IN VITRO ASSESSMENT OF SOME TRADITIONAL MEDICATIONS USED IN SOUTH AFRICA FOR PHARMACOKINETIC DRUG INTERACTION POTENTIAL

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8 YWYa VYf 2013

Declaration

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

Signature: .... Date: ....

February, 2013

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Abstract

Introduction

Earlier studies have shown the popularity of herbal products among people as traditional, complementary or alternative medication. One of the major clinical risks in the concomitant administration of herbal products and prescription medicine is pharmacokinetic herb-drug interaction (HDI). This is brought about by the ability of phytochemicals to inhibit or induce the activity of metabolic enzymes and transport proteins. The aim of this study was to investigate the potential of the crude extracts of popular medicinal herbs used in South Africa to inhibit major cytochrome P450 (CYP) enzymes and transport proteins through *in vitro* assessment.

Methods

Medicinal herbs were obtained from traditional medical practitioners and 15 were selected for this study. The selected herbal products were extracted and incubated with human liver microsomes to monitor the following reactions as markers for the metabolic activities of the respective CYP: phenacetin *O*-deethylation (CYP1A2), diclofenac 4'-hydroxylation (CYP2C9), *S*-mephenytoin 4'-hydroxylation (CYP2C19) and testosterone 6β-hydroxylation (CYP3A4). In addition, the influence of *Lessertia frutescens* (formerly *Sutherlandia frutescens*) and *Hypoxis hemerocallidea* was investigated on more isozymes: coumarin 7-hydroxylation (CYP2A6), bupropion hydroxylation (CYP2B6), paclitaxel 6α-hydroxylation (CYP2C8), bufuralol 1'-hydroxylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1) and midazolam 1'-hydroxylation (CYP3A4/5). The generation of the CYP-specific substrates/metabolites were monitored and quantified with the aid of LC-MS/MS. The metabolic clearance of midazolam using cryopreserved hepatocytes was monitored in the presence of *Lessertia frutescens* and *Hypoxis hemerocallidea*. The potential of both to inhibit human ATP-binding cassette (ABC) transporter activity was assessed using recombinant MDCKII and LLC-PK1 cells over-expressing human breast cancer resistant protein (BCRP) and human P-glycoprotein (P-gp), respectively. Similarly, the potential for interactions with human organic anion transporting polypeptide (OATP1B1 and OATP1B3) was assessed using recombinant HEK293 cells over-expressing OATP1B1 and OATP1B3, respectively.

Results

Bowiea volubilis, Kedrostis Africana, Chenopodium album, Lessertia frutescens (methanolic extract), *Hypoxis hemerocallidea, Spirostachys africana* and *Lessertia frutescens* (aqueous extract), in ascending order of potency demonstrated strong inhibition of CYP1A2 activity (IC_{50} = 1-100 μg/mL). Similarly, *Emex australis, Alepidea amatymbica, Pachycarpus concolor, Lessertia frutescens, Capparis sepiaria, Kedrostis africana and Pentanisia prunelloides inhibited* CYP2C9 with IC_{50} less than 100 μg/mL. The following demonstrated strong inhibition of CYP2C19 with IC_{50} values less than 100 μg/mL: *Acacia karroo, Capparis sepiaria*, *Chenopodium album*, *Pachycarpus concolor*, *Ranunculus multifidus, Lessertia frutescens* and *Zantedeschia aethiopica*. CYP3A4 was inhibited by *Lessertia frutescens*, *Hypoxis hemerocallidea, Spirostachys Africana, Bowiea volubilis, Zantedeschia aethiopica*, *Chenopodium album*, *Kedrostis Africana*, *Acacia karroo*, *Emex australis*, *Pachycarpus concolor*, *Ranunculus multifidus*, *Capparis sepiaria* and *Pentanisia prunelloides*. Time-dependent (irreversible) inhibition of CYP3A4/5 (KI = 296 μg/mL, kinact = 0.063 min^{-1}) and delay in the production of midazolam metabolites in the human hepatocytes, leading to a 40% decreased midazolam upscaled *in vivo* clearance, was observed with *Lessertia frutescens*. Further, *Lessertia frutescence* inhibited the activity of P-gp (IC_{50} = 324.8 μg/mL), OATP1B1 (IC_{50} = 10.4 μg/mL) and OATP1B3 (IC_{50} = 6.6 μg/mL). *Hypoxis hemerocallidea* inhibited the activity of OATP1B1 (IC_{50} = 118.7 μg/mL) and OATP1B3 (IC_{50} = 290.1 μg/mL) with no potent inhibitory effects on P-gp. None of the two inhibited the activity of BCRP within the tested concentrations.

Conclusion

The result indicates the potential for HDI between the selected medicinal herbs and the substrates of the enzymes investigated in this study, if sufficient *in vivo* concentrations are achieved.

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Abstrak

Inleiding

Vroeëre studies het aangedui dat die gebruik van plantaardige produkte as tradisionele, aanvullende en alternatiewe medikasie baie gewild is. Een van die grootste kliniese risiko's geassosieer met die gelyktydige gebruik van plantaardige produkte met voorskrifmedikasie is farmakokinetiese kruie-geneesmiddel interaksies (HDI). Hierdie interaksies word veroorsaak deur die vermoë van plantchemikalieë om die aktiwiteit van metaboliese ensieme en transportproteïene te inhibeer of te induseer. Die doel van hierdie studie is om ondersoek in te stel na die moontlikheid van onsuiwer ekstrakte van gewilde Suid-Afrikaanse medisinale kruie om die belangrikste sitochroom P450 (CYP)-ensieme en transportproteïene te inhibeer. Hierdie ondersoek sal plaasvind deur middel van *in vitro*-studies.

Metodes

Medisinale kruie is verkry vanaf tradisionele genesers, waaruit 'n totaal van 15 kruie geselekteer is vir gebruik tydens hierdie studie. Die geselekteerde kruie is geëkstraheer en met menslike lewermikrosome geïnkubeer om die volgende reaksies as merkers vir die metaboliese aktiwiteit van die onderskeie CYP-ensieme te moniteer: fenasetien-*O*-deëtilasie (CYP1A2), diklofenak-4'-hidroksilasie (CYP2C9), *S*-mefenitoïen-4'-hidroksilasie (CYP2C19) en testosteroon-6β-hidroksilasie (CYP3A4). Afgesien van die voorafgaande, is ook die invloed van *Lessertia frutescens* en *Hypoxis hemerocallidea* op verskeie ander iso-ensieme ondersoek. Hierdie iso-ensieme is soos volg: koumarien-7-hidroksilasie (CYP2A6), bupropioonhidroksilasie (CYP2B6), paklitaksiel-6α-hidroksilasie (CYP2C8), bufuralol-1'-hidroksilasie (CYP2D6), chloorsoksasoon-6-hidroksilasie (CYP2E1) en midasolaam-1'- hidroksilasie (CYP3A4/5). Die produksie van CYP-spesifieke substrate/metaboliete is gemoniteer en deur middel van LC-MS/MS-analises gekwantifiseer. Die metaboliese opruiming van midasolaam deur middel van krio-gepreserveerde hepatosiete is gemoniteer in die teenwoordigheid van *Lessertia frutescens* en *Hypoxis hemerocallidea*. Die moontlikheid van beide om menslike ATP-bindingskasset (ABC)-transporteerderaktiwiteit te inhibeer is bepaal deur die gebruik van rekombinante MDCKII- en LLC-PK1-selle wat onderskeidelik menslike borskanker-weerstandige proteïen (BCRP) en menslike P-glikoproteïen (P-gp) potensieel. Op 'n soortgelyke wyse is die moontlikheid vir interaksies met menslike organiese anion-transportpolipeptiede (OATP1B1 en OATP1B3) bepaal deur rekombinante HEK293-selle te gebruik wat onderskeidelik OATP1B1 en OATP1B3 potensieel.

Resultate

Bowiea volubilis, Kedrostis Africana, Chenopodium album, Lessertia frutescens (metanol-ekstrak), *Hypoxis hemerocallidea, Spirostachys africana* en *Lessertia frutescens* (water-ekstrak), in toenemende potensie, het sterk inhibisie van CYP1A2-aktiwiteit (IC_{50} = 1-100 µg/mL) getoon. In ooreenstemming met die voorafgaande resultate het *Emex australis, Alepidea amatymbica, Pachycarpus concolor, Lessertia frutescens, Capparis sepiaria, Kedrostis africana* en *Pentanisia prunelloides* CYP2C9 met IC_{50}–waardes van minder as 100 µg/mL geïnhibeer. Die volgende het sterk inhibisie van CYP2C19 met IC_{50}-waardes van minder as 100 µg/mL getoon: *Acacia karroo, Capparis sepiaria*, *Chenopodium album*, *Pachycarpus concolor*, *Ranunculus multifidus, Lessertia frutescens* en *Zantedeschia aethiopica*. CYP3A4 is deur *Lessertia frutescens, Hypoxis hemerocallidea, Spirostachys Africana, Bowiea volubilis, Zantedeschia aethiopica*, *Chenopodium album*, *Kedrostis Africana*, *Acacia karroo*, *Emex australis*, *Pachycarpus concolor*, *Ranunculus multifidus*, *Capparis sepiaria* en *Pentanisia prunelloides* geïnhibeer. Tydafhanklike (onomkeerbare) inhibisie van CYP3A4/5 (KI = 296 µg/mL, kinact = 0.063 min^{-1}) en vertraging in die produksie van midasolaammetaboliete in menslike hepatosiete wat aanleiding gee tot 'n 40% afname in midasolaam bepaal *in vivo* opruiming, is waargeneem met *Lessertia frutescens*. *Lessertia frutescens* het ook die aktiwiteit van P-gp (IC_{50} = 324.8 µg/mL), OATP1B1 (IC_{50} = 10.4 µg/mL) en OATP1B3 (IC_{50} = 6.6 µg/mL) geïnhibeer. *Hypoxis hemerocallidea* het die aktiwiteit van OATP1B1 (IC_{50} = 118.7 µg/mL) en OATP1B3 (IC_{50} = 290.1 µg/mL) geïnhibeer met geen betekenisvolle effekte op P-gp nie. Geen een van die twee het die aktiwiteit van BCRP geïnhibeer binne die konsentrasies waarin getoets is nie.

Gevolgtrekking

Die resultate van hierdie studie dui aan dat wanneer voldoende *in vivo*-konsentrasies bereik word, die moontlikheid vir kruie-geneesmiddel interaksies tussen die geselekteerde medisinale kruie en ensiemsubstrate 'n werklikheid word.

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Dedication

To

Mrs Avosewhe Fasinu (Nee Avosegbo)

The mother, who in the face of despair accepts the sole responsibility/single parenthood of the three-month old baby

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Research Output

Publications from this thesis (Appendix A)

1. **<u>Pius Fasinu</u>**, Patrick J Bouic and Bernd Rosenkranz (2012). Liver-based in vitro technologies for drug biotransformation studies – a review. *Curr Drug Metab,* 13:215-224 (Abstract in Appendix A1).

2. **<u>Pius Fasinu,</u>** Patrick J Bouic and Bernd Rosenkranz (2012). An overview of the evidence and mechanisms of herb–drug interactions *Frontier in Pharmacol*, 3:69. doi:10.3389/fphar.2012.00069 (Abstract in Appendix A2).

3. **<u>Pius Fasinu</u>,** Heike Gutmann, Hilmar Schiller, Alexander-David James, Patrick J Bouic and Bernd Rosenkranz (2013). The potential of *Sutherlandia frutescens* for herb-drug interaction. *Drug Metab Dispos*, 41(2):488-97. (Abstract in Appendix A3).

Conference proceedings and published abstracts from this thesis (Appendix B)

4. **<u>Pius Fasinu</u>**, Heike Gutmann, Hilmar Schiller, Bertrand-Luc Birlinger, Lisa Bijasson, Sylwia Faller, Heiner Seifart, Patrick Bouic and Bernd Rosenkranz. Herb-drug interaction potential of popular South African medicinal herbs: an in vitro assessment. Proceedings of the Annual Congress of the South African Society for Basic and Clinical Pharmacology in association with the Department of Family Medicine, University of Pretoria) and Toxicology Society of South Africa, 29 September – 2 October 2012 (Abstract in Appendix B1).

5. **<u>Pius Fasinu</u>**, Heiner Seifart, Patrick Bouic and Bernd Rosenkranz. The potential of H. hemerocallidea and S. frutescens to induce herb-drug interaction with antiretroviral drugs. Proceedings of the 56th Annual Academic Day, 15-16th August 2012, Tygerberg Campus, University of Stellenbosch, South Africa; Page 70. Available online: http://sun025.sun.ac.za/portal/page/portal/Health_Sciences/English/Annual_Academic_Day/AAD_Menu/Programme%20Book%20Akademiese%20Jaardag%202012.pdf (Abstract in Appendix B2).

6. **<u>Pius Fasinu</u>,** Hilmar Schiller, Heike Gutmann, Alexandra-David James, Cyrille Marvalin, Bertreand-Luc Birlinger, Lisa Bijasson, Sylwia Faller, Majorie Simon, Patrick Bouic and Bernd Rosenkranz. Herb-drug Interaction Potential of *Sutherlandia frutescens* and

Hypoxis hemerocallidea. Annual Novartis Next Generation Scientist Research Day, Basel, Switzerland, August 2012.

7. **Pius Fasinu**, Heiner Seifart, Patrick Bouic, and Bernd Rosenkranz. In vitro Investigation of the Effects of Commonly Used South African Medicinal Herbs on CYP1A2 activity Employing Human Liver Microsomes. Proceedings of the 6th International Conference on Pharmaceutical and Pharmacological Sciences, University of KwaZuluNatal, Durban South Africa, 25th – 27th September, 2011; Page 42. Available online: http://www.sapharmacol.co.za/CONGRESS_Site/html_pages/Docs/program.pdf (Abstract in Appendix B3).

8. **Pius Fasinu**, Heiner Seifart, Patrick JD Bouic and Rosenkranz B. In vitro investigation of herb-drug interaction potential: the influence of 15 commonly used South African medicinal herbs on CYP1A2 activity. Proceedings of the 55th Annual Academic Day, 17-18th August 2011, Tygerberg Campus, University of Stellenbosch, South Africa; Page 57. Available online. http://sun025.sun.ac.za/portal/page/portal/Health_Sciences/English/Annual_Academic_Day/Information/Programme%20Book%202011%20FINAL.pdf (Abstract in Appendix B4).

