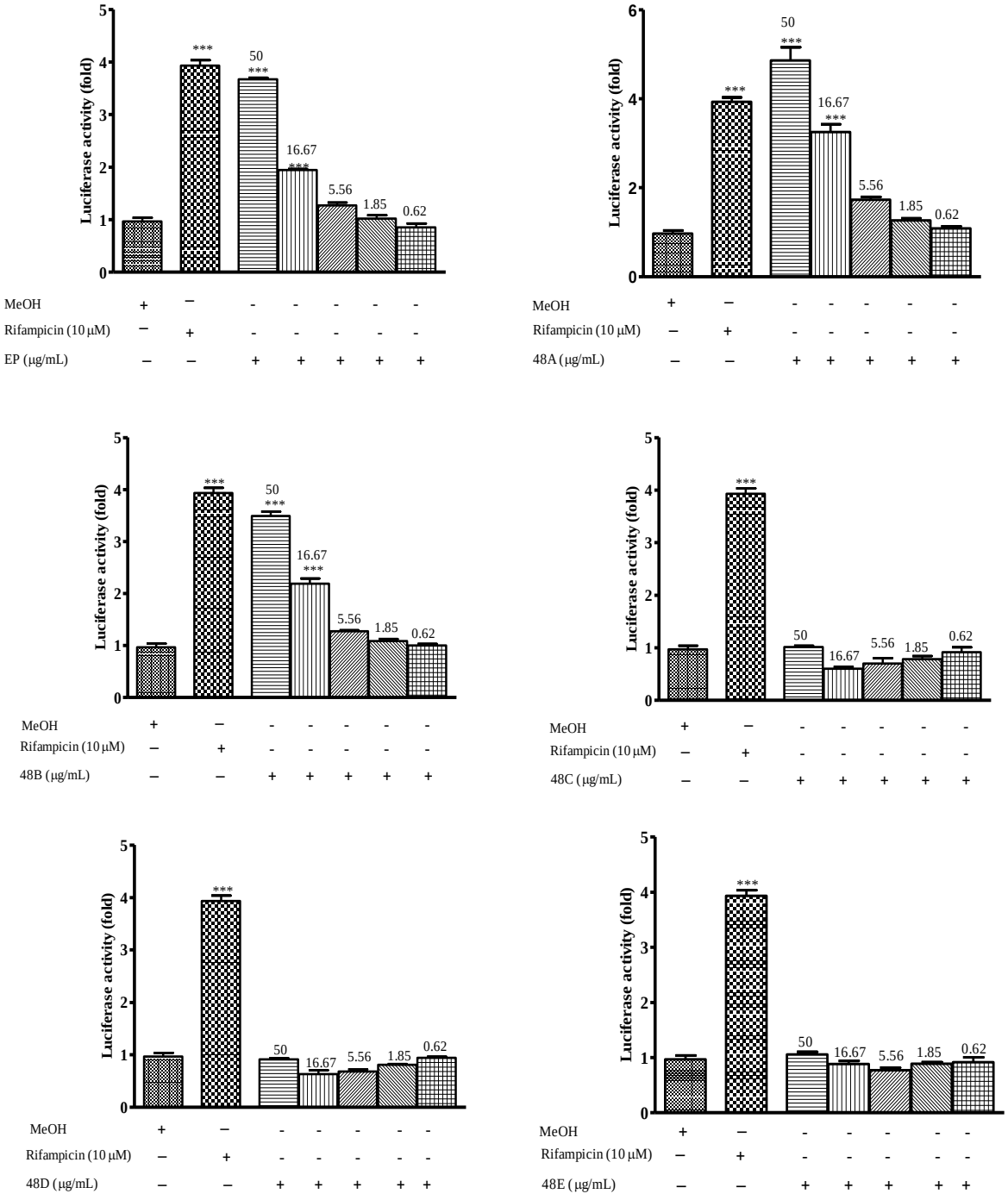


Figure 3.17 Concentration-dependent effect of Echinacea on PXR activation.

HepG2 cell line transfected with PXR was exposed to fractions of *Echinacea purpurea* at different concentrations for 24 hours. The fold induction relative to vehicle (methanol) was determined. Results are expressed as Mean ± SEM.

3.4.2.3 Time- dependent PXR activation

Unlike inhibition, the onset of PXR-mediated induction of DME or drug transporter genes is relatively slow and it may take days or even weeks for the full effects to manifest. The effects of various fractions on hPXR activation after 24 and 48 h treatment in HepG2 cells were analyzed. EP and 48A showed 8-10% increase in hPXR activation at 48 h compared to 24 h at concentrations ranging 1.85 – 50 µg/mL. No difference was noticed for 48B and 48C at 24 and 48 h (Figure 3.18A). One-tailed t test showed no significant effect of time on activation of hPXR by fractions except EP tested at 50 µg/mL and 16.65 µg/mL with P value of 0.047.

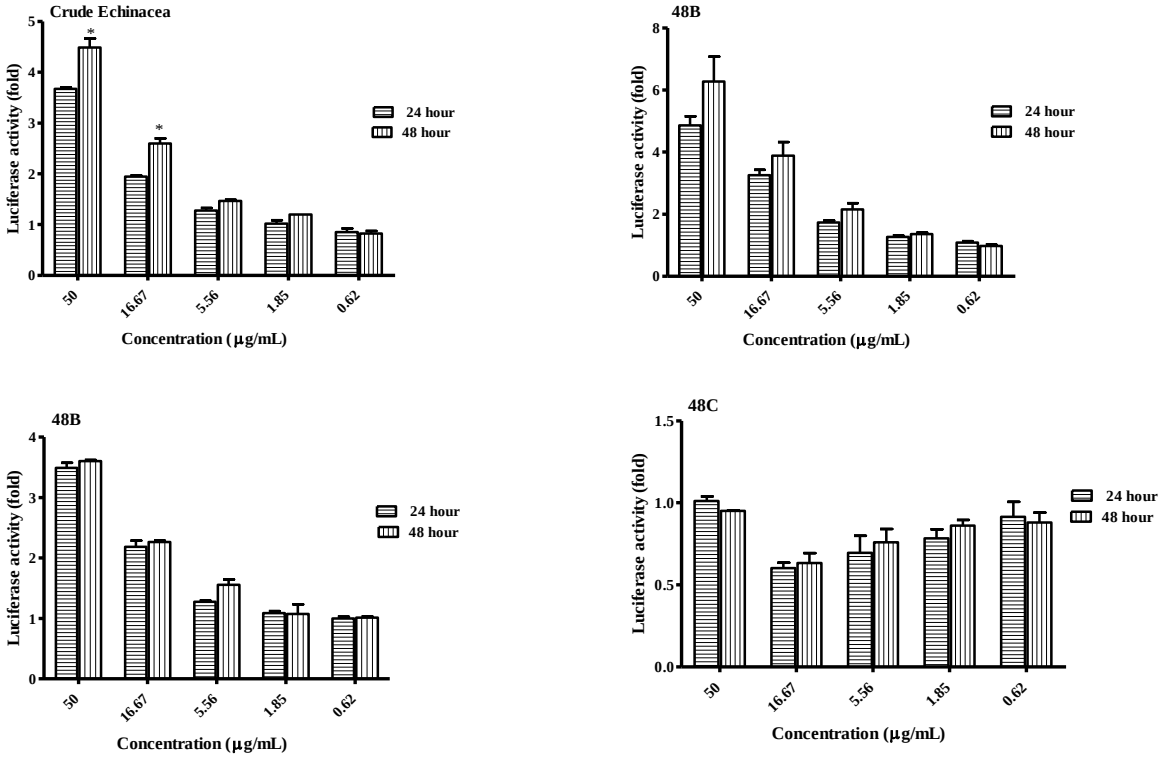


Figure 3.18: Time-dependent effect of Echinacea on PXR activation.

HepG2 cell line transfected with PXR was exposed to fractions *E. purpurea* at different concentrations for 24 and 48 hours to determine concentration and time dependent effect of test samples on induction in PXR. The fold induction relative to vehicle (methanol) was determined. Results are expressed as Mean ± SEM. One-tailed t test was performed to compare the PXR activation for 24 and 48 h, respectively. *P = 0.047 was noted for EP.

3.4.2.4 Regulation of CYP1A2, CYP3A4 and P-gp mRNA expression

Crude extracts and fractions of *E. purpurea* were tested for their potential to activate mRNA expression of CYP1A2, CYP3A4 and P-gp in HepG2 cells transfected with hPXR. The expression of mRNA was used to differentiate CYP or P-gp inducers and non-inducers based on the normalized ratio of the housekeeping gene after 48 h exposure of hPXR transfected HepG2 cells to the test samples using RT-qPCR (Figure 3.19). Test samples with mRNA expression ≥2-fold of the non-treated group were classified as inducers whilst others with mRNA expression cut-off <2-fold of non-treated group were tagged as non-inducers. Crude extract of *Echinacea* and fraction 48B indicated a non-inducing effect on CYP1A2 mRNA expression in PXR. However, crude extract of *Echinacea* capsule and active fraction 48A exhibited an induction effect on CYP1A2 mRNA expression at 50 µg/mL. The crude extracts of *Echinacea* plant material and the capsule demonstrated strong induction effect on CYP3A4 and P-gp mRNA expression in PXR. Additionally, a similar effect was observed for the active fraction 48A on both CYP3A4 and P-gp mRNA expressions.