Additional Outputs from Collaborative Research (Appendix C)

Publications

9. **Pius Fasinu**, Yahya E. Choonara, Riaz A. Khan, Lisa C. Du Toit, Pradeep Kumar, Valence M. K. Ndesendo, Viness Pillay (2012). Flavonoids and polymer derivatives as CYP3A4 inhibitors for improved oral drug bioavailability. *Journal of Pharmaceutical Sciences* 2012 Nov 27. doi: 10.1002/jps.23382 [Epub ahead of print] (Abstract in Appendix C1)..

10. **Pius Fasinu** and Bernd Rosenkranz (2012). Drug-drug interactions in ageing HIV-infected individuals. *African Journal of Pharmacy and Pharmacology* 6(38):2710-2723 (Abstract in Appendix C2).

11. Xolani W. Njovane, **Pius Fasinu**, Bernd Rosenkranz. Comparative evaluation of warfarin utilization in two primary health care clinics in the Cape Town area. *Cardiovascular Journal of Africa* 2012 Dec 3. [Epub ahead of print] (Abstract in Appendix C3).

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12. **Pius Fasinu**, Viness Pillay, Valence Ndesendo, Lisa du Toit and Yahya Choonara (2011). Diverse approaches for the enhancement of oral drug bioavailability. *Biopharmaceutics and Drug Disposition*; 32:185-209 (Abstract in Appendix C4).

Conference Proceedings

13. **Pius Fasinu.** Pharmacokinetic drug interaction as a route for improving oral drug bioavailability. Proceedings of the South African Congress for Pharmacology and Toxicology, Cape Town, 3rd – 6th October, 2010; Page 45; Available online: http://www.sapharmacol.co.za/CONGRESS_Site/html_pages/Docs/abstr2010.pdf (Abstract in Appendix C5).

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List of abbreviations

Abbreviation	Description
a	Maximal transporter inhibition
ABC	ATP-binding cassette
ABCB1	ATP-binding cassette, sub-family B, member 1 (P-glycoprotein)
ABCG2	ATP-binding cassette sub-family G member 2 (also known as breast cancer resistant protein)
ADME	Absorption, distribution, metabolism and excretion
AIDS	Acquired immunodeficiency syndrome
ARV	Antiretroviral drugs
ATO	Atorvastatin
ATP	Adenosine-5'-triphosphate
AZT	Azidothymidine (zidovudine)
cat. no.	Catalog number
CLh,b	Hepatic metabolic blood clearance
CLint	Intrinsic clearance
CYP	Cytochrome P450
DDI	Drug- drug interactions
DIG	Digoxin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
ESI(+)	Electrospray ionization in positive ion mode
ESI(-)	Electrospray ionization in negative ion mode
FBS	Fetal bovine serum
FTC	Fumitremorgin C
fumic	Fraction of unbound test substance in microsomal incubations
G418	Geneticin
GIT	Gastrointestinal tract
GSH	Glutathione
GST	Glutathione *S*-transferase
HBSS	Hank's balanced salt solution
HDI	Herb-drug interaction
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLM	Human liver microsomes

Abbreviation	**Description**
HP	*Hypoxis hemerocallidea* extracts
HPLC	High performance liquid chromatography
g	Gravitational acceleration constant
I	Inhibitor concentration
IC_{50}	Concentration of crude herbal extracts that causes 50% inhibition of the enzyme/transporter activity
Imax	Maximal inhibitor concentration
Ki	Inhibitor binding constant
KI	Inhibition constant (time-dependent inhibition)
kinact	Maximal rate of enzyme inactivation (time-dependent inhibition)
Km	Intrinsic transporter affinity or Michaelis-Menten constant
Ko143	(3S,6S,12aS)-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1',2':1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester
LC-MS	Liquid chromatography - mass spectrometry
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LSC	Liquid scintillation counting
LLC-PK1	porcine kidney cell line
LOQ	Limit of quantitation
n	Hill or slope factor
N	Number of replicates
NADPH	β Nicotinamide adenine dinucleotide phosphate, reduced form
NAT	*N*-acetyl transferase
n.i.	Not investigated
n/a	Not applicable
NaOH	Sodium hydroxide
NS	Not significant
MDCKII	Madin-Darby canine kidney II cells
MDZ	Midazolam
MDR1	Multidrug-resistant protein 1/ P-glycoprotein
MRM	Multiple reaction monitoring
MS, MS/MS	Mass spectrometry, tandem mass spectrometry
MW	Molecular weight
MXR, BCRP	Mitoxantrone resistant protein / Breast cancer resistant protein
m/z	Mass-to-charge ratio
NADPH	Dihydronicotinamide adenine dinucleotide phosphate

Abbreviation	Description
NRS	NADP-regenerating system
PBS	Phosphate buffered saline
P-gp	P-glycoprotein
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-2
post ctrl	Positive control
PSapp	Overall membrane permeability
PSapp,0	Initial membrane permeability of a probe substrate at 37°C
PSapp,i	membrane permeability in the presence of test inhibitor
PSapp,p	membrane permeability in the presence of positive control inhibitor
PSm	Non-specific (passive) membrane permeability
RA	Radioactivity
rpm	Rotations per minute
RSV	Rifamycin
Rt	Retention time
S	Substrate concentration
SD	Standard deviation
SJW	St John's wort
ST	Sulfotransferase
LT	*Lessertia frutescens* extracts
$T_{1/2}$	Half-life
THP	Traditional health practitioner
UDPGA	Uridine 5'-diphospho-α-D-glucuronic acid
UGT	Uridine diphosphate glucuronosyltransferase
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
V_0	Apparent (measured) uptake velocity
V_{max}	Maximal transporter activity or velocity
$^{v}/_{v}$	Volume per volume
WHO	World Health Organization
y	Relative transporter inhibition
y_0	relative baseline inhibition

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CHAPTER ONE

INTRODUCTION TO STUDY

1.1. Background of this Study

Traditional healing is fast becoming an integral component of healthcare all over the world (Ernst, 2004). In South Africa where traditional health practitioners (THPs) (also called *sangomas*) are the most popular type of healers who practice with some governmental regulations, it is estimated that up to 85% populace use herbal remedies, usually in combinations of two or more (Van Wyk et al., 1997; Moagi, 2009). THPs often give their patients medications of plant and animal origin (Muti) for the treatment of physical and mental illness, social disharmony and spiritual difficulties (Puckree et al., 2002). The formal health sector in South Africa and other African countries have shown continued interest in the role of THPs and in the popularity of their traditional herbal remedies with the ultimate aim of total integration of traditional medicine into health care (Pretorius, 1991; Wreford, 2005; Babb et al., 2007).

While traditional medicine entails a wide range of practices, the use of medicinal herbs forms the bedrock of African traditional medical practices. Medicinal herbs contain phytochemicals which include potent pharmacologically active substances such as flavonoids, alkaloids, furanocoumarins, terpenoids, anthocyanins, phytoestrogens, hypericins, saponins and a host of others.

For example, in South Africa, one of the well documented herbs employed in traditional healing is *Callilepsis laureola*. In a study conducted by Popat and co-workers (2001), the toxicity and the potential for pharmacodynamic drug interactions with this herb were reported. Although there are limited documented pharmacokinetic herb-drug interactions between common South African medicinal herbs and prescription drugs, Malangu (2008) reported that 9% of the interviewed HIV/AIDS patients who are on antiretroviral therapy consumed herbal medications. Such figures vary from study to study, depending on the region and the population group. A recent review on the use of African herbal medicines in the treatment of HIV and other chronic diseases by Mills and co-workers (2005a) concluded that more information about the potential for drug interactions of traditional medicines used by the THPs is required. This information will be important to assess the safety of the concomitant use of traditional herbal medicines in pharmacotherapy. The necessity of

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polypharmacy in the management of most diseases further increases the risk of herb-drug interactions in patients who are on concomitant traditional medications.

One of the major steps involved in the development of new drug candidates is the verification of their potential for interaction with other drugs (Veber et al., 2002; Umehara and Camenisch, 2011). Various *in vitro* liver-based methods have been developed for such purpose. *In vitro* investigations of drug metabolism provides information regarding the metabolic stability of the test drug, its potential for possible interactions with other compounds that have affinity for the same drug-metabolizing enzymes, and indication of its oral bioavailability and toxicological potential (White, 2000; Masimirembwa et al., 2001; Veber et al., 2002). One of the best characterized and validated models for such drug metabolism studies is the use of microsomes fractionated from the human liver subcellular organelles by differential ultracentrifugation (Wrighton et al., 1993; Ekins et al., 2000). A human liver microsomal fraction contains a full complement of cytochrome P450 (CYP) which makes it a suitable tool for studying CYP-catalyzed metabolite formation and inhibitory interactions. In addition to reliability and reproducibility of *in vitro* data generated from their use, human liver microsomes (HLM) are relatively easy to prepare, commercially available and stable on prolonged storage. This has made the application of liver microsomes the most widely used in metabolic and toxicological studies (Taavitsainen, 2001). Additionally, cryopreserved hepatocytes and transfected cell lines can be used to investigate drug metabolism and transport.

Although there are various *in vitro* techniques to investigate drug metabolism, the desired clinically relevant information on drug interactions has been reliably obtained through the utilisation of HLM, cell lines and hepatocytes, the results of which provide indications of *in vivo* behaviour (Umehara and Camenisch, 2011). Such *in vitro* investigations are optimized by either measuring the rate of disappearance of substrates in incubation mixtures or the rate of formation of a specific metabolite.

The current project addressed the drug interaction potential of selected medicinal herbs used by South African THP through *in vitro* liver-based techniques.

1.2. Rationale and Motivation for this Study

There has been a high increase in the consumption of herbal remedies across South Africa despite the availability of orthodox medications. This traditional practice cuts across social classes. A large number of patients who are on ARVs, anti-tubercular drugs and other medications are also on concurrent traditional therapy. The likelihood of herb-drug interaction is believed to be higher than that of drug-drug interaction (Fugh-Berman, 2000). This is because most therapeutic agents contain single chemical entities whereas herbal medications are made of complex mixture of pharmacologically active chemical agents, even in single herbal products. In addition to the insufficient knowledge of the efficacy of traditional medicine, the risk of pharmacokinetic drug interaction poses two major extreme challenges – pharmacotoxicity and treatment failure. The former can result from the inhibition of the metabolic enzymes while the latter may be the consequence of enzymatic induction leading to faster drug metabolism.

The human CYP enzyme family is responsible for the metabolism of a wide range of clinical drugs. For the determination of appropriate dosage regimens, the pharmacokinetic profiles of the individual drugs are taken into consideration including their metabolic clearance. Many drugs are prone to pharmacokinetic drug interactions in the presence of CYP inhibitors or inducers. It is therefore important to investigate the influence of commonly used medicinal herbs on the metabolic enzymes, especially CYP. Following satisfactory correlation between *in vitro* and *in vivo* animal and human studies, there is increasing acceptance of *in vitro* metabolic studies as alternative methods which can reduce, refine or replace the use of laboratory animals as advanced and promoted by the European Centre for the Validation of Alternative Methods (ECVAM) (Kenworthy et al., 1999; Casati et al., 2005; Nair et al., 2007).

Information on the safety and HDI potential of popular western and Chinese herbal products is available in the literature (see Chapter 2). The novelty of this study is its intention to investigate the suitability of concomitant use of orthodox and traditional medicines in South Africa, the potential for herb-drug interactions and the possible influence on therapeutic efficacy or toxicity. Information in this field is currently sparse and is in great demand for regulatory policy designs and integral medical practice.

The choice of medicinal herbs used in this study is dependent on the information obtained from THPs. This is because; they are the custodian of traditional medical practices. They enjoy patronage from a number of patients. Aqueous and methanolic extracts of the medicinal herbs were used because they represent what the patients are exposed to in real-

life situations. This is necessary in order to make the findings of this study relevant to the society.

1.3. Hypothesis

The primary hypothesis to be tested in this research is that, due to the overlapping substrate specificity of CYP enzymes and transport proteins, herbal products may interact with them, thereby inhibiting their metabolic/transport activity. This effect can provide an indication of *in vivo* potential for HDI with co-administered drugs that are CYP and/or transport protein substrates.

CYP and some transport proteins are richly expressed in the human intestines and the liver. CYP-mediated metabolism has significant influence on oral drug bioavailability and the systemic elimination of xenobiotics from the body. Alteration in CYP activities may lead to significant changes in the known pharmacokinetic profiles of drugs.

It is hypothesized that components of the crude extracts of commonly used medicinal herbs in South Africa may inhibit the activity of CYP isozymes and transport proteins involved in the disposition of drugs.

1.4. Aims and Objectives

The overall aim of this study was to assess the inhibitory effect of selected traditional medications employed in the management of various diseases in South Africa on the metabolic activity of CYP isozymes responsible for the metabolic clearance of conventional drugs through *in vitro* metabolic techniques. The study also aimed to assess the inhibitory effect of selected herbal extracts on drug transporters.

1.4.1. Specific Objectives

1. To provide a literature overview of known herb-drug interactions; and the various liver-based technologies for biotransformation studies.

2. To source from South African traditional healers, common herbal medications employed in the management of diseases, select such medicinal herbs with preference for those used in chronic diseases (in reflection of long-term use).

3. To prepare crude extraction of the selected herbs in close reflection of traditional use, for the *in vitro* interaction studies.

4. To assess the inhibitory effect of the crude extracts of the identified herbs on drug metabolism employing pooled HLM which express various CYP isozymes especially CYP1A2, CYP2C9, CYP2C19 and CYP3A4.

5. To perform a more extensive *in vitro* study with crude extracts of *Lessertia frutescens* (formerly *Sutherlandia frutescens*) and *Hypoxis hemerocallidea* (the two most popular medicinal herbs used by people living with HIV/AIDS in South Africa (Mills et al., 2005a)). The study included the investigation of the inhibitory effect on CYP2A6, CYP2B6, CYP2C8, CYP2D6 and 2E1 in HLM.

6. To investigate the inhibitory effects of the crude extracts of *Lessertia* and *Hypoxis* on CYP3A4-mediated metabolism of midazolam utilizing cryopreserved hepatocytes.

7. To investigate the inhibitory effects of the crude extracts of *Lessertia* and *Hypoxis* on efflux and uptake proteins employing cell lines stably expressing these drug transport proteins.

8. To analyse and interpret such influence as observed in 3, 4, 5 and 6 above, quantifying the influence on the metabolic/transport process in terms of concentrations (IC_{50}) required to inhibit 50% of the substrate concentration; and to put such figures in medical/safety perspective.

1.5. Potential Benefits of this Study

The results of *in vitro* HDI studies are important to provide indications for *in vivo* activity and further studies. Test compounds lacking inhibitory activity on metabolic enzymes *in vitro* are not likely to inhibit metabolism *in vivo*. *In vivo* studies can be used to confirm *in vitro* findings. The knowledge of the herb-drug interaction in the management of chronic diseases such as HIV/AIDS and tuberculosis in South Africa will be important in policy designs and provide the basis to apply caution in herb-drug use advocacy. Such knowledge will provide warning signals to healthcare providers where necessary and may encourage warning against the

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concomitant use of traditional medications and conventional drugs. The outcome of this study will add to the knowledge of the safety of the co-administration of traditional remedies and orthodox medications.

1.6. Ethical Consideration

This study was approved by the University of Stellenbosch Health Research Ethics Committee with Ethics Reference number N10/09/307 (Appendix D).

1.6.1. Sourcing of medicinal herbs

Material transfer agreement (MTA) guiding the collection and transfer of traditional medicinal product from traditional medical practitioners for research purposes was made and signed by both parties under the supervision of the legal advisory department of the University of Stellenbosch (Appendix E). Informed consent from the traditional medical practitioners who supplied the herbal specimens used in this study was obtained through the administration of approved semi-structured questionnaire (Appendix F).

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CHAPTER TWO

AN OVERVIEW OF HERB-DRUG INTERACTIONS, THEIR MECHANISMS AND CLINICAL SIGNIFICANCE[1]

2.0. Summary

This chapter details the clinical implications of concurrent herb-drug use with respect to herb-drug interaction (HDI). While the potential for HDI for a number of herbal products is inferred from non-human studies, certain HDIs are well established through human studies and documented case reports. A general overview along with the significance of pharmacokinetic and pharmacodynamic HDI is provided, detailing basic mechanisms and nature of evidence available. An increased level of awareness of HDI is necessary among health professionals and drug discovery scientists. With the increasing interest in plant-sourced drugs, the potential for HDI should be assessed in the non-clinical safety assessment phase of drug development process. More clinically relevant research is required in this area as current information on HDI is insufficient for clinical applications.

[1] This Chapter is an updated version of Fasinu PS, Bouic PJ, Rosenkranz B. An overview of the evidence and mechanisms of herb-drug interactions. mechanisms of herb-drug interactions. Front Pharmacol. 2012;3:69. Epub 2012 Apr 30.

2.1. Introduction

There is increasing consumption of medicinal herbs and herbal products globally, cutting across social and racial classes, as it is observed both in developing and developed countries (Hussin, 2001; Cheng et al., 2002; Bodeker, 2007; Mitra, 2007). Medicinal plants were the major agents for primary health care for many centuries before the advent of modern medicine (Sheeja et al., 2006). Their use however declined in most developed western countries during the last century's industrialization and urbanization (Ogbonnia et al., 2008). In the past two decades a new resurgence in medicinal plants consumption was observed. According to the WHO, about 70% of the world population currently uses medicinal herbs as complementary or alternative medicine (Wills et al., 2000). It is estimated that over 40% of the adult American population consume herbal products for one medical reason or the other (Tachjian et al., 2010). A recent study involving 2055 patients in the US revealed that the consumption pattern of traditional medications has no significant gender or social difference (Kessler et al., 2001). Consumption rate has been particularly high in Canada (Calixto, 2000), Australia (Bensoussan et al, 2004), as well as Europe where the highest sales of herbal products have been reported in Germany and France (Capasso et al., 2003). In Africa, there is continuous addition to the list of medicinal herbs, while consumption rate is also increasing.

The indications for herbal remedies are diverse (Ernst, 2005). Studies have shown that 67% of women use herbs for perimenopausal symptoms, 45% use it in pregnancy and more than 45% parents give herbal medications to their children for various medical conditions (Ernst, 2004). Regulations in most countries do not require the demonstration of therapeutic efficacy, safety or quality on the part of herbal remedies, as most of them are promoted as natural and harmless (Homsy et al., 2004; Routledge, 2008). It is pertinent however, that herbs are not free from side effects as some have been shown to be toxic (Perharic et al., 1993; Popat et al., 2001; Déciga-Campos et al., 2007; Patel et al., 2011). Habitual pattern of concomitant consumption of herbal and prescription medication has been reported. Kaufman and co-workers (2002) reported that 14-16% of American adult population consume herbal supplements often concomitantly with prescribed medications, while 49.4% of Israeli consumers of herbal remedies use them with prescription drugs (Giveon et al., 2004). This is significant bearing in mind that less than 40% of patients disclose their herbal supplement usage to their health care providers coupled with the fact that many physicians are unaware of the potential risks of herb-drug interactions (HDI) (Klepser et al., 2000).

HDI is one of the most important clinical concerns in the concomitant consumption of herbs and prescription drugs (Gouws et al., 2010; Tarirai et al., 2010; Müller and Kanfer, 2011). The necessity of polypharmacy in the management of most diseases further increases the risk of HDI in patients. The ability of intestinal and hepatic CYP to metabolize numerous structurally unrelated compounds, apart from being responsible for the poor oral bioavailability of numerous drugs is responsible for the large number of documented drug-drug and drug-food interactions (Quintieri et al., 2008). This is more so, considering that oral drug delivery is the most employed in the management of most disease conditions in which case, drug interaction alters both bioavailability and pharmacokinetic disposition of the drug. This alteration and the resulting poor control of plasma drug concentrations would particularly be of concern for drugs that have a narrow therapeutic window or a precipitous dose-effect profile (Aungst, 2000; Perucca, 2006). The risk of pharmacokinetic drug interaction poses two major therapeutic challenges – pharmacotoxicity and treatment failure. The former can result from the inhibition of the metabolic enzymes responsible for the metabolism and clearance of the drugs, while the latter may be the consequence of enzymatic induction leading to faster drug metabolism. This is in addition to the intrinsic pharmacodynamic actions of the herbal products themselves which may include potentiating, additive, antagonistic or neutralization effects.

Until recently, HDI was often unsuspected by physicians for several reasons. Most physicians lack adequate knowledge on herbal drugs and their potential for drug interactions (Suchard et al., 2004; Clement et al., 2005, Ozcakir et al., 2007; Fakeye and Onyemadu, 2008); herbal products also vary considerably in composition depending on the source and package (Liang et al, 2004; Sousa et al., 2011); most patients do not consider it necessary to disclose their herbal consumptions to physicians who themselves hardly inquire such (Cassidy, 2003; Howell et al., 2006; Kennedy et al., 2008; Chao et al., 2008). Further challenges with herbal medications include scientific misidentification, product contamination and adulteration, mislabeling, active ingredient instability, variability in collection procedures and failure of disclosure on the part of patients (Boullata and Nace, 2000). A fairly recent systematic review by Izzo and Ernst (2009) on the interactions between medicinal herbs and prescribed medications provides some more details on these.

Herbal products are made of complex mixtures of pharmacologically active phytochemicals (Mok and Chau, 2006), most of which are secondary metabolites generated through the shikimate, acetate-malonate and acetate-mevalonate pathways. These constituents include phenolics, (such as tannins, lignins, quinolones and salicylates), phenolic glycosides (such as flavonoids, cyanogens and glucosinolates), terpenoids (such as sesquiterpenes, steroids,

carotenoids, saponins and iridoids), alkaloids, peptides, polysaccharides (such as gums and mucilages), resins and essential oils which often contain some of the aforementioned classes of phytochemicals (Wills et al., 2000; Wang et al., 2008). This complexity increases the risk of clinical drug interactions.

2.2. Aim, Search Strategy and Selection Criteria

The current review is aimed at providing an overview of known and recently reported HDI with interest in the evidence available and the mechanism thereof. The review was systematically conducted by searching the databases of MEDLINE, PUBMED, EMBASE and COCHRANE libraries for original researches, and case reports on HDI using the following search terms or combinations thereof: 'drug-herb', 'herb-drug', 'interaction', 'cytochrome P450', 'plant', 'extract', 'medicinal' 'concomitant administration', 'herbal and orthodox medicines'. Relevant search terms were employed to accommodate the various individual medicinal herbs employed in Africa, America, Asia, Europe and Australia. The reported interactions and their mechanisms, with orthodox medications were searched and collated. Searches were not limited by date or place of publications, but to publications available in English language.

2.3. Results

2.3.1. Clinical presentation of herb-drug interactions

Clinical presentations of HDI vary widely depending on the herbs and the drugs concerned. Examples for relevant clinical presentations of HDI include the potentiation of the effects of oral corticosteroids in the presence of liquorice (*Glycyrrhiza glabra*) (Liao et al., 2010); potentiation of warfarin effects with resultant bleeding in the presence of garlic (*Allium sativum*) (Borrelli et al., 2007), dong quai (*Angelica sinensis*) (Page and Lawrence, 1999; Nutescu et al., 2006) or danshen (*Salvia miltiorrhiza*) (Chan, 2001); decreased blood levels of nevirapine, amitriptyline, nifedipine, statins, digoxin, theophyline, cyclosporine, midazolam and steroids in patients concurrently consuming St John's wort (SJW) (*Hypericum perforatum*) (De Maat et al., 2001; Henderson et al., 2002; Johne et al., 2002; Mannel, 2004; Borrelli and Izzo, et al., 2009), decreased oral bioavailability of prednisolone in the presence of the Chinese herbal product xiao-chai-hu tang (sho-saiko-to) (Fugh-Berman, 2000); ginseng (*Panaz ginseng*)-induced mania in patients on antidepressants (Engelberg et al.,

2001); production of extrapyramidal effects as a result of the combination of neuroleptic drugs with betel nut (*Areca catechu*) (Huang et al., 2003; Coppola and Mondola, 2011); increased blood pressure induced by tricyclic antidepressant-yohimbine (*Pausinystalia yohimbe*) combination (Tam et al., 2001), increased phenytoin clearance and frequent seizures when combined with Ayurvedic syrup shankhapushpi (Patsalos and Perucca, 2003), among other clinical manifestations. These clinical presentations reflect the mechanism the HDI – inhibition or induction of drug metabolic and/or transport pathways.

2.3.2. Evidence-based HDI studies and clinical relevance

HDIs have been reported through various study techniques. While these reports usually give evidence of potential interactions, the level of evidence varies often failing to predict the magnitude or clinical significance of such HDI. Apart from the specific limitations attributable to study methods employed (Table 1), major draw-backs in deducting relevant conclusions from reported HDI include misidentification and poor characterization of specimen, presence and nature of adulterants (some of which may be allergens), variations in study methodologies including extraction procedures, source location of herbs involved, seasonal variation in the phytochemical composition of herbal materials, under-reporting and genetic factors involved in drug absorption, metabolism and dynamics. Table 1 provides some limitations of the methods commonly applied in drug metabolism studies.

Table 1: Comparison of study methods available for HDI

Report/Study method	Comments	Advantages	Limitations to clinical inferences
In vitro studies	Usually involves deliberate investigations employing metabolic enzymes, tissues or organs e.g. CYP-transfected cell lines, hepatic subcellular fractions, liver slices, intestinal tissues	Provides information on potential HDI, easy to perform, good for high throughput screenings; Compared to *in vivo* animal studies, results are closer to human if human liver-based technologies are employed	Concentrations used in study may be at significant variance with clinical concentrations; other *in vivo* phenomena like protein binding and bioavailability are not accounted for; poor reproducibility of results; poor correlation to clinical situation
Non-clinical *in vivo* studies	Usually involves metabolic studies in animals, especially mammals	Concentration and bioavailability of active components are taken into consideration	Results are often difficult to interpret due to species variation; most results are from disproportionate and non-physiologic dosages
Case Reports	Patients diagnosed with HDI after history taking	Ideal in providing information on HDI	Hardly discovered by physicians; infrequent and under-reporting; difficult to ascertain the identity of the herb responsible for HDI in multiple herbal use
Human studies	Involves the use of human subjects	The ideal study, providing directly extrapolative data on interactions	Generally costly with stringent ethical considerations; most subjects are healthy leaving out the effects of pathologies on drug metabolism; genetic variation in enzyme activity

Recently, structured assessment procedures have been emerging in an attempt to provide levels of evidence for drug interactions. In addition to evidence of interaction, such assessments take into consideration the clinical relevance of the potential adverse event resulting from the interaction, the modification- and patient-specific risk factors, and disease conditions for which the interaction is important. Van Roon and co-workers (2005) developed a system of hierarchical evidence-based structured assessment procedure of drug-drug interaction. This can be applicable to HDI. This method particularly allows the extraction of HDIs that have been well established and those that are merely inferred from certain phytochemical characteristics. A modified form of this method as presented in Table 2 is applied in this literature review to provide the nature and level of evidence for the HDIs mentioned.

Table 2: Quality of HDI evidence for clinical risk assessment

Level	Description of evidence
1	Published theoretical proof or expert opinion on the possibility of HDI due to certain factors including the presence of known interacting phytochemicals in the herbs, structure activity relationship
2	Pharmacodynamic and/or pharmacokinetic animal studies; *in vitro* studies with a limited predictive value for human *in vivo* situation
3	Well documented, published case reports with the absence of other explaining factors
4	Controlled, published interaction studies in patients or healthy volunteers with surrogate or clinically relevant endpoint

2.3.3. Mechanisms of herb-drug interactions

The overlapping substrate specificity in the biotransformational pathways of the physiologic systems is seen as the major reason for drug-drug and HDI (Marchetti et al., 2007). The ability of different chemical moieties to interact with receptor sites and alter physiological environment can explain pharmacodynamic drug interactions, while pharmacokinetic interactions arise from altered absorption, interference in distribution pattern as well as changes and competition in the metabolic and excretory pathways (Izzo, 2005). The major underlying mechanism of pharmacokinetic HDI, like drug-drug interaction, is either the induction or inhibition of intestinal and hepatic metabolic enzymes particularly the CYP enzyme family. Additionally, similar effect on drug transporters and efflux proteins particularly the p-glycoproteins (P-gp) in the intestines is responsible in most other cases (Meijerman et al., 2006; Nowack, 2008; Farkas et al., 2010). The presystemic activity of CYP and efflux proteins often influence oral bioavailability, thus the modulating activity of co-administered herbal products has been shown to result in pronounced reduction or increase in the blood levels of the affected drugs (Brown et al., 2008).