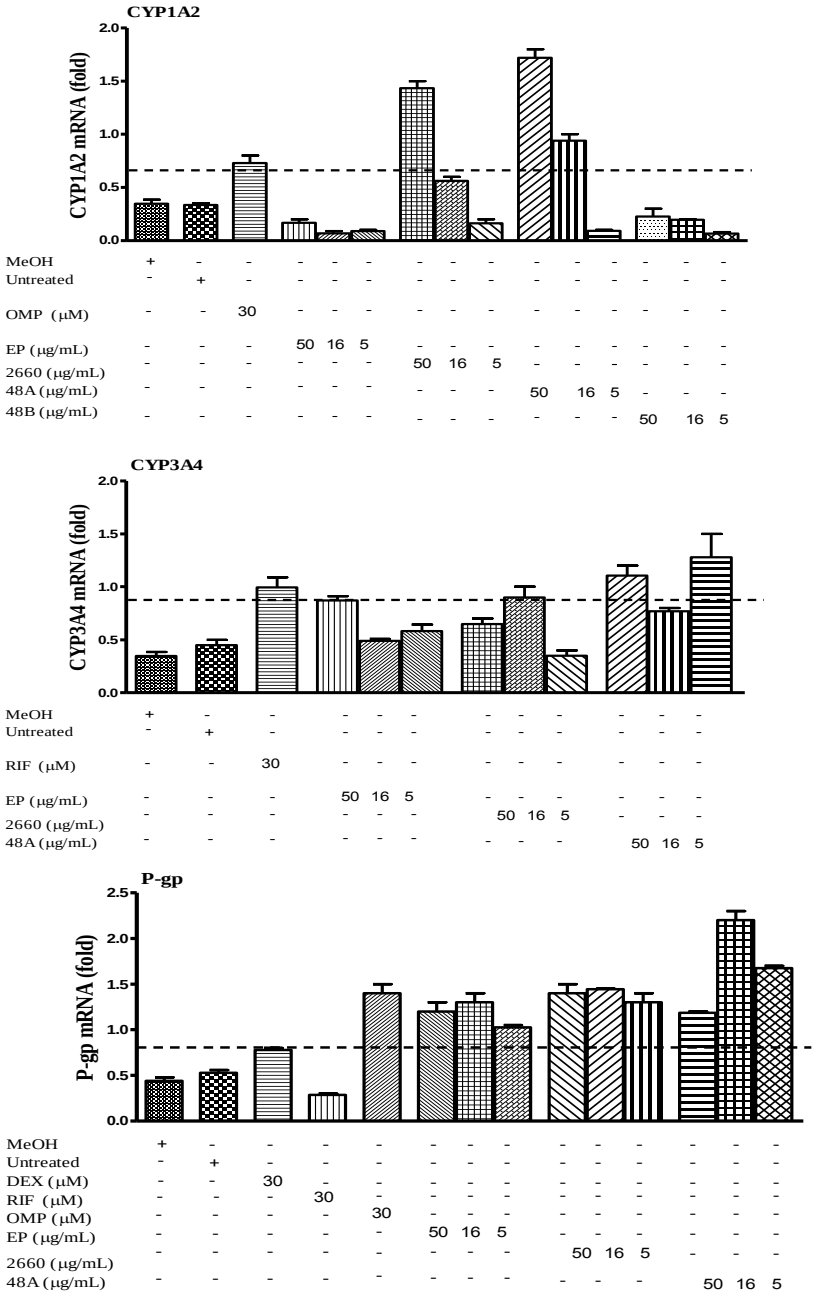


Figure 3.19: Analysis of Echinacea-mediated up-regulation of CYP3A4, CYP1A2 and P-gp mRNAs. HepG2 cells were transfected with *pSG5-PXR* expression plasmids (400 ng/well) using the manufacturer's instructions and exposed to different concentrations of Echinacea (5.6 – 50 μg/mL) for 48 h. mRNA expression of tested genes was determined using real-time RT-PCR and normalized to *HPRT* housekeeping gene. The effect of Echinacea on CYP1A2 (A), CYP3A4 (B) and MDR1 (C) mRNA expression is presented as -fold increase to HPRT. Test samples with mRNA expression ≥2-fold of untreated were considered as an inducer. OMP and DEX refer to omeprazole and dextromethorphan, respectively.

3.4.2.5 Measurement of CYP1A2 enzymatic activity

To examine the effect of the test samples on CYP1A2 and CYP3A4, the activities of the enzymes were tested in PXR-transfected HepG2 cells; omeprazole and rifampicin were used as inducers for CYP1A2 and CYP3A4, respectively using specific fluorescent substrates. Significant induction of CYP1A2 by Fraction 48A (EC_{50} = 41.01 µg/mL) and crude extract, 2660 (EC_{50} = 83.04 µg/mL) were observed (Figure 3.20). None of the fractions of *Echinacea* showed positive activity on CYP3A4 (data not shown).

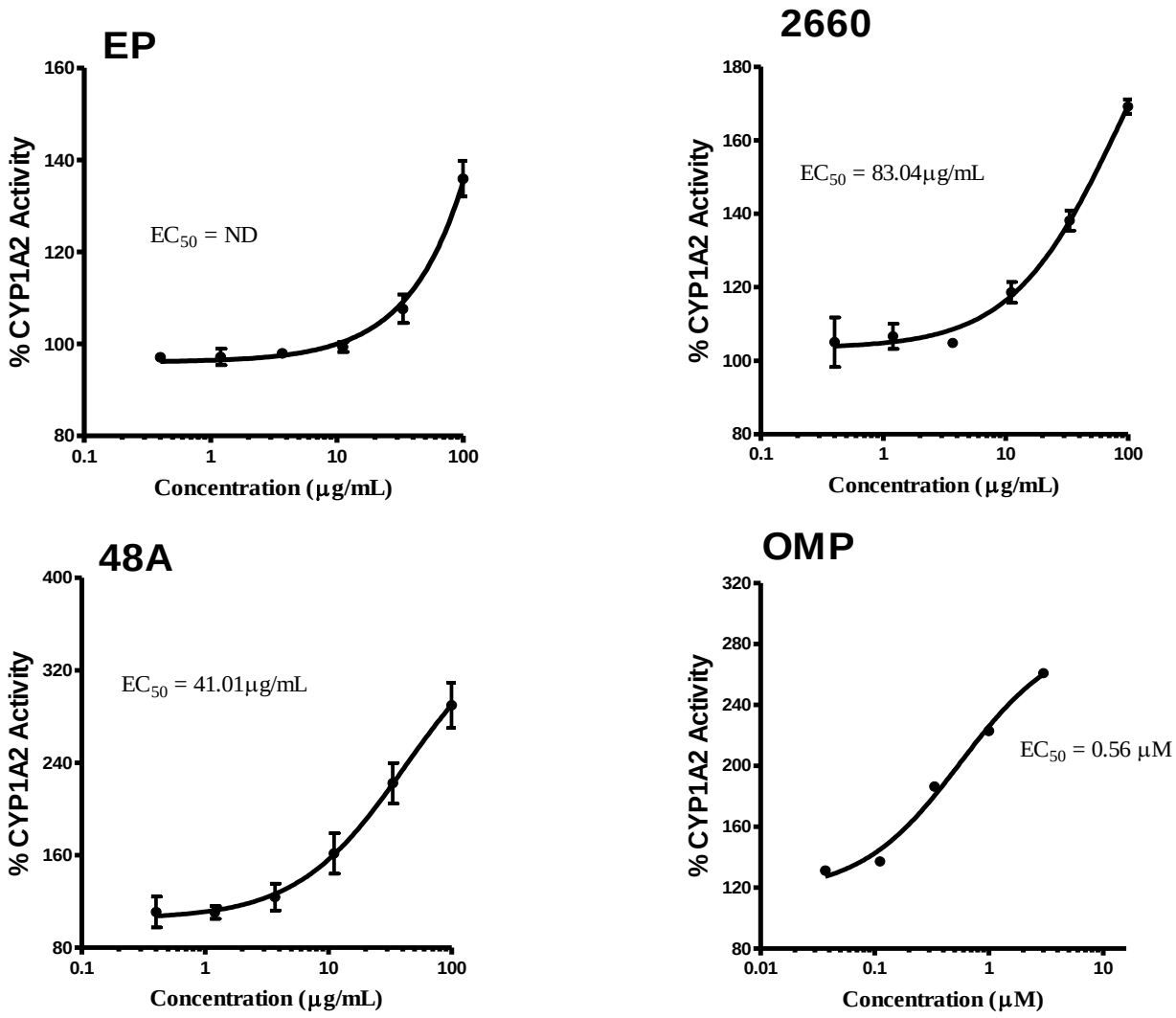


Figure 3.20 Determination of CYP1A2 activity in Echinacea-mediated activated PXR. Log transformations of test sample concentrations against % CYP1A2 activity after 30 mins incubation with CEC in hPXR-tranfected HepG2 cells was fitted using non-linear regression and EC_{50} determined.

3.4.2.6 *In vivo* prediction of HDI from *in vitro* data

One of the objectives of the present study is to rank the potential risk of HDI according to the US FDA guidelines based on the [I]/EC_{50} ratio where [I] is the gut or intra-hepatic concentration of the inducer (drug or herb) an individual is exposed to and EC_{50} is the half of the maximum inducer concentration. In the absence of knowledge of the chemical constituents in the herbs or how much would be absorbed to finally reach the liver and interact with the CYP, the worst case assumption that all herbal extract administered would be absorbed was made. The gut concentration was therefore estimated by dividing the administered dose by the estimated GIT volume of 250 mL. The intra-hepatic concentration of herbal extract was determined using equation 3.1. The estimated concentration for each herb is shown in Table 3.6A.