Potential for *in vivo* drug interactions are often inferred from *in vitro* studies with liver enzymes. *In vitro* results of HDI studies have provided indications of *in vivo* occurrence, although, the extent of clinical significance is poorly inferable (Rostami-Hodjegan and Tucker, 2007; Iwamoto et al., 2008; Xu et al., 2009; Umehara and Camenisch, 2011). Thus

most of the well-established HDIs, as will be seen in subsequent sections, were initially demonstrated through *in vitro* studies and confirmed *in vivo*.

The interaction of herbal products with hepatic enzymes can also result in hepatic toxicity (van den Bout-van den Beukel et al., 2008; Nivitabishekam et al., 2009; Dasgupta et al., 2010; Asdaq and Inamdar, 2010; Kim et al., 2010a.) Specific liver injury inducible by phytochemical agents includes elevation in transaminases (Zhu et al., 2004; Saleem et al., 2010), acute and chronic hepatitis (Stedman 2002; Pierard et al., 2009), liver failure (Durazo et al., 2004), veno-occlusive disorders (DeLeve et al., 2002), liver cirrhosis (Lewis et al., 2006), fibrosis (Chitturi and Farrell, 2000), cholestasis (Chitturi and Farrell, 2008), zonal or diffusive hepatic necrosis (Savvidou et al., 2007), and steatosis (Wang et al., 2009). Mechanisms of liver injury include bioactivation of CYP, oxidative stress, mitochondrial injury and apoptosis (Cullen, 2005).

2.3.3.1. Induction and Inhibition of metabolic enzymes

The CYP superfamily is generally involved in oxidative, peroxidative and reductive biotransformation of xenobiotics and endogenous compounds (Nebert and Russell, 2002; Hiratsuka, 2011). It is conventionally divided into families and subfamilies based on nucleotide sequence homology (Omura and Sato, 1962; Fasinu et al., 2012). There is a high degree of substrate specificity among the various families. CYP belonging to the families 1, 2 and 3 are principally involved in xenobiotic metabolism, while others play a major role in the formation and elimination of endogenous compounds such as hormones, bile acids and fatty acids (Norlin and Wikvall, 2007; Amacher, 2010). The most important CYP subfamilies responsible for drug metabolism in humans are 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5 (Ono et al., 1996; Wang and Chou, 2010).

Induction is the increase in intestinal and hepatic enzyme activity as a result of increased mRNA transcription leading to protein levels higher than normal physiologic values (Amacher, 2010). When this happens, there is a corresponding increase in the rate of drug metabolism affecting both the oral bioavailability and the systemic disposition (Argikar et al., 2009). In the formulation and dosage design of oral medications, allowance is often made for pre-systemic metabolism in order to achieve predictable systemic bioavailability. A disruption in this balance can result in significant changes in blood concentrations of the drugs. As shown in subsequent sections, some herbal products have been shown to be capable of inducing CYP. Concomitant administration of enzyme-inducing herbal products and

prescription drugs can therefore result in sub-therapeutic plasma levels of the latter with therapeutic failure as a possible clinical consequence.

Apart from enzyme induction, herbal products can also inhibit enzyme activities. The inhibition of CYP and other metabolic enzymes is usually competitive with instantaneous and inhibitor concentration-dependent effects (Zhang and Wong, 2005). Most inhibitors are also substrates of CYP (Zhou, 2008). This phenomenon alters pharmacokinetic profiles of xenobiotics significantly. As a result of the suppression of the anticipated pre-systemic intestinal and hepatic metabolism, unusually high plasma levels of xenobiotics are observed. Toxic manifestation could be the ultimate effect of this observation. An equally clinically important consequence of enzyme inhibition is drug accumulation due to subdued hepatic clearance. These effects will be of particular concern in drugs with narrow therapeutic window or steep dose-response profiles.

SJW is widely used for its antidepressant properties (Lawvere and Mahoney, 2005; Høyland, 2011). It is a potent inducer of CYP3A4 and depending on the dose, duration and route of administration, it may induce or inhibit other CYP isozymes and P-gp (Markowitz et al., 2003b; Roby et al., 2000; Tannergren et al., 2004; Komoroski et al., 2004; Madabushi et al., 2006). Studies from case reports indicate that, due to its inducing effects on CYP3A4, it significantly reduces the plasma levels of CYP3A4 substrates including cyclosporine, simvastatin, indinavir, warfarin, amitriptyline, tacrolimus, oxycodone and nevirapine (Johne et al., 2002; Henderson et al., 2002; Nieminen et al., 2010; Vlachojannis et al., 2011). It has been reported that the alteration in the blood serum concentration of cyclosporine due to SJW has led to organ rejection in patients (Ernst, 2002; Murakami et al., 2006). Reports of breakthrough bleeding and unplanned pregnancies due to interaction between SJW and oral contraceptives have been documented (Hu et al., 2005). The group of drugs with a high potential for clinically significant pharmacokinetic drug interaction with SJW is the antidepressants as SJW itself is consumed by patients with depression. Its concomitant use with SSRIs like sertraline and paroxetine has been reported to result in symptoms of central serotonergic syndrome (Barbenel et al., 2000; Dannawi, 2002; Spinella and Eaton, 2002; Birmes et al., 2003; Bonetto et al., 2007). Amitriptyline is a substrate to both CYP3A4 and intestinal P-gp. The risk of therapeutic failure is thus high due to induction of CYP3A4-dependent metabolism activities resulting in poor oral bioavailability. In a study by Johne and co-workers (2002), a 21% decrease in the area under the plasma concentration-time curve of amitriptyline was observed in 12 depressed patients who were concomitantly administered with extracts of SJW and amitriptyline for 2 weeks.

It has also been said to increase the incidence of hypoglycemia in patients on tolbutamide without apparent alteration in the pharmacokinetic profile of tolbutamide (Mannel, 2004). It also inhibits the production of SN-38, an active metabolite of irinotecan, in cancer patients (Caraci et al., 2011).

Other CYP and P-gp substrates whose pharmacokinetic profile have been altered by SJW include anticoagulants like phenprocoumon and warfarin; antihistamines like fexofenadine; antiretroviral drugs including protease inhibitors and reverse transcriptase inhibitors; hypoglycemic agents such as tolbutamide; immunosuppressants like cyclosporine, tacrolimus and mycophenolic acid; anticonvulsants such as carbamazepine; anticancer like irinotecan; bronchodilators like theophylline; antitussives like dextromethorphan; cardiovascular drugs like statins, digoxin and dihydropyridine calcium channel blockers; oral contraceptives; opiates like methadone and loperamide; and benzodiazepines including alprazolam and midazolam (Greeson et al., 2001; Di et al., 2008; Hojo et al., 2011).

Following a single dose administration of 300mg standardized extracts of SJW containing 5% hyperforin to humans, a maximum plasma concentration of 0.17-0.5μM hyperforin was obtained. Compared with *in vitro* inductive concentrations, *in vivo* extrapolation suggests a high possibility of *in vivo* pharmacokinetic drug interaction (Agrosi et al., 2000). Bray and co-workers (2002) confirmed through animal studies that SJW modulates various CYP enzymes. Dresser and co-workers (2007) demonstrated that SJW is capable of inducing CYP3A4 in healthy subjects through the observation of increased clearance of midazolam. Thus animal and human studies further confirm SJW as containing both inhibitory and inducing constituents on various CYP isozymes. These effects depend on dosage and duration of administration, and are species- and tissue-specific. While the individual phytochemical constituents of SJW have elicited varying effects on the metabolic activity of the CYP isozymes, whole extracts and major constituents especially hyperforin have been reported to induce the metabolic activities of CYP1A2, 2C9, 2C19, 2D6 and 3A4 as shown in *in vitro* studies and *in vivo* studies (Lee et al., 2006; Madabushi et al., 2006, Hokkanen et al., 2011).

Ginkgo biloba have been reported to induce CYP 2C19-dependent omeprazole metabolism in healthy human subjects (Yin et al., 2004). Piscitelli and co-workers in a garlic-saquinavir interaction study reported 51% decrease in saquinavir oral bioavailability caused by the presence of garlic and attributable to garlic-induced CYP3A4 induction (Pisciteli et al., 2002). Its effects on the pharmacokinetics of warfarin have been reported in animal models (Taki et al., 2011)

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Although grapefruit juice is not consumed for medicinal purposes, the discovery of the inhibitory activity of its flavonoid contents on CYP has led to further researches in medicinal herbs which have revealed HDI potentials in flavonoid-containing herbal remedies (Choi and Burm, 2006; Palombo, 2006; Paine et al., 2008; Quintieri et al., 2008, Alvarez et al., 2010). A related CYP inhibitor is rotenone. By interfering with the electron transfer of the heme iron, rotenone, a naturally occurring phytochemical found in several plants such as the jicama vine plant is known to inhibit CYP activity (Sanderson et al., 2004). Resveratrol, a natural polymer, and tryptophan, an amino acid have been documented as potent CYP inhibitors (Rannug et al., 2006). Some herbal medications and their phytochemical constituents capable of interacting with CYP are presented in Table 3. A more detailed involvement of CYP in HDI is detailed in some recently published reviews (Delgoda and Westlake, 2004; Pal and Mitra, 2006; Cordia and Steenkamp, 2011; Liu et al., 2011).

Table 3: Some herbal products known to interact with CYP and efflux proteins

Medicinal Plant and parts used	Scientific name	Major constituents	Mechanism of drug interactions	Candidates for interactions	LE[1]	References
Cranberry (fruit extract)	*Vaccinium macrocarpon*	Anthocyanins, flavonoids	Inhibition of CYP enzymes and P-gp	Warfarin, CYP1A2, 2C9 and 3A4 substrates	4[2]	Uesawa and Mohri, 2006; Lilja et al., 2007; Li et al., 2009; Kim et al., 2010b; Roberts and Flanagan, 2011; Hamann et al., 2011
Dong quai (root)	*Angelica sinensis*	Flavonoids, coumarins	Inhibition of CYP1A2, 3A4 and P-gp	CYP substrates	3	Page and Lawrence, 1999; Scott and Elmer, 2002; Tang et al., 2006; Sevior et al., 2010
Gan cao (root)	*Glycyrrhiza uralensis*	Glycyrrhizin	CYP2C9 and 3A4 induction	Warfarin, lidocaine, CYP2C9 and 3A4 substrates	2	Mu et al., 2006; Tang et al., 2009
Garlic (bulb)	*Allium sativum*	Allicin, phytoncide	CYP 3A4 and P-gp induction	Saquinavir, warfarin, CYP2D6 and 3A4 substrates	4[3]	Piscitelli et al., 2002; Markowitz et al., 2003a; Cox et al., 2006; Berginc and Kristl, 2012
Germander (leaves)	*Teucrium chamaedrys*	Saponins, flavonoids, diterpenoids	Production of toxic CYP3A4-induced metabolites	CYP3A4 inducers like phenobarbital, rifampicin	3	De Berardinis, et al., 2000; Savvidou et al., 2007
Ginseng (root)	*Panax ginseng*	Ginsenosides	Inhibition and induction of CYP2C9, 2C19, 2D6 and 3A4 activity	Imatinib, CYP2E1 and 2D6 substrates	4[4]	Gurley et al., 2005a; Bilgi et al., 2010; Malati et al., 2011
Grape seed (seed oil)	*Vitis vinifera*	Proanthocyanidin, resveratrol	Decreased CYP2C19, 2D6 and 3A4 activity	CYP2C19, 2D6 and 3A4 substrates	4[5]	Nishikawa et al., 2004
Kava kava (root)	*Piper methysti cum*	Kavalactones	Decreased CYP1A2, 2D6, 2E1 and 3A4 activity	CYP substrates	4[6]	Gurley et al., 2005b; Teschke, 2010; Sarris et al., 2011
Liquorice (root)	*Glycyrrhizia glabra*	Glycyrrhizic acid	Inhibition of CYP2B6, 2C9 and 3A4	CYP2B6, 2C9 and 3A4 substrates	4[7]	Kent et al., 2002; Al-Deeb et al., 2010; Methlie et al., 2011
St John's wort (aerial parts)	*Hypericum perforatum*	Hyperforin, hypericin, flavonoids	Inhibition and induction of CYP and P-gp	Orally administered CYP substrates	4[8]	Johne et al., 2002; Komoroski et al., 2004 Hu et al., 2005; Hafner et al., 2009; Lau et al., 2011

[1]LE – Level of evidence; [2]A daily consumption of 1 cup of cranberry juice for 3 days; [3]Garlic extract (3x 600 mg twice daily of for 14 days); [4]Daily ingestion of *Panax ginseng* via energy drinks for the past 3 months; [5]Continuous ingestion of grape seed over time; [6]28-day administration; [7]3-day administration; [8]Single 300 mg dose

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Phase II metabolic enzymes including uridine diphosphoglucuronosyl transferase (UGT), *N*-acetyl transferase (NAT), glutathione *S*-transferase (GST) and sulfotransferase (ST) catalyse the attachment of polar and ionizable groups to phase I metabolites aiding their elimination. While cytochrome P450-mediated HDI have been extensively investigated in various studies, the effects of herbal extracts on phase II enzymes have not been adequately studied. However, there is sufficient evidence to suggest the potential of phase II enzymes to induce clinically significant HDI.