With both the estimated EC_{50} and [I] values, the [I]/EC_{50} induction ratio was used to rank the herbs with respect to risk for HDI based on FDA guidelines where, I/EC_{50} >1.0 is associated with high risk for DDI, I/EC_{50} = 0.1-1 is associated with intermediate risk for DDI and I/EC_{50} <0.1 is unlikely to result in DDI (Table 3.6B) (Hewitt *et al*., 2007).

Table 3.6A: Calculation of herbal medicine concentration in the gut

Fraction	Yield (%W/W)	Estimated extract per dose (mg/mL)	GIT concentration (µg/mL)	Intra-hepatic concentration (µg/mL)
EP	58.38	186.80	747.2	767.07
2660	23.17	74.14	296.58	316.45
48A	4.19	13.41	53.63	73.50

Usual human dose for Echinacea capsule (320 mg single dose)

Table 3.6B: *in vivo* prediction of HDI from *in vitro* data

Test sample	EC_{50} (µg/mL)	Induction potency $[I]_{gut}/EC_{50}$	Induction potency $[I]_{in}/EC_{50}$	Risk for HDI
2660	83.04	3.57	3.81	Likely
48A	41.01	1.3	1.79	Likely

Induction potency of EP not determined, intra-hepatic concentration ($[I]_{in}$) = C_{max} + k_aF_aD/Qh and k_aF_aD/Qh = 19.87

DISCUSSION

Evaluation of the potential of a new drug candidate to cause drug-drug or herb-drug interactions has become an integral part of drug development and regulatory review prior to market approval. Although drug interactions can be evaluated using specific clinical studies in healthy subjects or patients, *in vitro* approaches are now adopted as a critical first step in the assessment of drug interaction potential through specific pathways. Results obtained from *in vitro* studies can be employed to predict *in vivo* interaction and guide the need for further *in vivo* studies. The application of luciferase reporter genes for investigation of mechanisms underlying the induction potential of drugs or xenobiotics has become a routine practice.

Human PXR-transfected HepG2 cells have been utilized as an *in vitro* tool to evaluate the potential of new drug candidates to cause clinically significant induction of DME or transporter proteins (Chu *et al*., 2009). As the use of herbal medicines either as nutritional supplements or medication is on the increase and well established in developing and developed countries, concerns on the risk of herb-mediated induction of CYPs and drug transporters studies have increased. St. John's wort (SJW) and garlic are examples of herbal products that have drawn a lot of attention because of HDI. Previous studies have demonstrated that concurrent use of SJW and garlic can significantly reduce plasma concentration of unboosted protease inhibitors (Piscitelli *et al*., 2000a; Piscitelli *et al*., 2000b). PXR was identified as a key mediator in SJW interaction with conventional drugs (Mannel, 2004). Other herbal products such as *Ginkgo biloba*, Tian Xian and *Coleus forskohlii* have been reported as herbs that activate PXR (Ding and Staudinger, 2005; Li *et al*., 2009). In recent times, *E. purpurea* has become a popular herbal supplement used as immunostimulant in HIV/AIDS patients. Although *in vitro* studies have reported *E. purpurea* ability to induce CYP3A4 and P-gp employing different models, the underlying mechanism is unknown. In this study, we showed that *E. purpurea* up-regulates the CYP1A2, CYP3A4 and MDR1 (P-gp) gene expression via activation of PXR nuclear receptor.

Echinacea purpurea demonstrated a non-cytotoxic effect on HepG2 cell. Additionally, it was found in this study that 0.8% methanol had no significant effect on PXR activation. This makes methanol a good solvent to dissolve *E. purpurea* capsule and fractions. The final concentration did not exceed 0.8% in the experiments. Hence subsequent outcomes observed in the assays were

presumably due to activation or induction effect of *Echinacea* on PXR. Initial screening of the methanolic extract of *E. purpurea* (EP) plant material and capsule indicated significant activation of PXR with 3.5- and 2.0-fold increase in luciferase activities, respectively (Figure 3.16C). Any herbal supplement showing ≥2 fold increase in luciferase activity is considered to be an activator of PXR with the potential to cause HDI (Shimizu, 2010). However, unlike conventional medications, herbal supplements consist of several phytochemical compositions which may differ depending on the place of collection, the season and extraction process. Profiling the chemicals in an herbal supplement is therefore a critical step in understanding the mechanism for HDI. In this study, silica gel fractionation was used to collect six different fractions of increasing polarity. Each fraction was evaluated for its potential to activate PXR. The non-polar fractions; 48A and 48B were the most potent activators of PXR with fold increase in luciferase activity of 5 and 3.5, respectively. The activation effects of 48A and 48B on PXR were significant at 16.67 µg/mL and 50 µg/mL although dose-dependent activation was observed. However, the activation of PXR by 48C, 48D and 48E2 were non-significant when compared with the control vehicle. The fractions 48A and 48B of *E. purpurea* root showed evidence of alkamides as the principal phytochemicals in a chromatographic scanning (Bauer and Remiger, 1989). Chloroform is the best solvent for the extraction of alkamide, even though both methanol and ethanol can be used. It is therefore not surprising that chloroform fraction 48A showed the strongest activation of PXR.

The activation of CYP and drug transporter is time- and concentration- dependent. Therefore the effect of time on the activation of PXR by the fractions of *Echinacea* was investigated. The results showed 30-50% increase in PXR activation at 48 hours compared to 24 hours by EP, Fractions 48A and 48B at 50 µg/mL and 16.67 µg/mL. Comparison of PXR activation between 24 and 48 h indicated significant difference at 50 µg/mL for the crude EP. The observed significant time-dependent activation of PXR by the crude EP is an indication of its potential to cause HDI during chronic administration.

To analyze the effect of *E. purpurea* on expression of CYP1A2, CYP3A4 and P-gp genes, primers of respective DME and transporter were introduced into PXR co-transfected HepG2 cell model using the protocol supplied by the manufacturer. In this study, it was observed that, *Echinacea* significantly potentiates up-regulation of CYP1A2, CYP3A4 and MDR1 (P-gp) in

PXR co-transfected HepG2 cell model. The outcome of this study agrees with that of Naspinski *et al.*, (2008) who reported that PXR up-regulates the expression of CYP1A2 and CYP3A4. However, in their study, the expression of MDR1 (P-gp) was down-regulated by PXR co-transfected HepG2. Thus the current study speculate that up-regulation of CYP1A2, CYP3A4 and MDR1 mRNA genes to be presumably the underlying mechanism of *Echinacea*-mediated induction reported in humans and other *in vitro* studies (Gorski *et al*., 2004; Gurley *et al*., 2004; Yale and Glurich, 2005; Penzak *et al*., 2010). However, the observed differences in the activation of CYP1A2 genes by the *Echinacea* capsule compared to the EP authenticated plant material warrants further investigation to ascertain a complete phytochemical profile. Cases of capsules and tablets of herbal supplements surreptitious with conventional drugs such as benzodiazepine, fluoxetine and furosemide as weight loss products have been reported (de Carvalho *et al*., 2011).

The adoption of current industry and FDA guidelines for classification of herbal supplements' potential to cause drug interactions comes with a number of challenges. Firstly, in drug development, pure compounds are evaluated whereas for herbal supplements, mixtures of unknown chemical composition are used, making it impossible to know the identity or the concentration of the inducing or activating components. The use of the total GIT concentration of the herbal extract (μg/mL) as the assumed absorbed amount and also responsible for interacting with the CYPs is therefore a gross exaggeration of the likely concentration of inducer or activator components to interact with CYPs. Accounting for plasma protein binding is also not possible with herbal extracts. Secondly, the EC_{50} used for herbal extracts is an apparent value associated with complex mixtures of chemicals hence likely to also be a gross exaggeration of these kinetic parameters. Thirdly, industry uses the metabolism based DDI studies to both guide the molecular design of NCE devoid of potential DDI risks during the early hit and lead identification and lead optimization stages. At candidate drug selection stage, the DDI data is used to select compounds devoid of such DDI risk or the design of *in vivo* DDI studies to determine the extent of the risk of DDI in compounds which still bear the CYP induction effects. For herbal extracts already in use, the HDI studies are therefore mainly to assess risk, guide the design of *in vivo* HDI studies and possibly revise product labels to highlight the risk of co-administering some herbs with certain conventional drugs. The effect of SJW on the protease inhibitor, indinavir is a good example of the likely use of results from this study through the

observations done in reverse, that is, observations of pharmacokinetic interactions *in vivo* (Piscitelli *et al*., 2001) and working backwards to *in vitro* studies to understand the underlying mechanism, and eventually have product label recommendation to avoid such HDI (Clauson *et al*., 2008). The mechanistic approach of using *in vitro* systems used in this study allows rapid evaluation of many herbal medicines and re-labelling of products with respect to HDI which can guide patients and clinicians in avoiding drug-herb combinations likely to result in HDI (Awortwe *et al*., 2014b).

Several approaches based on results from the PXR reported gene assay can be used to risk rank the potential of fractions tested to cause HDI. The I/EC_{50} induction ratio was used to rank the herbs with respect to risk for HDI based on FDA guidelines where, I/EC_{50} >1.0 is associated with high risk for DDI, I/EC_{50} = 0.1-1 is associated with intermediate risk for DDI and I/EC_{50} <0.1 is unlikely to result in DDI on CYP1A2. The inclusion of inducer or activator concentration in both the gut and the intra-hepatic systems for the risk ranking of compounds reduces the tendency of false positive or false negative estimation. *Echinacea* capsule (2660) and 48A was ranked with high risk to cause HDI with a ratio of 3.57 and 1.3 in the gut and 3.81 and 1.79 in the intra-hepatic systems, respectively for CYP1A2. The risk ranking correlated with the earlier prediction observed when mRNA expression of CYP1A2 based on positive control.

PART FIVE

3.5 RESULTS

3.5.1 Time-dependent Calcein-AM uptake

Evaluation of the influence of herbal extracts (100 µg/mL) on P-gp employing calcein-AM uptake demonstrated that TO, KC, EP and LFT are time-dependent inhibitors of the transporter protein in co-transfected MDCKII with fluorescence of >2-fold above the untreated cells. However, these herbs were mild inhibitors of P-gp compared to verapamil at 60 minutes as shown in Figure 3.21. Methanol extracts of HHC and MO had calcein fluorescence units below the untreated cells.

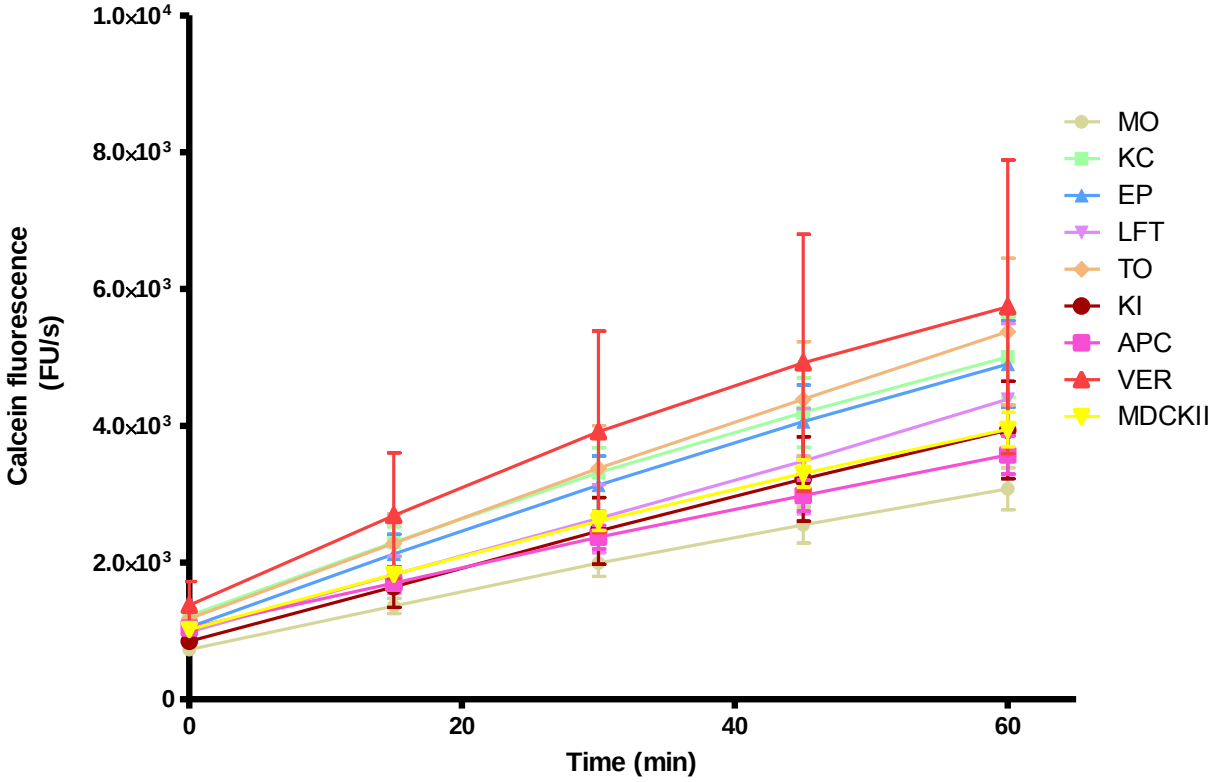


Figure 3.21 Influence of the methanol extracts of various herbal medicines on P-gp inhibition in hMDR1-MDCKII cell lines determined based on calcein fluorescence. Data expressed as Mean ± SEM of duplicate determinations.

3.5.2 Concentration-dependent Calcein-AM uptake

The herbal extracts were screened for their ability to increase calcein-AM uptake expressed as a percentage of the wild type of MDCKII. All the herbal extracts except HHC exhibited at least 110% increased uptake of Calcein-AM at a given concentration within the range of 1-100 µg/mL after 1 hour preincubation. Nevertheless, none of these herbal extract indicated concentration-dependent uptake of calcein-AM in MDCKII (Figure 3.22).

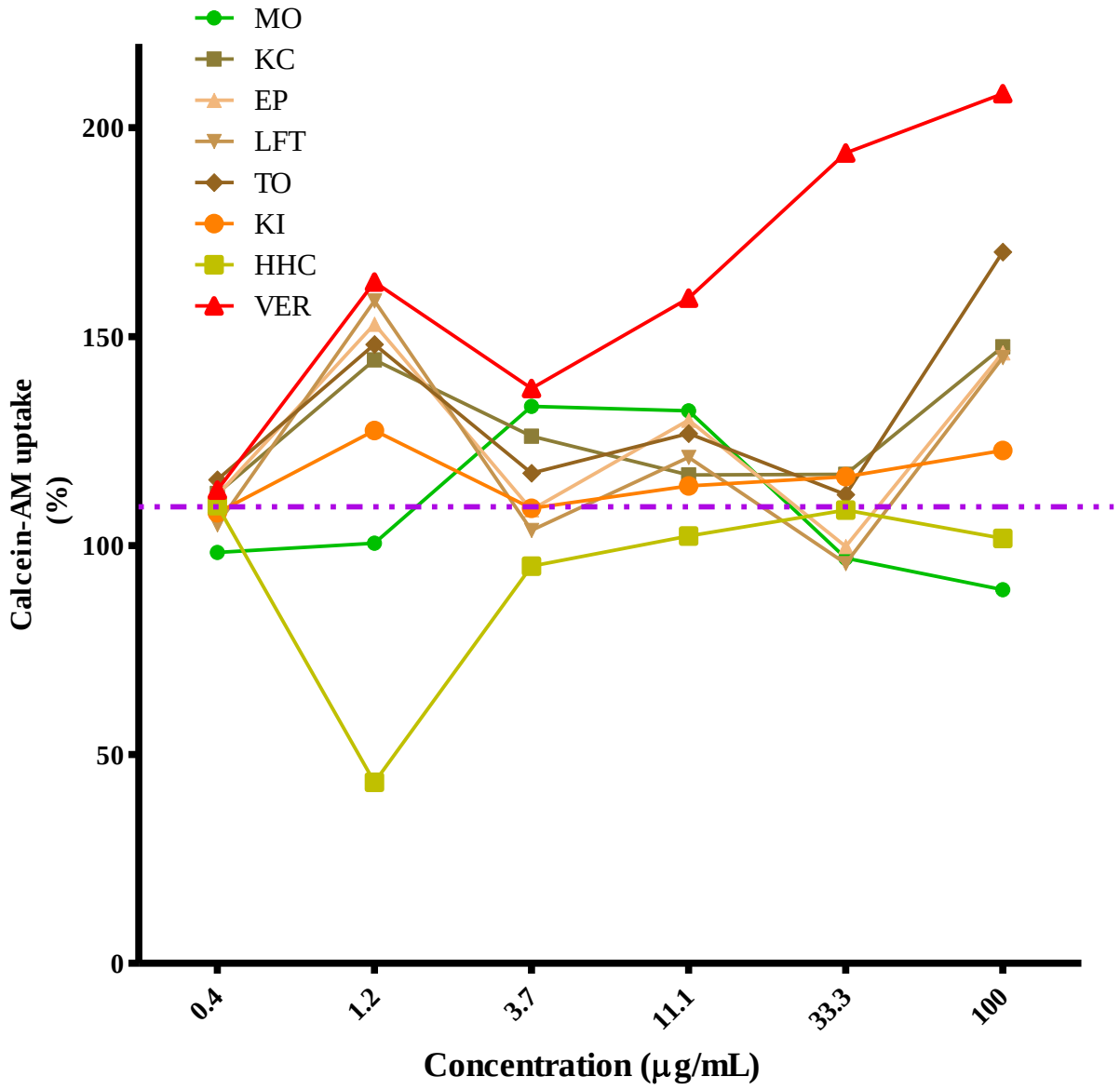


Figure 3.22 Concentration-dependent uptake of Calcein-AM in hMDR1-MDCKII cell lines used as an index of inhibitory effect of various herbal extracts on P-gp. Data expressed as Mean of duplicate determinations.

However, verapamil (positive control) demonstrated a dose-dependent effect on calcein-AM uptake in MDCKII cell lines with EC_{50} value of 23.0 ± 2.4 (Figure 3.22).