In a study carried out in rat models by Sheweita and co-workers (2002), extracts of hypoglycemic herbs, *Cymbopogon proximus, Zygophylum coccineum* and *Lipinus albus* reduced the activity of GST and GSH. Curcumin, from *Curcuma longa*, an herbal antioxidant with anti-inflammatory and antitumor properties increased the activity of GST and quinone reductase in the ddY mice liver (Iqbal et al., 2003). Valerian, a herbal sleeping aid has demonstrated the potential of inducing HDI through the inhibition of UGT. Up to 87% of inhibition of UGT activity by valerian extract was reported in an *in vitro* study utilizing oestradiol and morphine as probe substrates (Alkharf and Frye, 2007). Kampo, a traditional Japanese medicine made of a mixture of several medicinal herbs has shown inhibitory effects on some phase II enzymes. In an *in vitro* study by Nakagawa and co-workers (2009), nine out of 51 components of kampo medicine elicited more than 50% inhibition of UGT2B7-mediated morphine 3-glucuronidation. In the same study, extracts of kanzo (*Glycyrrhizae radix*), daio (*Rhei rhizona*) and keihi (*Cinnamomi cortex*) elicited more than 80% inhibition of morphine AZT glucuronidation. This result agrees with Katoh et al., (2009) who carried out similar studies on rhei, keihi and ogon (*Scutellariae radix*).

Apart from the well-known effects of *Ginkgo biloba* on CYP enzymes as illustrated earlier (section 2.3.3.1), its extracts have demonstrated potent inhibition of mycophenolic acid glucuronidation investigated in human liver and intestinal microsomes (Mohamed and Frye, 2010).

In a study to investigate the influence of 18 herbal remedies on the activity of human recombinant sulfotransferase 1A3 employing dopamine and ritodrine as substrates, extracts of grape seed, milk thistle, gymnema, SJW, ginkgo leaf, banaba, rafuma and peanut seed coat showed potent inhibition with IC_{50} values lower than putative gastrointestinal concentrations (Nagai et al., 2009). Similarly, Mohamed and Frye (2011a) reported the inhibition of UGT1A4 by green tea derived epigallocathechin gallate; of UGT 1A6 and UGT1A9 by milk thistle; of UGT1A6 by saw palmetto; and of UGT 1A9 by cranberry. A recent publication presents evidence of potential HDI mediated by UGT (Mohamed and Frye, 2011b).

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Certain phytochemicals including coumarin, limettin, auraptene, angelicin, bergamottin, imperatorin and isopimpinellin have been reported to be capable of inducing hepatic GST activities (Kleiner et al., 2008). While the clinical significance of these findings is yet to be determined, it is noteworthy that phase II metabolic enzymes may play significant roles in HDIs.

2.3.3.2. Inhibition and induction of transport and efflux proteins

The ATP-binding cassette (ABC) family of drug transporters plays significant roles in the absorption, distribution and elimination of drugs. P-gp, the most studied member of this family is a 170-kDa plasma glycoprotein encoded by the human MDR1 gene (also known as ATP-binding cassette, sub-family B, member 1 (ABCB1)). It is constitutively expressed in a number of body tissues and concentrated on the apical epithelial surfaces of the bile canaliculi of the liver, the proximal tubules of the kidneys, the pancreatic ductal cells, the columnar mucosal cells of the small intestine, colon and the adrenal glands (Marzolini et al., 2004; Degorter et al., 2011). It is actively involved in drug absorption, distribution and elimination from the intestines the liver, kidneys and the brain. Specifically these proteins are involved in the processes of hepatobiliary, direct intestinal and urinary excretion of drugs and their metabolites (Szakács et al., 2008). Thus, the modulation of P-gp or competitive affinity as substrates for its binding sites by co-administered herbs presents a potential for alteration in the pharmacokinetic profile of the drug.

Pharmacokinetic interaction occurs when herbal drugs inhibit or decrease the normal activity level of drug transporters through a competitive or non-competitive mechanism. Interactions can also occur through the induction of transport proteins via the increase of the mRNA of the relevant protein. Studies have identified a number of clinically important P-gp inhibitors including phytochemicals – flavonoids, furanocoumarins, reserpine, quinidine, yohimbine, vincristine, vinblastine among others (Krishna and Mayer, 2001; Zhou et al., 2004; Patanasethanont et al., 2007; Iwanaga et al., 2010; Eichhorn and Efferth, 2011; Yu et al., 2011). Borrel and co-workers (1994) reported that mobile ionophores such as valinomycin, nonactin, nigericin, monencin, calcimycin, and lasalocid inhibit the efflux of anthracycline by P-gp whereas channel-forming ionophores such as gramicidin do not (Larsen et al., 2000). A number of herbal products which interact with CYP also have similar effects on transport proteins (Table 3). The transport proteins are actively involved in the pharmacokinetics of

anti-cancer drugs and account for one of the well-known mechanisms of multiple resistance of cancerous cells to chemotherapeutic agents (Bebawy and Sze, 2008; Bosch, 2008; He et al., 2011). The influence of some herbs on transport proteins with respect to anti-cancer drugs is presented in Table 4. Clinically relevant interactions between herbal medicine and chemotherapeutic agents are detailed in a recent review by Yap and co-workers (2010).

Table 4: Influence of herbal products on transport proteins

Drug transporter	Anti-cancer substrates	Interacting herbal products	LE*	References
P-glycoprotein (ABCB-1, MDR-1)	Actinomycin D, daunorubicin, docetaxel, doxorubicin, etoposide, irinotecan, mitoxantrone, paclitaxel, teniposide, topotecan, vinblastine, vincristine, tamoxifen, mitomycin C, tipifarnib, epirubicin, bisantrene	*Rosmarinus officinalis*	2	Oluwatuyi et al., 2004; Nabekura et al., 2010
MRP-1 (ABCC-1)	Etoposide, teniposide, vincritine, vinblastine, doxorubicin, daunorubicin, epirubicin, idarubicin, topotecan, irinotecan, mitoxantrone, chlorambucil, methotrexate, melphalan	*Curcuma longa*	2	Shukla et al., 2009
MRP-2 (ABCC-2)	SN-38G (metabolite of irinotecan), methotrexate, sulfinpyrazone, vinblastine	*Inchin-ko-to*	2	Okada et al, 2007
BCRP (ABCG-2, MXR)	9-aminocamptothecin, daunorubicin, epirubicin, etoposide, lurtotecan, mitoxantrone, SN-38, topotecan	Flavonoid-containing herbs such as *Glycine max* (soybean), *Gymnema sylvestre* and *Cimicifuga racemosa* (black cohosh)	2	Merino et al., 2010; Tamaki et al., 2010

*LE = Level of evidence
ABC = ATP-binding cassette; BCRP = breast cancer resistance protein; MDR = multidrug resistance gene; MRP = multidrug resistance-associated protein; MXR = mitoxantrone resistance-associated protein

2.3.3.3 Alteration of gastrointestinal functions

Besides their influence on the intestinal metabolic enzymes and efflux proteins, herbal medications can alter the absorption of concomitantly administered medicines through a number of mechanisms. Changes in the gastrointestinal pH and other biochemical factors

can alter dissolution properties and the absorption of pH-dependent drugs such as ketoconazole and itraconazole. Complexation and chelation, leading to the formation of insoluble complexes and competition at the sites of absorption especially with site-specific formulations can greatly affect the absorption of medicines. Anthranoid-containing plants – cassia (*Cassia senna*), Cascara (*Rhamnus purshiana*), rhubarb (*Rheum officinale*) and soluble fibres including guar gum and psyllium can decrease drug absorption by decreasing GI transit time. They are known to increase GIT motility. During concomitant use with prescribed medication, significant alteration in the absorption of the latter has been reported due to decreased GI transit time (Fugh-Berman, 2000).

Izzo and others (1997) demonstrated that anthranoids could be harmful to the gut epithelium by inhibiting Na^+/K^+ ATPase and by increasing the activity of nitric oxide synthase. This significantly increased intestinal transit due to the alteration in the intestinal water and salt absorption and the subsequent fluid accumulation. In a study conducted by Munday and Munday (1999), a garlic-derived compound was shown to increase the tissue activities of quinone reductase and glutathione transferase in the gastrointestinal tract of the rat. In view of their roles in metabolism, both enzymes are considered chemoprotective especially from chemical carcinogens. In addition to CYP and P-gp mediated mechanisms, ginseng may cause pharmacokinetic HDI through its inhibitory effects on gastric secretion (Suzuki et al., 1991). The potential of rhein and danthron to increase the absorption of furosemide, a poorly water-soluble drug, has been demonstrated through *in vitro* studies (Laitinen et al., 2007). In a study carried out in mice, a Chinese herbal plant, *Polygonum paleaceum*, showed the potential to depress the motility of the gastrointestinal tract, inhibit defecation reflex and delay gastric emptying (Zhang, 2002). A similar study demonstrated the inhibitory effects of two Chinese traditional herbal prescriptions, *Fructus aurantii immaturus* and *Radix paeoniae alba* on gastrointestinal movement (Fang et al., 2009).

The absorption of drugs such as phenoxymethylpenicillin, metformin, glibenclamide and lovastatin may be reduced by high-fiber herbal products through the sequestration of bile acids (Colalto, 2010). Mochiki and co-workers (2010) reported the ability of *kampo*, a traditional Japanese medicine, to stimulate elevated intestinal blood flow, and to induce increased secretion of gastrointestinal hormones including motilin, vasoactive intestinal peptide, and calcitonin gene-related peptide. Similarly, another traditional Japanese medicine has been shown to increase the intestinal secretion of ghrelin, a hunger-related hormone, leading to delayed gastric emptying (Tokita et al., 2007; Kawahara et al., 2009; Hattori, 2010; Matsumura et al., 2010). Also, Qi and co-workers (2007) demonstrated the capability of Da-Cheng-Qi-Tang, a traditional Chinese herbal formula, to increase plasma

motilin, enhance gastrointestinal motility, improve gastric dysrhythmia, and reduce gastroparesis after abdominal surgery. These effects have the potential of reducing the intestinal transit time of concurrently administered drug, with the risk of reduced absorption.

2.3.3.4. Alteration in renal elimination

This involves herbal products capable of interacting with renal function, leading to altered renal elimination of drugs. Such interaction can result from the inhibition of tubular secretion, tubular reabsorption or interference with glomerular filtration (Isnardd et al., 2004). In addition to this group of herbal products are those products consumed as diuretics. The mechanism of herbal diuresis is complex and non-uniform. Certain herbs increase the glomerular filtration rate but do not stimulate electrolyte secretion while some others act as direct tubular irritants (Crosby et al., 2001; Al-Ali et al., 2003; Musabayane et al., 2005). Some herbs capable of interacting with renal function and drug elimination are presented in Table 5.

Table 5: Some herbal remedies capable of interacting with other drugs via alteration in renal functions

Medicinal plants	Brief description	Mechanism	LE	References
Aristolochia fangchi	A Chinese weight loss/slimming herbal remedy	It contains aristolochic acid which forms DNA adducts in renal tissues leading to extensive loss of cortical tubules	4[1]	Martinez et al., 2002; Lai et al., 2010
Djenkol bean (*Pithecellobium lobatum*)	A pungent smelling edible fruit, used for medicinal purposes in Africa	It contains djenkolic acid which is known to be nephrotoxic	3	Luyckx and Naicker, 2008; Markell, 2010
Impila (*Callilepis laureola*)	A South African herb whose root is used in traditional medicine	Its consumption has been shown to cause damage to the proximal convoluted tubules and the loop of henle, shown to be hepatotoxic	3	Steenkamp and Stewart, 2005
Licorice root (*Glycyrrhiza glabra*)	A herbaceous and leguminous plant native to Europe and parts of Asia, whose root and extracts are used in chronic hepatitis and other ailments	Glycyrrhizic acid in licorice root is hydrolyzed to glycyrrhetenic acid which is an inhibitor of renal 11-hydroxysteroid dehydrogenase leading to a pseudoaldosterone- like effect. The resulting accumulation of cortisol in the kidney stimulates the aldosterone receptors in cells of the cortical leading to increased BP, sodium retention and hypokalaemia. This may potentiate the action of drugs such as digoxin	4[1]	Isbrucker and Burdock 2006; Kataya et al., 2011
Noni fruit (*Morinda citrifolia*), alfalfa (*Medicago sativa*), Dandelion (*Taraxacum officinale*), horsetail (*Equisetum arvense*), stinging nettle (*Urtica dioica*)	These plants and their extracts are used variously in traditional medicine, and have been shown to contain very high potassium levels	Hyperkalemic, hepatotoxic	3	Mueller et al., 2000; Saxena and Panbotra, 2003; Stadlbauer et al., 2005; Jha, 2010
Rhubarb (*Rheum officinale*)	Used as laxative	High oxalic acid content may precipitate renal stone formation and other renal disorders	1	Bihl and Meyers, 2001
Star fruit (*Averrhoa carambola*)	A tree popular in Southeast Asia and South America employed traditionally as antioxidant and antimicrobial	Oxalate nephropathy		Chen et al., 2001; Wu et al., 2011
Uva ursi (*Arctostaphylos uva ursi*), goldenrod (*Solidago virgaurea*), dandelion (*Taraxacum officinale*), juniper berry (*Juniperus communis*), horsetail (*Equisetum arvense*), lovage root (*Levisticum officinale*), parsley (*Petroselinum crispum*), asparagus root (*Asparagus officinalis*), stinging nettle leaf (*Urtica dioica*), alfalfa (*Medicago sativa*)	Various plants used as diuretics.	Plants have diuretic properties* and may increase the renal elimination of other drugs	1	Dearing et al., 2001; Wojcikowski et al., 2009
Wild mushrooms	Some species of widely consumed wild mushroom especially C*ortinarius* have been shown to be nephrotoxic	Contain orellanine shown to be nephrotoxic	3	Mount et al., 2002; Wolf-Hall, 2010

*Some of these herbs exert their diuretic effects via extra-renal mechanisms with no direct effects on the kidneys (see Dearing et al., 2001); [1]Long-term use;
LE = Level of evidence

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2.3.3.5. Pharmacodynamic synergy, addition and antagonism

HDI can occur through the synergistic or additive actions of herbal products with conventional medications as a result of affinities for common receptor sites (Ma et al., 2009). This can precipitate pharmacodynamic toxicity or antagonistic effects (Table 6). Like most other herbs, SJW contains complex mixture of phytochemicals including phenylpropanes, naphthodanthrones, acylphloglucinols, flavonoids, flavanol glycosides and biflavones. Hyperforin is known to inhibit the reuptake of neurotransmitters (dopamine, serotonin, and noradrenalin) and is believed to be the main bioactive moiety responsible for the antidepressant activity of SJW.