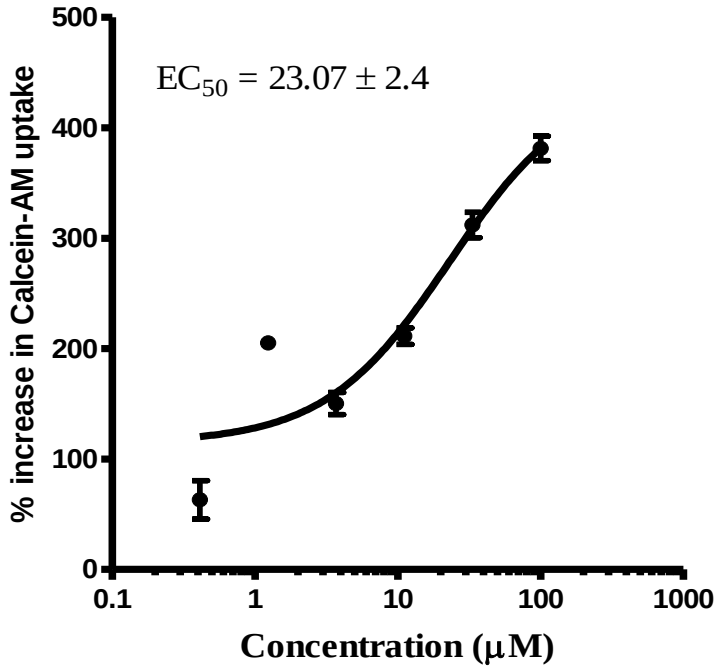


Figure 3.23 Dose-response curves of P-gp inhibition by Verapamil determined by calculating the per cent uptake of calcein AM into hMDR1-MDCKII cells. Equations used in evaluating EC_{50} and % increase in uptake of calcein-AM were described in materials and methods section. The data are represented as mean ± SEM of 2 independent experiments (n=2 in each experiment).

3.5.3 P-gp inhibition by ^{3}H-digoxin uptake in hMDR1-MDCK-II cells

The herbal medicines demonstrated varying degrees of inhibition on transport of ^{3}H-digoxin from basolateral to apical in hMDR1-MDCKII cell monolayer. *K. integra* and *E. purpurea* indicated strong inhibiton on ^{3}H-digoxin transport with IC_{50} values of 18.24 ± 2.52 µg/mL and 24.47 ± 4.97 µg/mL, respectively. *M. oleifera* exhibited moderate inhibitory effect on ^{3}H-digoxin transport in hMDR1-MDCKII with IC_{50} value of 35.45 ± 3.91 µg/mL. However, *K. integra* indicated a weak inhibition on transport of ^{3}H-digoxin in MDR1-MDCKII cells as shown in Figure 3.24.

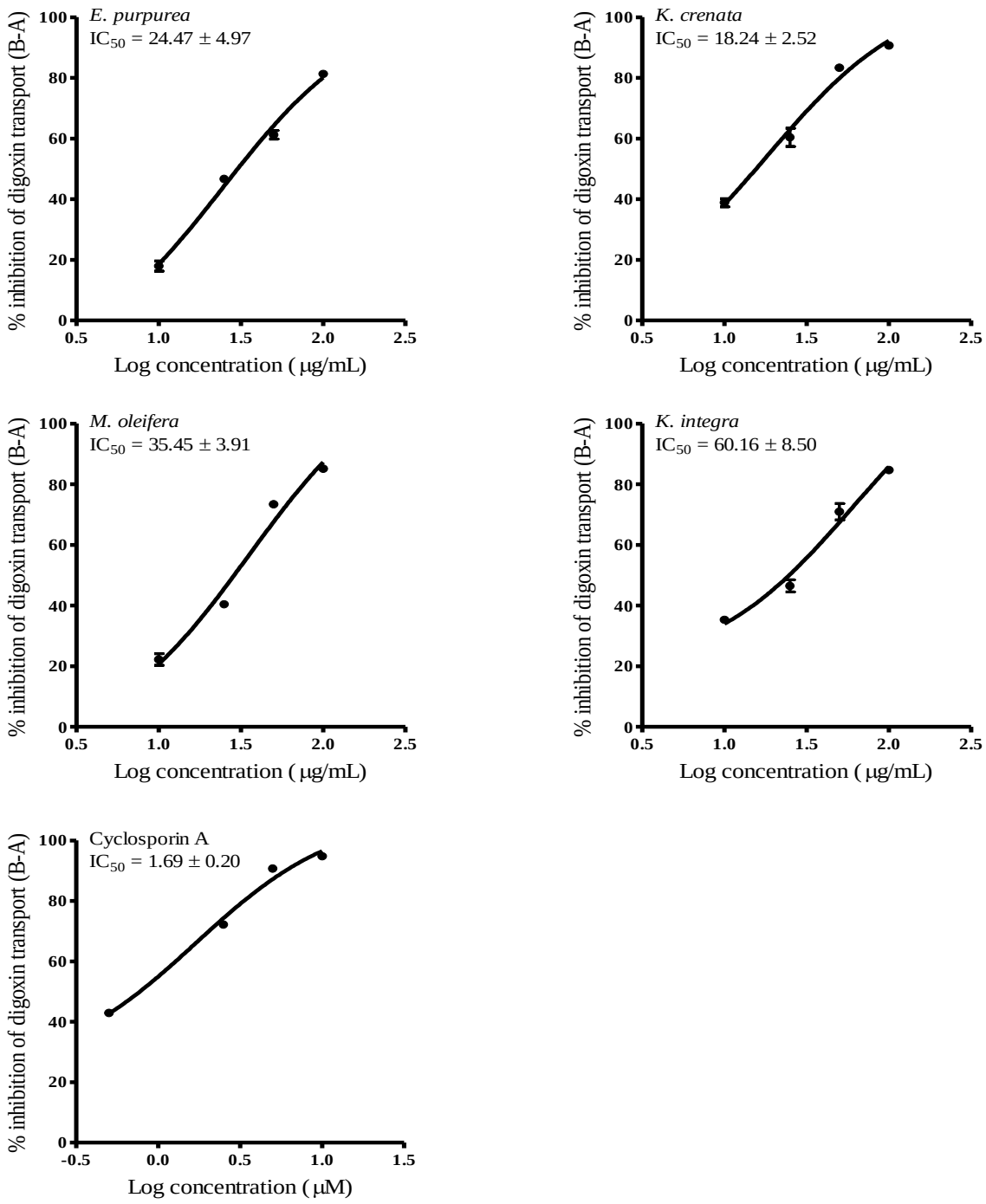


Figure 3.24: Dose-response curve of P-gp inhibiton by selected herbal medicines determined by calculating the basolateral to apical transport (%) of ^{3}H-digoxin across hMDR1-MDCKII cell monolayer. The data are represented as Mean ± SEM of 2 independent experiments. (n=1 for each experiment).

DISCUSSION

P-glycoprotein is considered as the most critical ABC transporter involved in intestinal absorption, biliary and urinary excretion and brain distribution of drugs. Thus, drug interactions mediated by P-gp may cause significant pharmacokinetic changes in systemic drug exposure or tissue specific distribution. Recently, the international transporter consortium released a white paper containing the decision tree for evaluation of clinically relevant P-gp mediated DDI studies. This white paper reported the relevance of using more than single substrates for P-gp mediated DDI evaluation presumably due to the multiple binding sites within the transporter protein (Giacomini *et al*., 2010). In evaluating the potential of the various herbal extracts used in this study to inhibit P-gp, the calcein acetoxymethyl ester (Calcein-AM) fluorescent probe substrate and digoxin radiolabelled substrates were used.

Calcein is a fluorescent dye designed for intracellular quantification of Ca^{2+} and the acetoxymethyl ester moiety is transported by MDR1 hence it was proposed to be a useful probe substrate for P-gp mediated inhibition evaluation. Calcein-AM is P-gp transporter substrate with high permeability through cell membranes. Within the cell, it is rapidly cleaved to free calcein, a low permeable fluorescent molecule which is non-substrate of P-gp. Thus, cells expressing P-gp accumulate low amounts of free calcein, whilst increased fluorescent is an indication of P-gp blockade (Szerémy *et al*., 2011).

Screening of the herbal extracts using the calcein-AM implicated MO and HHC as non-inhibitors of P-gp with calcein fluorescent units below that of the untreated MDR1 cells. Conversely, extracts; KC, LFT, EP and TO exhibited moderate inhibitory effects on P-gp with calcein fluorescent units between verapamil (positive control) and the untreated MDR1 (negative control). However, the inhibitory effect of these herbal extracts were non-concentration dependent as illustrated in Figure 3.22. Verapamil demonstrated strong inhibition on P-gp with EC_{50} value of 23.07 ± 2.4 µg/mL.

E. purpurea and *K. crenata* demonstrated strong inhibition on P-gp by reducing transport of digoxin in hMDR1-MDCKII cell monolayer. Additionally, *M. oleifera* and *K. integra* were observed as intermediate and weak inhibitor of digoxin transport across basolateral to apical in hMDR1-MDCKII. Digoxin is considered as a sensitive marker for P-gp due to its narrow therapeutic index (Giacomini *et al*., 2010). However, most drugs possess wider therapeutic index

and thus less likely to interact with other medications or herbal medicines which are P-gp modulators. Hence, the inhibition profile of various herbal extracts exhibited on transport of digoxin across hMDR1-MDCKII cell monolayer should be interpreted with caution to avoid false prediction.

CHAPTER FOUR

4. CONCLUSIONS, STUDY LIMITATIONS AND RECOMMENDATIONS

4.1 Conclusions

The objectives of this thesis were to: 1) investigate inhibition of CYPs and P-glycoprotein by herbal medicines namely; *Moringa oleifera*, *Kalanchoe integra*, *Kalanchoe crenata*, *Echinacea purpurea*, *Lessertia frutescens*, *Hypoxis hemercallidea* and *Taraxacum officinale* in recombinant CYPs, HLM, hepatocytes and MDCKII-MDR1 cells, 2) rank the potential risk of each herbs to cause HDI employing FDA guideline for DDI (2012), 3) investigate the degree of inhibition of CYP by the above mentioned herbal medicines after permeation through PAMPA and 4) investigate the modulation of PXR and regulation of CYP3A4, CYP1A2 and P-gp genes in activated PXR by the selected herbal medicines.