Table 6: Examples of pharmacodynamic interactions between herbal products and conventional drugs

Medicinal plant	Major active ingredients	Indications	Mechanism of action	Drug candidates for potential interactions	LE	References
*Adonis vernalis (*pheasant's eye, red chamomile)	Adoniside	Cardiotonic	Cardiostimulant	Cardiovascular drugs	1	Lange, 2000
Allium sativum (Garlic)	Allins	Hypercholesterolemia, prevention of arteriosclerosis	Antihypertensive, antidiabetic, antiplatelet, antilipidemic	Propranolol, antihypertensives, hypoglycemic agents, anticoagulants	3	Bernajee and Maulik, 2002;Asdaq et al., 2009; Asdaq and Inamdar, 2011
Anabasis sphylla	Anabesine	Skeletal muscle relaxant	Nicotinic receptor agonist which at high doses produces a depolarizing block of nerve transmission	Muscle relaxants	1	Taylor, 2000
Angelica sinensis (Dong quai)	Phytoestrogens, flavonoids, coumarins	Gynecological and circulation disorders	Estrogenic, vasorelaxant, anti-inflammatory	Contraceptives, vasodilators, anticoagulants, antiplatelets	3	Goh and Loh, 2001; Circosta et al., 2006
Anisodus tanguticus	Anisodine , Anisodamine	Used in treating acute circulatory shock in China	Anticholinergic	Cholinomimetics	1	Fabricant and Farnsworth, 2001
*Areca catechu (*Betel nut)	Arecoline	Muscular relaxant	Direct acting cholinergic agonist	Cholinergic agents, CNS drugs	4	Boucher and Mannan, 2002
Aspilia Africana	Alkaloids, tannins	Malaria	Antagonism	Artemisinin, chloroquine	1	Waako et al., 2005, Abii and Onuoah, 2011
Atropa belladonna (Deadly nightshade)	Atropine	Motion sickness, GIT disorders	Anticholinergic	Cholinergic drugs	1	Ulbricht et al., 2008
Black henbane, stinking nightshade, henpin	Hyoscyamine	GIT disorders	Anticholinergic	Cholinergic drugs	3	Gilani et al, 2008
Calabar bean	Physostigmine		Cholinesterase inhibitor	Cholinergic drugs	3	Hsieh et al., 2008
Callilepsis laureola	Atractyloside	GIT disorders, fertility,	Hepatotoxicity	Liver-dependent	3	Stewart et al.,

Medicinal plant	Major active ingredients	Indications	Mechanism of action	Drug candidates for potential interactions	LE	References
(Impila)		cough, worm infestations		metabolism		2002
Camellia sinensis (Green tea)	Polyphenols, caffeine	Cardiovascular diseases, prevention of cancer	Antioxidants, CNS stimulants, antilipidemic	Sedatives, hypnotics and anxiolytics	1	Ferrara et al., 2001
Camellia sinensis, Theobroma cacao, Thea species	Caffeine	CNS stimulant	CNS stimulant	CNS drugs	1	Ashihara and Crozier 2001
Carica papaya (Papaya)	Papain	GIT disorders	Alteration in platelet functions	Anticoagulants, antihypertensives	2	Ono et al., 2000
Catha edulis (Khat)	Catinone	Loss of energy	CNS stimulant, indirect sympathomimetic	Antihypertensives, antiarrhythmic, vasodilators	1	Al-Habori, 2005
Chelidonium majus (Greater celandine)	Alkaloids	Gallstones, dyspepsia	Hepatotoxicity	Liver-dependent metabolism	3	Crijns et al., 2002; Gilca et al., 2010
Cissampelos pareira (Velvet)	Cissampeline	Skeletal muscle relaxant	Muscle relaxants	Muscle relaxants	2	Bafna and Mishra, 2010
Convallaria majalis (Lily-of-the-valley)	Convallatoxin	Cardiotonic	Cardiostimulant	Cardiovascular drugs	3	Knight and Walter, 2002
Cyamopsis tetragonolobus (Guar gum)	Galactomannan, lipids, saponin	Diabetes, obesity, hypercholesterolemia	Hypoglycemic, antilipidaemic	Hypoglycemic agents	2	Mukhtar et al., 2006
Digitalis lanata (Grecian foxglove, woolly foxglove)	Acetyldigoxin, digitalin, digoxin, digitoxin, gitalin, lanatosides	Cardiotonic	Positive ionotrope	Cardiovascular drugs	1	Wood et al., 2003
Echinacea species	Alkamides, phenols, polysaccharides	Upper respiratory tract infections	Immunostimulants	Immunosuppressants	3	Barnes et al., 2005
Eleutherococcus senticosus (Siberian ginseng)	Eleutherosides	Loss of energy and memory, stress, male sexual dysfunction	Immunomodulatory, anti-inflammatory, antitumor	Immunosuppressants	4	Szołomicki et al., 2000
Ephedra species (Ma-huang)	Ephedrine	Weight loss	Hepatotoxicity	CNS drugs	3	Shekelle et al., 2003
Ginko biloba (Ginko)	Flavonoids, ginkgolides, ginkgolic acid	Cardioprotection, dementia, antioxidant	Alteration in platelet function	Anticoagulants, antiplatelets	3	Yagmur et al., 2005
Glycine max (Soya)	Phytoestrogens	Menopausal symptoms, prevention of heart diseases and cancer	Hepatoprotective, anti-osteoporosis	Contraceptives	4	Albert et al., 2002

Medicinal plant	Major active ingredients	Indications	Mechanism of action	Drug candidates for potential interactions	LE	References
Glycyrrhiza glabra (Liquorice)	Glycyrrhizinic acid	Gastric ulcer, catarrhs, inflammation	Antiulcer, aldosterone-like effects, (mineralocorticoid actions) expectorant, anti-inflammatory	Diuretics, antihypertensives,	3	Armanini et al., 2002
Harpagophytum procumbes (Devils claw)	Harpagophy cumbens	Musculoskeletal and arthritic pain	Anti-inflammatory, anti-arrhythmic, positive inotropic	Anti-arrhythmic	3	Galindez et al., 2002
Heliotropium species, *senecio* species, *symphytum crotalaria* (Pyrrolizidines)	Pyrrolizidine alkaloids	Herbal teas and enemas	Hepatotoxicity	Liver-metabolized drugs	2	Huxtable and Cooper, 2000
Jaborandi, Indian hemp	Pilocarpine	Purgative	Parasympathomimetic	Cholinergic drugs	3	Agra et al., 2007
Jimsonweed	Scopolamine	Sedative	Sedative	Cardiovascular drugs	2	Ayuba and Ofojekwu, 2005
Khetin	Kheltin	Asthma	Bronchodilator	Anti-asthma drugs	1	Ziment and Tashkin, 2000
Larrea tridentata (Chaparral)	Lignans, flavonoids, volatile oils, amino acids	RTI, chicken pox, TB, STI, pain, TB, weight loss	Estrogenic activity, hepatotoxicity	Steroids	3	Arteaga et al., 2005)
Lyceum barbarum (Chinese wolfberry)	Glycoproteins, polysaccharides, vitamin C	Energy replenishing agent, Diabetes, liver and kidney diseases	Hypoglycemic, immunostimulants	Hypoglycemic agents, immunosuppressants	3	He and Liu, 2005
Lycopodium serratum (Jin Bu huan)	Tetrahydropalmatine	Sedative, analgesic	Hepatotoxicity	CNS drugs	3	Emma, 2008
Mentha pulegium (Pennyroyal)	Pulgenone	Abortifacient, herbal tonic	Hepatotoxicity	Most drugs	2	Sztajnkrycer et al., 2008
Octea glaziovii	Glasiovine	Antidepressant	Antidepressant	CNS drugs	3	Maridass and De Britto, 2008
Ouabain tree	Ouabain	Cardiotonic	Cardiostimulant	Cardiovascular drugs	1	Schoner, 2000
Panax ginseng (Ginseng)	Triterpene saponins (ginsenosides)	Loss of energy and memory, stress, male sexual dysfunction	Immunomodulatory, hypoglycemic	Immunosuppressants, hypoglycemic agents	3	Wilasrusmee et al., 2002; Ni et al., 2010
Peumus boldus (Boldo)	Boldine	Indigestion, constipation, hepatic disorders	Diuretic, choleretic, cholagogue	Diuretics, laxatives	2	De Almeida et al., 2000
Piper methysticum (Kava)	Kavapyrones	Anxiety, insomnia	Anxiolytic, anesthetic, muscle relaxants	Sedative/hypnotic/anxiolytics	2	Feltenstein et al., 2003

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Medicinal plant	Major active ingredients	Indications	Mechanism of action	Drug candidates for potential interactions	LE	References
Rauwolfia canescens; Rauwolfia serpentina	Deserpidine, reserpine	Antihypertensive, tranquilizer	Antihypertensive	Cardiovascular drugs	3	Emilio et al., 1998
Rhamnus purshiana (Cascara)	Anthracene glycosides	laxative	Increasing GIT motility	Orally administered drugs	1	Fugh-Berman, 2000
Salvia miltiorriza (Danshen)	Tanshinones, phenolic compounds	Cardiovascular diseases	Vasorelaxants, anti-platelets	Warfarin, vasodilators, anticoagulants	3	Shi et al., 2005; Wu and Yeung, 2010
Squill	Scillarin A	Cardiotonic	Sedative	Cardiovascular drugs	1	Marx et al., 2005
Tamarindus indica (Tamarind)	Saponins, flavonoids, sesquiterpenes, tannins	Stomach disorder, jaundice	Alteration in platelet functions	Anticoagulants	3	Scott et al., 2005
Tanacetum parthenium (Feverfew)	Parthenolide, tanetin	Headache, fever, arthritis	Inhibition of serotonin and prostaglandin release, thus altering platelet function	Antiplatelets, anticoagulants	2	Rogers et al., 2000
Ternstroemia pringlei	Essential oils: monoterpenes	Sedative	Sedative synergy	Sedatives, hypnotics	2	Balderas et al., 2008
Trigonella foenum-graecum (Fenugreek)	Alkaloids, flavonoids, saponins	Diabetes, hypercholesterolemia	Antilipidemic, hypoglycemic, cholagogue	Oral hypoglycemic agents	2	Vats et al., 2002; Tripathi and Chandra, 2010; Moorthy et al., 2010; Baquer et al., 2011
Vaccinium macrocarpon	Anthocyanins, flavonoids	Antioxidant	VKORC1* genotype dependent interaction	Warfarin	4	Mohammed et al., 2008
White false hellebore	Protoveratrines A, B	Antihypertensives	Antihypertensive	Cardiovascular drugs	3	Gaillard and Pepin, 2001
Yohimbe	Yohimbine	Aphrodisiac	Vasodilatory	Cardiovascular drugs	2	Ajayi et al., 2003
Zingiber officinale (Ginger)	Zingerone, gingerols	Nausea, dyspepsia	Antiemetic, antiplatelet, antiulcer, anti-inflammatory	Diclofenac, anticoagulants	3	Thomsom et al., 2002; Lala et al., 2004; Young et al., 2006

*VKORC1 = Vitamin K epoxide reductase complex subunit 1; LE = Level of evidence

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2.4. Conclusion

Concomitant use of herbs and conventional drugs may present with untoward events. Available evidence indicates various mechanisms through which this can occur. By interacting with conventional medication, herbal remedies may precipitate manifestations of toxicity or in the other extreme, therapeutic failure. A good knowledge of the potential of commonly consumed herbal medicines to interact with prescription medicines, irrespective of the nature of evidence available, will equip health professionals in their practice. Apart from those demonstrated in significant numbers in human subjects, not all reported HDIs are clinically significant. As such, more clinically relevant research in this area is necessary.

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CHAPTER THREE

AN UPDATE ON LIVER-BASED *IN VITRO* TECHNOLOGIES FOR DRUG BIOTRANSFORMATION STUDIES[2]

3.0. Summary

Although various other body organs are involved in drug biotransformation, the liver is the predominant organ of metabolism for a wide range of endogenous compounds and xenobiotics. The enzymes contained in the cytochrome P450 superfamily present predominantly in the liver have been identified as the single most important catalyst of drug metabolism and have formed the bedrock of most matured technologies for *in vitro* drug biotransformation studies. With the development of a number of liver-based technologies, *in vitro* metabolism has gained significant popularity in the past three decades. This has come in response to several demanding factors including the waning relevance of data from animal studies; the high cost and stringent regulatory and ethical requirements, as well as safety issues involved with studies using human subjects; and the need for high throughput due to the wide range of chemical entities for routine tests. *In vitro* liver-based metabolic technologies have found ready application in generating the desired information on the substrate and inhibitor specificity of most metabolic enzymes. The technologies available vary from whole liver to subcellular fractions. These include isolated fresh liver; liver slices; primary, cultured and cryopreserved hepatocytes; microsomes; cytosolic fractions; and purified or heterologously expressed drug-metabolizing enzymes. The choice of a particular method depends on the metabolic questions to be answered. The determining factors for such choices could be drug interaction studies, metabolic profiling and metabolite identification, sequential and/or parallel oxidative and conjugative biotransformation in the same system among others. A common advantage to all the *in vitro* technologies is the reduced complexity of the study system. In this review, the general principles of *in vitro* enzyme kinetics are discussed. The review also discusses the major features, advantages and disadvantages of the various liver-based *in vitro* methodologies for drug metabolism study.

[2] This Chapter is an updated version of Fasinu P, Bouic PJ, Rosenkranz B. Liver-based *in vitro* technologies for drug biotransformation studies - a review. Curr Drug Metab. 2012 Feb;13(2):215-24.

3.1. Introduction

Drug biotransformation study is an integral part of preclinical screening for new drug candidates (Pelkonen and Raunio, 2005). This assessment usually involves both *in vitro* and *in vivo* models in animal species where the main pharmacokinetic, pharmacodynamic and toxicological profiles are investigated (Singh 2006). Biotransformation involves the physiologic conversion of nonpolar, lipophilic and pharmacologically active drugs into polar, hydrophilic, inactive or nontoxic molecules. In certain instances as with prodrugs like tamoxifen, enalapril, levodopa among others, products of biotransformation can be bioactive, and further routes of disposition are responsible for their clearance. The liver is responsible for the biotransformation of the majority of drugs. Other organs including the kidneys, skin, lungs and intestine contribute significantly to the biotransformation of xenobiotics (Krishna and Klotz, 1994; De Kanter et al., 2002).

The biotransformation pathway of a drug could be mediated by any or combination of phase I (hydrolysis, oxidation and reduction), phase II (conjugations) and phase III (further modification, efflux transport, excretion) reactions (Xu et al., 2005). Phase I biotransformation is mediated predominantly by the cytochrome P450 (CYP) enzyme superfamily present in the liver, intestines, kidney, lung and other organs. This superfamily of hemoproteins is expressed throughout the phylogenic spectrum and is responsible for catalytic metabolism of a wide range of endogenous and foreign chemicals. Initially thought to be hepatic, CYP has been found to be significantly expressed in other body organs including the intestines, spleen and the kidneys (Ingelman-Sundberg, 2004). Phase II enzymes including uridine diphosphoglucuronosyl transferase (UGT), *N*-acetyl transferase (NAT), glutathione *S*-transferase (GST) and sulfotransferase (ST) mediate the attachment of polar and ionizable groups to phase I metabolites aiding their elimination while drug transporters (phase III) exert their influence on the absorption, distribution and elimination of drugs (Petzinger and Geyer, 2006).

The use of human subjects for comprehensive clinical pharmacology studies seems to be ideal. However, the risks involved, and the stringent regulatory demands make this practically challenging with attendant high cost and long time duration. Results from animal studies pose challenges regarding their clinical significance. Current guidelines for drug development encourage the use of *in vitro* systems for qualitative and supportive assessments. In general, *in vivo* studies are deemed necessary only after positive *in vitro* results. The European Centre for the Validation of Alternative Methods (ECVAM) promotes the scientific and regulatory acceptance of alternative methods which reduce, refine or replace the use of laboratory animals (Eric et al., 2001).

Several *in vitro* absorption, distribution, metabolism and excretion (ADME) models utilized in studying hepatic drug biotransformation were developed in the 80s and 90s and have since gained prominence in the drug discovery process towards a ''fail early, fail cheaply'' philosophy (Ekins et al., 2000; Brandon et al., 2003). Such models which range from whole cell systems (intact perfused liver, human hepatocytes cultures, hepatic and transfected cell lines) to enzyme preparations (liver microsomes and isolated enzymes cultures) are now increasingly applied for quantitative and qualitative assessment in preclinical drug development, post-approval routine checks, identification of metabolic determinant factors and prediction of drug-drug, herb-drug and food-drug interactions. Apart from helping researchers and drug discovery scientists extract important information on chemical and metabolic characteristics from early screening in the drug discovery phase, results from *in vitro* investigations are relevant determinants for clinical trials of new drugs. Results have been utilized for physiology-based pharmacokinetic modeling (Baneyx et al., 2012; Heikkinen et al., 2012). In addition, they help to predict drug-drug interactions at metabolic levels. One of the most important features of *in vitro* drug biotransformation research is how to make reliable extrapolations relevant to clinical practice (Wrighton et al., 1995; Obach et al., 1997; Hewitt et al., 2001). This has been achieved with a number of the liver-based technologies.

Each *in vitro* model presents with its own peculiar advantages and limitations. The choice of method however is determined mainly by the goal of evaluation. Factors to be considered in the choice of an *in vitro* model include *in vivo* resemblance, ethical considerations, cost and availability. Further, the choice of a particular method depends on the research question to be answered: the metabolites formed, the enzymes involved, the enzyme-substrate specificity, presence of active metabolites and/or intermediate substrate, influence of test drugs on enzyme activity and the relevance to man (Eddershaw and Dickins, 1999; Gosal et al., 2003; Gomez-Lechon, 2004; Walsky and Obach, 2004).

The aim of the current review is to provide an update on the various *in vitro* liver-based models currently utilized in drug biotransformation studies. The major features of each *in vitro* technology are highlighted.

3.2. Principles of *in vitro* metabolism

Depending on the throughput demand, biotransformation assays in most *in vitro* models can be conducted manually or robotically. Some of the *in vitro* models require exogenous supply of cofactors for activity. These include primary hepatocytes, cell lines, liver slices and

isolated perfused liver. The cofactors employed consists of NADP-regenerating system (NRS) (phase I) or uridine-5'-diphospho-α-D-glucuronic acid (UDPGA) (phase II glucuronidation). NADPH is required for the measurement of oxidase activity catalyzed by CYP, flavin-containing monooxygenases, NADPH-CYP reductase and many other oxidase enzymes. The choice of incubation conditions is believed to have significant influence on the outcome of *in vitro* metabolism (Venkatakrishnan et al., 2001; Kalvas et al., 2001; Bjornsson et al., 2003; Pelkonen et al., 2010). The *in vivo* physicochemical environment can be mimicked by incubating at pH values similar to the *in vivo* conditions. For insoluble test compounds and substrates, care should be taken in the choice of lipophilic solvents. Studies have demonstrated that beyond certain concentrations, organic solvents such as ethanol, dimethyl sulfoxide, isopropanol, methanol, acetonitrile, dimethyl formamide, acetone and polyethylene glycol can greatly interfere with the metabolic activities of *in vitro* enzymes (Tang et al., 2000; Mountfield et al., 2000; Easterbrook et al., 2001; Uchaipichat et al., 2004). Thus solvent effects are often part of the optimization process for *in vitro* metabolism. It is generally recommended that organic solvents should not exceed 1% of total incubation mixture (Yan and Caldwell, 2005).

The metabolic activities of *in vitro* models are assessed through the rate of metabolite formation or substrate depletion (Jones and Houston, 2004). Most single-substrate enzymatic reactions follow the Michaelis-Menten principle. The initial rate of enzymatic reactions is usually linearly proportional to the substrate concentration. The initial reaction rate, V_0 depends on the position of the substrate-binding equilibrium and the rate of metabolite formation (Kou et al., 2005). Beyond the substrate concentration where reaction rate is maximal (V_{max}), there is loss of linearity between the rate of metabolite formation and substrate concentration [S]. The substrate concentration corresponding to half the V_{max} is referred to as the Michaelis-Menten constant (K_m) (Hsu et al., 2001). The Michaelis-Menten equation as expressed in Equation 3.1 is the basis of most single-substrate (one enzyme model) enzyme kinetics. V is measured in nmole/hr/mg or nmole/min/mg protein for HLM or nmole/hr/nmole CYP when activities of CYP isoforms are involved.

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$ *Equation 3.1*

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Where V_0 is the initial rate of metabolism; V_{max}, the maximum rate of metabolism; [*S*], the substrate concentration and K_m, the Michaelis-Menten constant.

The rate of product formation depends on both the enzyme and substrate concentrations reaching its peak at equilibrium where the saturation of the enzyme binding site yields the highest rate of product formation. A typical Michaelis-Menten curve is therefore non-linear and various attempts have been made to modify it in a way that enzyme kinetic parameters can be estimated at a linear glance. One of the most common linearized modifications to the Michaelis-Menten curve is the Hanes-Woolf Plot. It is based on the rearrangement of Michaelis-Menten equation as depicted in Equation 3.2.

$$\frac{[S]}{V} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}}$$ *Equation 3.2*

As presented in Equation 3.2, a plot of the ratio of the initial substrate concentration to the reaction velocity (*[S]/V*) against [*S*] will yield a linear graph with $1/V_{max}$ as the slope, a *y*-intercept of K_m/V_{max} and an *x*-intercept of $-K_m$. The Hanes-Woolf Plot has been used for rapid and easy determination of major kinetic parameters such as the K_m and the V_{max}.

When 2 or more CYP isoforms with distinct affinities are involved in catalyzing a given metabolic pathway, a biphasic model is used as expressed in equation 3.3.

$$V = \frac{V_{max1}[S]}{K_{m1}+[S]} + \frac{V_{max2}[S]}{K_{m2}+[S]}$$ *Equation 3.3*

where K_{m1} and K_{m2} are affinity constants.

Softwares are now available for easier and more accurate determination of enzyme kinetic parameters. An example is the SigmaPlot® Enzyme Kinetic Software (SigmaPlot: Enzyme Kinetic Module, Systat Software Inc., Chicago, IL, USA) which determines Michaelis Menten kinetics through its non-linear and linearized plots including Lineweaver-Burk, Eadie-Hofstee, Scatchard, Hill and Hanes-Woolf.

The influence of test chemical inhibitors is assessed in the drug metabolism incubation mixture by co-incubating the enzyme-expressed *in vitro* system with test compound in the presence of various CYP-isoform-specific inhibitors. IC_{50} and K_i values are then determined. The IC_{50} is defined as the inhibitor concentration that causes 50% inhibition of substrate metabolism. It is calculated as shown in equation 3.4 below.

$$V = \frac{V_o}{1+[\frac{[I]}{IC_{50}}]^S}$$ *Equation 3.4*

Where V_o is the uninhibited velocity; V, the observed velocity; [*I*], the inhibitor concentration and *S*, the slope factor (Bloomer et al., 1994). Non-linear regression equations can also be generated by profiling the inhibitor concentration against observed inhibition. Such equation can be used to determine the IC_{50}.

K_i is a measure of the affinity of the compound to the enzyme determined graphically. The values of IC_{50} and K_i are determined by taking the concentration of marker substrates around their corresponding K_m values and about 0.5-4-fold of their corresponding K_m values respectively. Enzyme inhibition can also be expressed as percentage of control (Wang et al., 2010).

The use of *in vitro* technologies in drug biotransformation studies has thus evolved with standard procedures. The specific CYP and UGT activity can be measured with various substrate models as shown in Tables 7 and 8.

Table 7: A summary of recommended test substrates and inhibitors for various CYP isozymes (Brandon et al., 2003; Yan and Caldwell, 2005; Hariparsad et al., 2006)

Isoform	Occurrence	Acceptable test substrates	Metabolic reaction	Inhibitor	inducer
1A1	Mainly extrahepatic	Ethoxyresorufin	7-ethoxyresorufin O-deethylation	α-Naphtoflavone	Polycyclic hydrocarbons, Rifampicin, 3-methyl-cholanthrene
1A2	Liver	Phenacetin Caffeine	Phenacetin O-deethylation Caffeine N3-demethylation	Furafylline	
2A6	Liver	Coumarin	Coumarin C7-hydroxylation	Sulfaphenazole	Pyrazole Barbiturates
2B1/2	Liver	Pentoxyresorufin	Pentoxyresorufin O-dealkylation		
2B6	Liver	Mephenytoin Bupropion	(S)-mephenytoin N-desmethylation Bupropion hydroxylation	Sertraline	
2C8	Liver, intestine	Paclitaxel	Paclitaxel C6-α-hydroxylation	Glitazones	Rifampicin Phenobarbital
2C9	Liver, intestine	Warfarin Diclofenac Tolbutamide	(S)-warfarin C6-, C7 hydroxylation Diclofenac 4'-hydroxylation Tolbutamide *p*-CH_3-hydroxylation	Sulfaphenazole	Rifampicin Phenobarbital
2C18/19	Liver	Mephenytoin	(S)-mephenytoin C4'-hydroxylation	Ticlopidine Ketoconazole	Rifampicin Carbamazepine
2D6	Liver, intestine, kidney	Bufuralol Dextromorphan Codeine	Bufuralol C1'-hydroxylation Dextromorphan O-demethylation Codeine O-demethylation	Quinidine Haloperidol	
2E1	Liver, intestine, leukocytes	Chlorzoxazone Lauric acid	Chlorzoxazone C6-hydroxylation Lauric acid C (ω1)-hydroxylation	Diethyl-dithiocarbamate	Ethanol
3A4	Liver, GI tract,	Midazolam Testosterone Dihydropyridines	Midazolam C1'-hydroxylation Testosterone C6-β-hydroxylation Hydroxylation	Ketoconazole Furanocoumarins Grapfruit juice Flavonoids	Rifampicin Barbiturates
4A11	Liver, kidney	Lauric acid	Lauric acid ω-hydroxylation	17-Octadecynoic acid	

Table 8: Suggested isoform-selective *in vitro* probe substrates for hepatic UGTs

UGT Isoforms	Probe substrates	References
UGT1A1	Bilirubin	Levesque et al., 2007
	Estradiol	Mano et al., 2007
	Etoposide	Burchell et al., 2005
UGT1A3	R-Lorazepam	Court, 2005
	26, 26, 26, 27, 27, 27-hexafluoro-1α, 25-dihydroxyvitamin D_{3},	Kasai et al., 2005
UGT1A4	Trifluoperazine,	Uchaipichat et al., 2006
	Imipramine	Qian et al., 2004
	Lamotrigine	Argikar et al., 2009
	Tamoxifen	Kaku et al., 2004
UGT1A6	Serotonin	Krishnaswamy et al., 2004
UGT1A9	Propofol	Su et al., 2010
	7-hydroxy-mycophenolic acid	Girard et al., 2006
UGT2B4	Codeine	Hanioka et al., 2008
	Hyodeoxycholic acid	Barre et al., 2007
UGT2B7	Zidovudine	Uchaipichat et al., 2008
	Morphine	Stone et al., 2003
	Epirubicin	Innocenti et al., 2001
UGT2B15	Androstanediol	Soars et al., 2003
	S-oxazepam	He et al., 2009
	Tamoxifen	Oleson and Court, 2008
	Rofecoxib	Court, 2006
UGT2B17	Dihydrotestosterone	Setlur et al., 2010

3.3. Various technologies for liver based *in vitro* metabolism

3.3.1. Isolated Perfused Liver

The isolated perfused liver is considered theoretically as the most similar to the *in vivo* situation. However, only animal liver in small scale has been employed. The model usually involves the perfusion of isolated liver with Krebs-Henseleit buffer as perfusate (Lafranconi and Huxtable 1984; Jia and Liu, 2007). The three-dimensional cytoarchitecture, the presence of non-hepatic cells and transporters make this model the closest in similarity to the *in vivo* environment. The presence of functional bile canaliculi also makes this model suitable for bile collection and analysis (Groneberg et al., 2002). However this model is difficult to handle, has very short viable period of three hours due to the poor perfusion of the cells by oxygen and nutrients, poor reproducibility and low throughput. In addition, human liver is difficult to source (Brandon et al., 2003).