Based on the findings of the study, the following conclusions can be drawn:

1. The inhibition potential of herbal medicines on CYPs depends on the type of solvent and method used for the preparation of the herbal extract, and the kind of *in vitro* drug metabolism model applied. According to this study, preparation of plant material employing the hot aqueous extract method as practiced by the THPs presented mild inhibitory effect on the activity of CYPs in recombinant. The use of different organic solvents like methanol and chloroform enhanced inhibitory activity of herbal extracts on CYPs due to high recovery of phytochemicals. The prediction of *in vivo* HDI from *in vitro* data in methanol extracts confirmed over-estimation when compared with the aqueous extracts. Examination of the likelihood of herbal extracts to inhibit CYP in HLM indicated weak inhibition compared to the outcome observed in recombinant CYPs which may be attributed to presence of other phase I enzymes in HLM. Confirmatory tests using the cocktail approach also indicated reduced HDI compared to the conventional assays. However, the herbal extracts screened in this assay especially *M. oleifera*, *L. frutescens* and *E. purpurea* are reversible inhibitors of CYP3A4, CYP2C19 and CYP1A2 regardless

of the solvents and *in vitro* system applied. Thus these three herbal medicines have high risk to cause HDI in patients on other medications metabolized by CYP3A4, CYP2C19 and CYP1A2.

2. The introduction of absorption model, PAMPA, into the screening of herbal medicines for their potential to inhibit CYPs has shown the tendency for reduction in HDI prediction when an absorption model is introduced during the conduct of *in vitro* HDI studies. Such false negative predictions observed can be attributed to the inclusion of impermeable phytochemicals in the conventional *in vitro* assays which does not reflect *in vivo* scenario.

3. The inhibitory effect of most herbal medicines becomes pronounced after prolonged exposure to patients. Long term exposure of herbal medicines; *M. oleifera* and *K. crenata* has demonstrated high risk of CYP3A4 inactivation. *Lessertia frutescens* tea cut has indicated that although some herbal medications might be safe for acute usage, prolonged intake of such medication might affect the metabolic activity of CYP negatively later in life.

4. Prediction of metabolic clearance in HLM and Hepatocytes has shown the strong inhibitory effect of *M. oleifera* and *E. purpurea* on CYP3A4 activity when these herbal medicines are consumed concurrently with conventional drugs that are metabolized by the enzyme. The significant inhibition of CYP3A4 in hepatocytes by these two herbal medications observed is an indicative of their high risk to cause HDI in humans.

5. The PXR co-transfected HepG2 cell activation study has demonstrated that *E. purpurea* up-regulates expression CYP1A2, CYP3A4 and MDR1 genes in activated PXR. Hence caution must be exercised in patients concurrently taking orthodox medications and *E. purpurea*.

6. *Echinacea purpurea* and *Kalanchoe crenata* are strong inhibitors of P-gp whilst *Moringa oleifera* and *Kalanchoe integra* have shown mild inhibition on P-gp transport of digoxin. Thus these four herbal preparations have the potential to reduce plasma concentration of P-gp substrates when administered concurrently.

Figure 4.1 provides an overview of the work carried out in this study. The study has demonstrated the overlap between the various *in vitro* models used. Design of study in this manner offers a comprehensive examination of HDI potential of herbal medicine, avoiding the possibility of missing vital information. Such process has been used in many pharmaceutical companies at early drug discovery and development to screen new candidate drugs and proven to be beneficial. The outcome of studies involving herbal medications design using this approach will form basis for justification for possible studies in health subjects.

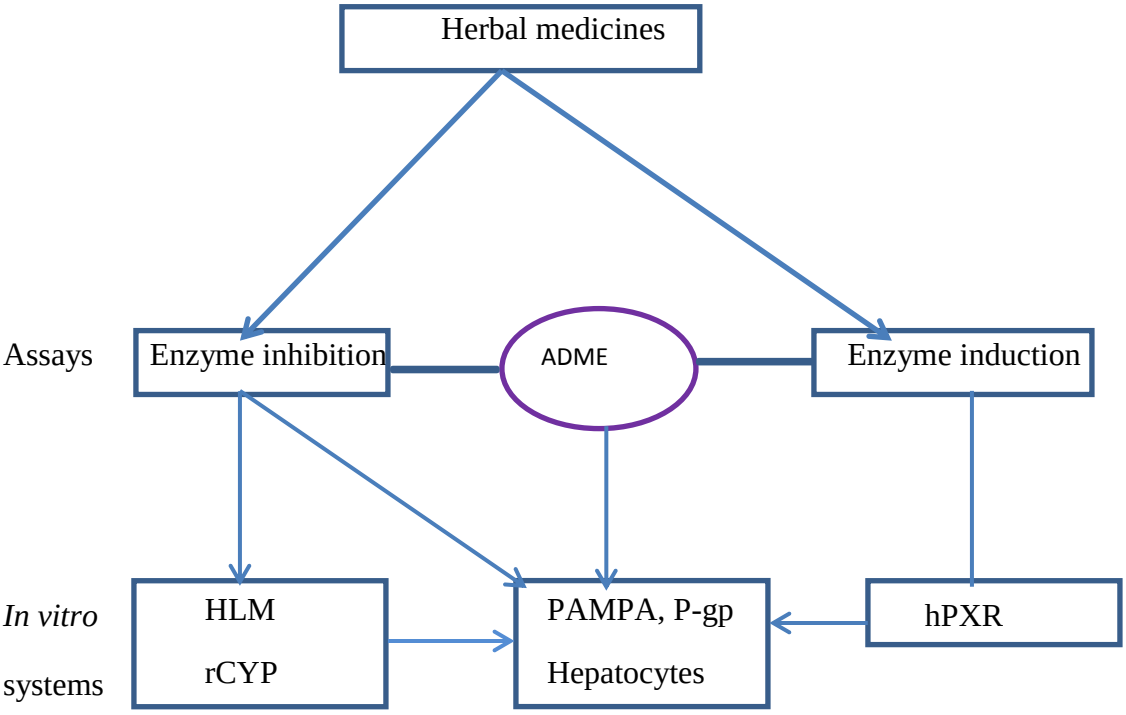


Figure 4.1 Summary of *in vitro* assays carried out in this study

4.2 Study limitations

Traditionally, different extraction conditions (temperature, duration and solvent) are applied for the preparation of herbal medicines. The variations in the extraction procedures culminate to consumption of different amount and phytoconstituents of plant extract by the patients. However, the methodologies employed in the laboratory to process the herbal extracts are different from the non-standardized methods used by the THPs. As a consequence, the magnitude of interaction of the herbal extracts with intestinal CYPs and transporters determined *in vitro* might be difficult to compare with that obtained in humans.

With the exception of *Lessertia frutescens*, the effects of herbal medicines on the CYPs and the P-glycoprotein were evaluated on crude extracts and fractions only. Due to logistical constraints, the inhibition and/or induction effect(s) of pure compounds of the other herbal extracts on CYPs and transportes could not be investigated.

The use of healthy human subjects is considered as the ideal approach to generate clinically significant pharmacokinetic HDI information on specific herbal medicines. However, due to the risk associated with such studies in humans and the rigorous ethical and regulatory requirements, the use of *in vitro* models has become the best alternative. In this study, human recombinant CYPs, human liver microsomes, cryopreserved hepatocytes and transfected mammalian cell lines were used under different experimental conditions. In all these *in vitro* systems, the herbal extracts are in direct contact with the enzymes/transporters and their respective substrates. This deviates from the *in vivo* system where intestinal pH and membrane barriers, non-specific plasma protein binding, organ perfusion and blood circulation affect the concentration and the phytoconstituents of the herbal extracts that enters the enzyme/transporter sites for effective interactions to occur. Additionally, in an attempt to predict *in vivo* HDI from *in vitro* data adopting the FDA guidelines, several assumptions were deduced to make the data meaningful. The soluble herbal extracts in the GIT consisted of polyphtoconstituents with different permeability coefficients. A reasonable proportion of these constituents may be impermeable via the intestinal membrane, and therefore complete bioavailability of the contents of the soluble herbal extracts is likely impossible. Thus although information from *in vitro* HDI studies provide

the basis for the need to conduct *in vivo* studies, studies in healthy human subjects are the definitive evidence for clinically significant HDI.

4.3 Recommendations

The use of *in vitro* liver-based models for screening of new candidate drugs and herbal medicines is considered as gold standard for drug interaction studies. However, due to the increase consumption of herbal medicines among HIV/AIDS patients, *in vivo* studies in healthy human subjects is recommended to provide better insight with respect to HDI potential of the selected herbal medicines used in this study. Studies of such nature provide the relevance of the observed inhibition of drug metabolizing enzymes and transporter clinically employing sophisticated mathematical models such as physiologically based pharmacokinetic (PBPK) modelling. Furthermore, there is the need for identification and isolation of active phytoconstituents based on the bioassay guided fractions documented in this study. This will be beneficial for structure elucidation and the possibility of drug discovery. The outcome of such studies will be important in understanding the safety and toxicity of phytoconstituents.

CHAPTER FIVE

5. REFERENCES

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APPENDIX 1: Abstracts of papers published from this thesis

Appendix 1.1: *In vitro* evaluation of reversible and time-dependent inhibitory effects of *Kalanchoe crenata* on CYP2C19 and CYP3A4 activities

Drug Metabolism Letters, 2015, *9*, 000-000 1

In Vitro Evaluation of Reversible and Time-Dependent Inhibitory Effects of *Kalanchoe crenata* on CYP2C19 and CYP3A4 Activities

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Abstract: *Kalanchoe crenata* popularly known as "dog's liver" is used in most African countries for the treatment of chronic diseases such as diabetes, asthma and HIV/AIDS related infections. The evaluation of *K. crenata* for herb-drug interactions has not been reported. This study therefore aims to evaluate the risk of *K. crenata* for herb-drug interaction *in vitro*. Crude methanol and fractions of *K. crenata* were incubated and preincubated with recombinant human CYP2C19 and CYP3A4. Comparative studies were conducted in both human liver microsomes and recombinant human CYP to ascertain the inhibition profile of the crude extract and the various fractions. The cocktail approach of recombinant human CYPs was conducted to confirm the inhibition potential of the fractions in the presence of other CYPs. The results showed significant time-dependent inhibition of tested samples on CYP3A4 with crude methanol (39KC), fractions 45A, 45B and 45D given IC_{50} fold decrease of 3.29, 2.26, 1.91 and 1.49, respective. Time dependent kinetic assessment of 39KC and 45D showed K_I and k_{inact} values for 39KC as 1.77µg/mL and 0.091 min^{-1} while that of 45D were 6.45 µg/mL and 0.024 min^{-1}, respectively. Determination of k_{inact} based on IC_{50} calculations yielded 0.015 and 0.04 min^{-1} for 39KC and 45D, respectively. Cocktail approach exhibited fold decreases in IC_{50} for all test fractions on CYP3A4 within the ranges of 2.10 – 4.10. At least one phytoconstituent in the crude methanol extract of *Kalanchoe crenata* is a reversible and time-dependent inhibitor of CYP3A4.

Keywords: *Kalanchoe crenata*, reversible inhibition, time-dependent inhibition, recombinant human CYPs, human liver microsomes.

APPENDIX 1.2 *Echinacea purpurea* up-regulates CYP1A2, CYP3A4 and MDR1 genes expression by activation of pregnane X receptor pathway

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RESEARCH ARTICLE

Echinacea purpurea up-regulates CYP1A2, CYP3A4 and MDR1 gene expression by activation of pregnane X receptor pathway

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Abstract

1. This study investigated the mechanism underlying Echinacea-mediated induction of CYP1A2, CYP3A4 and MDR1 in terms of human pregnane X receptor (PXR) activation.
2. Crude extracts and fractions of *Echinacea purpurea* were tested for PXR activation in HepG2 cells by a reporter gene assay. Quantitative real-time PCR was carried out to determine their effects on CYP1A2 and CYP3A4 mRNA expressions. Capsules and fractions were risk ranked as high, intermediate and remote risk of drug-metabolizing enzymes induction based on EC_{50} values determined for respective CYPs.
3. Fractions F_1, F_2 and capsule (2660) strongly activated PXR with 5-, 4- and 3.5-fold increase in activity, respectively. Echinacea preparations potentiated up-regulation of CYP1A2, CYP3A4 and MDR1 *via* PXR activation.
4. Thus *E. purpurea* preparations cause herb–drug interaction by up-regulating CYP1A2, CYP3A4 and P-gp *via* PXR activation.

Keywords

Activation, cytochrome P450 enzymes (CYPs), *E. purpurea*, human liver carcinoma (HepG2), human pregnane xenobiotic receptor (hPXR)

History

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APPENDIX 1.3 Application of Caco-2 cell line in herb-drug interaction studies

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Application of Caco-2 Cell Line in Herb-Drug Interaction Studies: Current Approaches and Challenges

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ABSTRACT - The Caco-2 model is employed in pre-clinical investigations to predict the likely gastrointestinal permeability of drugs because it expresses cytochrome P450 enzymes, transporters, microvilli and enterocytes of identical characteristics to the human small intestine. The FDA recommends this model as integral component of the Biopharmaceutics Classification System (BCS). Most dedicated laboratories use the Caco-2 cell line to screen new chemical entities through prediction of its solubility, bioavailability and the possibility of drug-drug or herb-drug interactions in the gut lumen. However, challenges in the inherent characteristics of Caco-2 cell and inter-laboratory protocol variations have resulted to generation of irreproducible data. These limitations affect the extrapolation of data from pre-clinical research to clinical studies involving drug-drug and herb-drug interactions. This review addresses some of these caveats and enumerates the plausible current and future approaches to reduce the anomalies associated with Caco-2 cell line investigations focusing on its application in herb-drug interactions.

APPENDIX 1.4 Inhibition of Major drug metabolising CYPs by common herbal medicines used by HIV/AIDS patients in Africa-Implication for herb-drug interactions

Drug Metabolism Letters, 2013, *7*, 83-95

Inhibition of Major Drug Metabolizing CYPs by Common Herbal Medicines used by HIV/AIDS Patients in Africa– Implications for Herb-Drug Interactions

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Abstract: The purpose of this study was to evaluate the potential risk of common herbal medicines used by HIV-infected patients in Africa for herb-drug interactions (HDI). High throughput screening assays consisting of recombinant Cytochrome P450 enzymes (CYPs) and fluorescent probes, and parallel artificial membrane permeability assays (PAMPA) were used. The potential of herbal medicines to cause HDI was ranked according to FDA guidelines for reversible inhibition and categorization of time dependent inhibition was based on the normalized ratio. CYPs 1A2 and 3A4 were most inhibited by the herbal extracts. H. *hemerocallidea* (IC_{50} = 0.63 µg/mL and 58 µg/mL) and *E. purpurea* (IC_{50} = 20 µg/mL and 12 µg/mL) were the potent inhibitors of CYPs 1A2 and 3A4 respectively. *L. frutescens* and H. *hemerocallidea* showed clear time dependent inhibition on CYP3A4. Furthermore, the inhibitory effect of both H. *hemerocallidea* and *L. frutescens* before and after PAMPA were identical. The results indicate potential HDI of H. *hemerocallidea*, *L. frutescens* and *E. purpurea* with substrates of the affected enzymes if maximum *in vivo* concentration is achieved.

Keywords: Cytochrome P450s, fluorometric assay, herb-drug interactions, parallel artificial membrane permeability assays.

APPENDIX 1.5 Prediction of reversible and TDI potential of CYP3A4 by *L. frutescens*

Planta Med 2014; 80 - PU8
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Prediction of reversible and time-dependent inhibition potential of CYP3A4 by *Lessertia frutescens* fractions and possibility of herb-drug interactions with anti-HIV drugs

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Congress Abstract

Lessertia frutescens is a popular medicinal product used in sub-Saharan Africa by patients with HIV-infection, malignancies or other diseases. Non-nucleotide reverse transcriptase inhibitors (NNRTIs) (nevirapine and efavirenz) and protease inhibitors (PIs) (ritonavir) are mainly biotransformed by CYP3A4. Consequences of CYP3A4 inhibition by *Lessertia frutescens* could cause inefficacy or systemic toxicity when coadministered with NNRTIs or PIs. Six concentrations of each of the tested Lessertia fractions (100 – 1.0µg/mL) were co-incubated or pre-incubated with 25 pmol/mL of CYP3A4 enzyme and 10µM of 7-benzyloxy-4-trifluoromethylcoumarin (BFC) as a substrate. Based on FDA guidelines, the potential risk for herb drug interaction (HDI) *in vivo* was predicted for the fractions using IC_{50} values. Time-dependent inhibition (TDI) was also assessed using IC_{50} shift with ≥1.5-fold reduction considered significant. *Lessertia frutescens* fractions; MeOH (100%), $CHCl_3$ (100%), $CHCl_3$:MeOH (90:10%), $CHCl_3$/MeOH/$H_2$0 (78.0:19.5:2.5%) inhibited CYP3A4 with IC_{50} of 26.9 ± 5.2, 12.0 ± 2.4, 6.0 ± 0.8, and 16.0 ± 1.8 respectively. An IC_{50} shift of 3.6, 1.3, 1.8 and 2.9 fold decrease was observed for MeOH (100%), $CHCl_3$(100%), $CHCl_3$:MeOH (90:10%), $CHCl_3$/MeOH/$H_2$0 (78.0:19.5:2.5%) respectively, when these fractions were preincubated with the enzyme. Fractions of *Lessertia frutescens* have the potential to cause both reversible and time-dependent inhibition on CYP3A4 and could affect the metabolism of NNRTI and PI upon concomitant administration.

APPENDIX 1. 6 Integration of Parallel Artificial Membrane Permeability Assays into HTS of DME

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INTEGRATION OF PARALLEL ARTIFICIAL MEMBRANE PERMEABILITY ASSAYS (PAMPA) INTO HIGH THROUGHPUT SCREENING OF MEDICINAL HERBS FOR POTENTIAL INHIBITION OF DRUG METABOLISING ENZYMES: FOCUS ON CYP1A2 AND CYP 3A4

Awortwe C., Fasinu P., Bouic P., Masimirembwa C., Rosenkranz B.

Use of medicinal herbs in HIV-infected patients is a major concern to Clinicians due to the potential for herb-drug interactions (HDI). Screening of HDI is usually performed using CYPs. This method, may give false positive results by excluding the effect of variable herb or drug absorption in the GIT. PAMPA is used to monitor transcellular permeation of drugs [1]. The system is applied in this assay to screen medicinal herbs for potential HDI using recombinant CYPs. As a follow up of previous study [2], two point screening assays (0.2 mg/ml and 2.0 mg/ml) of each herbal extract were performed using CYP1A2 and CYP3A4 with respective fluorescent probe substrates: 2.5 pmol/mL of CYP1A2 (7-ethoxy-3-cyanocoumarin (CEC), 3 mM) and 25 pmol/mL of CYP3A4 (7-benzyloxy-4-trifluoromethylcoumarin (BFC), 10 mM). Percentage inhibition of respective CYPs was determined. 2.0 mg/mL of each medicinal herb was passed through PAMPA, and collected from the acceptor compartment after 5 h. The herb collected from the acceptor compartment was tested for inhibitory effects on CYP1A2 and CYP3A4. Concentration dependent, 0.2 and 2 mg/ml, per cent inhibition on CYP activity by *Hypoxis* (73.3 ± 1.5, 81.0 ± 2.7), *Lessertia* (17.3 ± 8.7, 53.7 ± 1.4), *Echinacea* (71.9 ± 2.2, 89.5 ± 3.6) and *Moringa* (26.5 ±16.0, 21.8 ±9.7) for CYP1A2 and, *Hypoxis* (27.6 ± 4.1, 67.7 ± 5.6), *Lessertia* (57.2 ± 4.0, 66.3 ± 0.9), *Echinacea* (22.7 ± 4.1, 30.6 ± 2.6) and *Moringa* (23.9 ± 1.4, 62.4 ± 1.8) for CYP3A4 were observed. The inhibitory potency of *Echinacea* on CYP1A2 reduced from 28.1 ± 2.2 to 68.4 ± 3.2 and that of *Hypoxis* on CYP3A4 from 65.5±4.1 to 71.1 ±11.9 after permeation through PAMPA. The inhibitory potency of a perpetrator (inhibitor) on CYPs depends on the phytochemical constituents permeable through the membrane.

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APPENDIX 1.7 Risk assessment of two medicinal herbs used in HIV-infected patients for potential inhibition on CYP3A4

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RISK ASSESSMENT OF TWO MEDICINAL HERBS USED IN HIV-INFECTED PATIENTS FOR POTENTIAL INHIBITION ON CYP3A4

Awortwe C., Fasinu P., Bouic P., Masimirembwa C., Rosenkranz B.

Background: *Lessertia frutescens* and *Moringa oleifera* are popular medicinal herbs used among HIV-infected patients in sub-Saharan Africa. Non-nucleotide reverse transcriptase inhibitors (nevirapine and efavirenz) and protease inhibitors (ritonavir) are primarily metabolized by CYP3A4. Consequences of CYP3A4 inhibition by these herbal products could range from therapeutic failure to severe systemic toxicity.

Methods: Two point screening assays (0.2 mg/ml and 2.0 mg/ml) were conducted on CYP3A4 using the fluorescent probe substrates; 25 pmol/mL of CYP3A4 (7-benzyloxy-4-trifluoromethylcoumarin (BFC), 10 mM). The percentage residual activity of the respective enzymes was determined. IC50 values of *Lessertia* and *Moringa* were determined and the potential risk for herb-drug interaction *in vivo* evaluated based on FDA guidelines (1). Time-dependent inhibition (TDI) was also carried out and outcome classified into non-TDI (>0.9), grey zone (0.7–0.9) and clear TDI (<0.7) using normalized ratios (2).

Results: Both extracts showed concentration dependent inhibition; *Lessertia frutescens* (20 ± 1.2 and 50 ± 0.8) and *Moringa oleifera* (11 ± 3.7 and 49 ± 4.7) respectively on CYP3A4. The IC50 values for *Lessertia* and *Moringa* were 740 ± 12.5 µg/ml and 300 ± 9.0 µg/ml respectively. The *in vivo* prediction of potential risk for HDI showed *Lessertia frutescens* and *Moringa oleifera* cause possibly (10.7) and likely (0.35) inhibition respectively on CYP3A4. Normalized ratios confirmed the possibility of *Lessertia frutescens* generating clear TDI and *Moringa oleifera* producing non-TDI.

Conclusion: *Lessertia frutescens* has the greater tendency to cause inhibition on CYP3A4 upon concomitant administration with non-nucleotide reverse transcriptase inhibitors (nevirapine and efavirenz) and protease inhibitors (ritonavir). Hence caution must be exercised to prevent therapeutic failure in HIV-infected patients using *Lessertia frutescens*.

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APPENDIX 2: Ethics approval

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot · your knowledge partner

Approved with Stipulations
New Application

03-Dec-2012
Awortwe, Charles A
Stellenbosch, WC

Ethics Refernce #: S12/09/249

Title: **Pharmacokinetic herb-drug interaction study of slected Traditional Medicines used as Complementary and Alternative Medicine (CAM)for HIV/AIDS**

Dear Mr Charles Awortwe,

The **New Application** received on **20-Sep-2012**, was reviewed by Health Research Ethics Committee 1 via Committee Review procedures on **28-Nov-2012**.

Please note the following information about your approved research protocol:

Protocol Approval Period: **28-Nov-2012 -28-Nov-2013**

Present Committee Members:
Kinnear, Craig CJ
Seedat, Soraya S
Mukosi, M
Theunissen, Marie ME
Kearns, E
Meintjes, WAJ Jack
Mohammed, Nazli
Weber, Franklin CFS
Nel, Etienne EDLR
Sprenkels, Marie-Louise MHE
Rohland, Elvira EL
Theron, Gerhardus GB
Els, Petrus PJJS
De Roubaix, Malcolm JAM
Hendricks, Melany ML
Welzel, Tyson B
Barsdorf, Nicola

The Stipulations of your ethics approval are as follows:

This approval is subject to approval by the CPE Scientific Committee.

Please remember to use your **protocol number** (**S12/09/249**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired.
The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at 0219389657.

Included Documents:
Protocol
Application Form
Investigators declaration

Sincerely,

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Health Research Ethics Committee 1

APPENDIX 3: Metabolic clearance of TST

Appendix 3.1A: Intrinsic clearance of testosterone in the absence and presence of herbal extracts in human hepatocytes

Test samples	$t_{½}$ (min)[1)]	CL_{int} *in vitro* ((µL/min)/million cells)[2)]	CL_{int} *in vivo* ((mL/min)/kg body mass)[3)]	$CL_{h,b}$ ((mL/min)/kg body mass)[4,5)]	$CL_{h,b}$ (% hepatic blood flow)
TST	565.3	2.45	6.24	4.79	23.16
TST+ KI	783.3	1.77	4.50	3.70	17.87
TST + MO	1,390.8	1.00	2.54	2.26	10.91
TST + EP	1,532.2	0.90	2.30	2.07	10.01
TST + KC	618.1	2.24	5.71	4.47	21.61

1) Half-lives were evaluated by linear extrapolation
2) $(\ln 2/T_{1/2})$ / viable cells x 1000
3) $(CL_{int} / 1000)$ * (cells / g liver) * (g liver / kg body weight)
4) $CL_h = (Qh * CLint, scaled) / (Qh + CL_{int}, scaled)$, Qh: hepatic blood flow; (well-stirred liver model)
5) Qh (mL/min/kg body weight): 55 (rat); 30.9 (dog); 43.6 (monkey) ; 20.7 (human)

Appendix 3.1B: Intrinsic clearance of testosterone in the absence and presence of herbal extracts in HLM

Test samples	t½ (min)[1)]	CL_{int} *in vitro* ((µL/min)/microsomal protein (mg))[2)]	CL_{int} *in vivo* ((mL/min)/kg body mass)[3)]	$CL_{h,b}$ ((mL/min)/kg body mass)[4,5)]	$CL_{h,b}$ (% hepatic blood flow)
TST	439.7	2.64	3.05	2.66	12.84
TST+ KI	1,901.2	0.61	0.71	0.68	3.29
TST + MO	928.0	1.25	1.44	1.35	6.52
TST + EP	1,164.3	1.00	1.15	1.09	5.27
TST + KC	512.2	2.26	2.62	2.32	11.22

APPENDIX 4: Estimation of GIT concentrations of herbal extracts

Appendix 4.1A: Calculation of aqueous herbal extracts concentration in the gut

Herbal extracts	% yield (W/W)	Usual human dose (single; mg)	Estimated extract per dose (mg/mL)	Putative GIT concentration (µg/mL)
T30	22.989	350	80.46	321.84
T41	N.A	350	350.00	1400
T66	7.451	4000	298.04	1192.16
T79	10.917	300	32.75	131
T50	N.A	300	300.00	1200
T70	N.A	500	500.00	2000
T60	N.A	320	320.00	1280
T80	N.A	400	400.00	1600

Appendix 4.1B: Calculation of methaolic herbal extracts concentration in the gut

Herbal extracts	% yield (W/W)	Usual human dose (single; mg)	Estimated extract per dose (mg/mL)	Putative GIT concentration (µg/mL)
HHC	17.3	350	60.55	242.20
HHL	27.0	350	94.50	378.00
EP	18.6	320	59.52	238.08
KI	12.5	NA	NA	NA
MO	14.0	400	56.00	224.00
LFT	16.0	300	48.00	192.00
LFC	12.2	300	36.60	146.40

Putative GIT concentration [I] = Estimated extract per dose/volume of GIT fluid (250 mL).

Estimated extract per dose = Yield (W/W %) x single dose of herbal formulation recommended in human.