3.3.2. Liver slices

Liver tissue slices have been widely used for biotransformation studies. First developed in 1920s by Otto Heinrich Warburg, its use and reliability have further been improved by the development of high-precision tissue slicers (De Kanter et al., 1999; Edwards et al., 2003; Olinga et al., 1997, 2008). Unlike in perfused liver, the preparation of liver slices does not require harmful proteases (Seglen, 1973). This model offer intact cellular interaction, normal spatial arrangement and the possibility of morphological studies. Like the perfused liver, it has functional drug metabolizing enzymes, transporters and bile canaliculi. It has reasonably higher throughput than perfused liver. However, some of the disadvantages with this model include: lack of optimal cryopreservation procedure making it unavailable commercially, considerable decrease in CYP expression (>50%) within 24 hours of preparation, loss of hepatic function after 10 days, inadequate tissue penetration of the test medium, damaged cells on the outer layer and the necessity of expensive equipment (Lerche-Langrand and Toutain, 2000). In addition, viable cell enrichment is not possible with liver slices, the liver slices are not inducible by CYP inducers, biles collection for analysis may not be possible as in perfused liver and the presence of necrotic cells may affect drug transport studies (Olinga et al., 2001; Pan et al., 2002).

3.3.3. Hepatocytes

3.3.3.1. Primary hepatocytes

In vitro models of the liver employing isolated primary hepatocytes have been popularized for various screening purposes. In addition to their application in drug biotransformation and toxicity studies, they are important tools for studying drug efficacy, hepatic proliferation and as supportive tools in other bioartificial studies (Wang et al., 2004). Primary hepatocytes have strong resemblance of *in vivo* situation with metabolic results closely correlated to *in vivo* yields. They reflect the heterogeneity of CYP-expression in human liver and are thus popularly employed for *in vitro* drug biotransformation studies (Hewitt et al., 2007). Different methods have been developed to obtain hepatocytes for drug biotransformation (Guguen-Guillou et al., 1982; Smedsrod and Pertoft, 1985; Baccarani et al., 2003; McGinnity et al., 2004). An important feature of primary hepatocytes is the preservation of drug metabolizing enzymes at *in vivo* levels. Further advantage is the relative ease to use and reasonably higher throughput (Wang et al., 2002). However, lack of cell polarity, cell-cell and cell-matrix contacts limits the *in vivo* resemblance of isolated hepatocytes in drug biotransformation research. Isolated hepatocytes also have limited viability (2-4hrs) (Bi et al., 2006).

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3.3.3.2. Cultured hepatocytes

Isolated hepatocytes can be maintained in monolayer cultures for a maximum of four weeks. This offers the advantage of longer viability compared to primary hepatocytes (Guillouzo et al., 1990; Wang et al., 2008). This longer viability period with the prolonged functional regulatory pathways enables the utilization of this model for assessing upregulation/downregulation of metabolic enzymes. Cultured hepatocytes gradually lose viability and liver-specific functions including decreased CYP expression with time (Luttringer et al., 2002). Another major challenge with the use of hepatocyte is the dependence of its availability on the handiness of fresh liver tissue. This makes it impossible to perform studies on demand, or to repeat studies with hepatocytes from same donor. To solve these challenges, cryopreservation procedures have been developed for hepatocytes, retaining the activity of most phase I and phase II enzymes (Zaleski et al., 1993; Ostrowska et al., 2009). This has made the commercial availability of human hepatocytes possible and accessibility independent of fresh liver tissue availability. The hepatocytes preserved in this form are a potential tool for chronic toxicity and drug-drug interaction studies (Hewitt et al., 2001).

Hepatocyte cultures may require special supplements in media and, depending on the technology used to preserve the tissue function, cultured hepatocytes may maintain differentiated function and possess a functional bile canaliculi (LeCluyse et al., 2000). One of the major challenges with the use of human hepatocytes is the considerable inter-individual variation carried over from the source of the liver. This can be overcome by using mixtures of heptocytes from multiple donors (Hewitt et al., 2007). Human hepatocytes do not reflect completely the *in vivo* environment as non-hepatic cells like Kupffer cells which may be necessary for cofactor supply are absent. Although hepatocytes often give better *in vitro–in vivo* correlations than cell lines and subcellular fractions, perfused cell lines and liver slices are considered better in this regard (Boess et al., 2003).

3.3.4. Liver cell lines

Liver cell lines are often used as *in vitro* models for drug metabolism study but they are not as popular as other models. Their cellular characteristics are de-differentiated and they lack complete expression of all families of metabolic enzymes (Hoekstra and Chamuleau, 2002; Strick-Marchand and Weiss, 2002). They can be obtained as isolated cells from primary tumors of the liver parenchyma seen after chronic hepatitis or cirrhosis (Knasmuller et al., 2004). Commercially available cell lines include Hep G2, Hep 3B, C3A, PLC/PRF/5, SNU-398, SNU-449, SNU-182, SNU-475 and SK-Hep-1 among others. Each of these cell lines

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expresses various metabolic enzymes to different degrees. The most widely used and best characterized liver cell line is the hepatoma cell line Hep G2 (Brandon et al., 2003).

The metabolic activity of liver cell lines is generally low compared to freshly isolated human hepatocytes (Khetani and Bhatia, 2008). Liver cell lines require culture medium, the composition of which also greatly influences the metabolic activity (Xu et al., 2005). The main advantages of liver cell lines compared to other *in vitro* models are their ease to culture and their possession of relatively stable enzyme concentration (Khalil et al., 2001). On the other hand, the absence or low expression of most phase I and phase II enzymes is a major disadvantage. Metabolites are not easily detected and the activities of individual CYP and other enzymes are difficult to investigate. These factors limit the application of liver cell lines in drug biotransformation studies (Mersch-Sundermann et al., 2004).

3.3.5. Human liver S9 fractions

The liver S9 fractions are obtained after the centrifugation (9000g) of the supernatant yielded from an initial centrifuged (1000g) liver homogenates. They contain both microsomal and cytosolic fractions expressing a wide range of metabolic enzymes – CYP, flavin-monooxygenases, carboxylesterases, epoxide hydrolase, UDP-glucuronosyl transferases, sulfotransferases, methyltransferases, acetyltransferases, glutathione S-transferases, among others. They have been widely employed for metabolic, toxicity and mutagenicity studies mainly in combination with the Ames test (Hakura et al., 2001; Slaughter et al., 2003; Chalbot and Morfin, 2005). For *in vitro* activity, an exogenous supply of a NADPH-regenerating system or NADPH solution is required to provide the energy demand of the CYP enzymes. Similarly, for phase II reactions, uridine diphosphoglucuronic acid (UDPGA) and alamethicin for UGT; acetyl coenzyme A (acetyl CoA), dithiothreitol (DTT) and acetyl CoA regenerating system for NAT; adenosine-3’-phosphate 5’-phosphosulphate (PAPS) for ST; and glutathione for GST are necessary (Hakura et al., 2003).

S9 fractions offer a more complete representation of the metabolic profile compared to microsomes and cytosol, as they contain both phase I and phase II activity. The major disadvantage is the lower enzyme activity in the S9 fraction compared to microsomes or cytosol which may leave some metabolites unnoticed (Hakura et al., 1999).

3.3.6. Human liver cytosolic fractions

The human liver cytosol is obtained by differential centrifugation of whole liver homogenate, like microsomes or from the cytosolic (soluble) fraction of insect cells infected with recombinant baculovirus. It expresses the soluble phase II enzymes like N-acetyl transferase, glutathione S-transferase, sulfotransferase, carboxylesterases, soluble epoxide hydrolases, diamine oxidases, xanthine oxidases and alcohol dehydrogenase.

Human liver cytosolic fractions are used often in drug biotransformation research for enzyme-specific studies (Maser et al., 2000; Walsh et al., 2002). Their use requires cofactors like acetyl coA, dithiothreitol and acetyl coA-regenerating system for NAT, adenosine-3'-phosphate-5'-phosphosulfate for ST, glutathione for GST activity and S-adenosyl methionine (SAM) for thiopurine methyltransferase (TPMT). Human liver cytosol expressing the specific NAT isozymes in cytosol without the other soluble phase II enzymes is commercially available. This allows the study of the influence of both NAT1 and NAT2 isozymes on the biotransformation pathways employing this model (Li et al., 2005).

The most important advantage offered by this model is the presence of only few cystolic enzymes at concentrations higher than those in the liver S9 fraction. It allows individual study of the metabolic activity of NAT, ST and GST enzymes depending on the cofactors added. This limited enzyme expression also account for the main disadvantage, as other enzymes especially the UGT, which is located on the endoplasmic reticulum, are absent and cannot be investigated via this model (Li et al., 2005; Hariparsad et al., 2006).

3.3.7. Human liver microsomes (HLM)

HLM is the most popular model and is the most widely utilized *in vitro* model providing affordable tool for metabolic profiling and drug interaction studies (Buenz et al., 2007). Microsomes from the liver and small intestines are employed to investigate the potential for pre-systemic metabolism following oral drug administration while those from small intestine, kidney are often utilized to evaluate extrahepatic metabolism (Bu, 2006). Liver microsomes consist of vesicles of the hepatocyte endoplasmic reticulum prepared by differential centrifugation, and can be collected from liver preparations including fresh human liver, liver slices, liver cell lines and primary hepatocytes (Asha and Vidyavathi, 2010). They are used for the evaluation of phase I enzymes because of the rich expression of CYP, flavin monoxygenases, carboxyl esterases, epoxides, hydrolase and UDP glucuronyl transferases (Newton et al., 2005). The CYP retain their activity for many years in microsomes at low

temperatures (-80°C) (Pearce et al., 1996). HLM provides one of the most convenient ways to study CYP metabolism with high throughput.

Typical HLM incubation mixture consists of test compound, NADPH-regenerating system (NRS) and buffers like potassium or sodium phosphate. The reaction is initiated by the addition of NRS, incubation at 30°C in a shaking water bath or shaking incubator. The metabolic reaction is terminated by the addition of a stop solution comprising ice-cold acetonitrile, trichloroacetic acid, ice-cold carbonate buffer, sodium hydroxide or hydrochloric acid, in order to precipitate the microsomal protein. The reaction mixture is then centrifuged and analyzed (Asha and Vidyavathi, 2010). Exogenous cofactors are required for *in vitro* microsomal activity, the choice of which depends on the desired enzyme activity. For UGT activity, a glucuronyl moiety donor usually UDPGA is required. For CYP monooxygenase reactions, NADPH-regenerating system consisting of β-NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase is required. Alternatively, NADPH may be added directly to the incubation mixture to supply the required energy for metabolism. For UGT activity, pore-forming agents like alamethicin are often necessary to facilitate drug access to the UGTs (Yamazaki, 1997; Chen et al., 2007). HLM is employed in the drug discovery process for metabolite identification, comparison of metabolism by different species, prediction of *in vivo* clearance, reaction phenotyping and metabolic pathway identification (Bourrie et al., 1996; Li, 2001; Venkatakrishnan et al., 2001; Lu et al., 2003). The activity of HLMs can vary substantially between individuals. This problem however can be successfully solved by the application of microsomes pooled from a large bank of individual liver tissues while individual microsomes are used to study inter-individual variability in drug metabolism. Microsomes from single-sex sources are also available and are employed in the evaluation of gender-based discrepancies in drug metabolism (http://www.xenotechllc.com).

The utilization of HLM offers major advantages including ease of application, affordability, well established procedure and the possibility of its use in studying inter-individual and population-based metabolic variation. It provides qualitative estimations of *in vivo* drug metabolism and is convenient tools for high throughput screening of compounds, lead optimization studies during drug development and drug interaction studies.

The use of HLM is however limited in its quantitative clinical extrapolative relevance. Thus, results obtained from HLM cannot be used for quantitative estimations of in vivo human biotransformation (Brandon et al., 2003). This is because the microsomal fraction is enriched with CYPs and UGTs and there is no competition with other enzymes. This leads to relatively higher biotransformation rates in microsomes compared to the human *in vivo* situation, but also compared to primary hepatocytes and liver slices.The absence of other

enzymes like NAT, GST and ST, and cytosolic cofactors present in intact cells limits the metabolic reactions. This will necessarily lead to a reduction in the expected 'metabolic competition' with limited number of metabolites. Secondly, the fraction of drug bound to plasma versus microsomal protein is an important determinant of extent of drug metabolism *in vivo*. The impact of this factor is difficult to assess *in vitro*. Also, the direct contact and accessibility of the drugs and their metabolites to the microsomal enzymes in the incubation mixture is not a true reflection of the *in vivo* situation (Asha and Vidyavathi, 2010).

3.3.8. Recombinant human CYP and UGT enzymes

3.3.8.1. Transgenic cell lines

Transgenic cell lines are obtained by the recombinant expression of phase I and/or phase II enzymes in a cell line. They are enzyme-induced cells, often used in cytotoxic studies whose preparation involves the production of individual enzymes in the endoplasmic reticulum of an eukaryote host cell including yeast and mammalian cells (Donato et al., 2008). All human CYPs and UGTs have been expressed in this way to overcome the limitations of liver cell lines. Stable expression of human CYPs in a human cell line was first successfully performed in 1993 by Crespi and co-workers. Various other expression of liver enzymes in cell lines have been developed including the human lymphoblast stably expressing CYP1A2, CYP2A6, CYP2E1 and CYP 3A4 (Snawder et al., 1994; Sawada and Kamataki, 1998; Tiano et al., 2003; Zhuge et al., 2003; 2004). Cell lines may be transfected at high efficiency using protoplast fusion, centrifugation of lysozyme-treated bacteria bearing the desired vector with the parent cells in the presence of polyethylene glycol protoplast fusion (Crespi et al., 1997). The main advantages of transgenic cell lines are the ease of culturing, high expression of CYP and UGT enzymes and the possibility of single enzyme study as in supersomes. However, they are more expensive than most other enzyme-based technologies. They also do not completely reflect the *in vivo* situation (Ferro and Doyle, 2001).

3.3.8.2. Non-mammalian cells containing expressed CYP and UGT enzymes

CYP expression for this model includes bacterial expression in *Escherichia coli* and yeast cells. This model is the final and most focused 'enzyme only' system in which the most popular is the baculovirus-based model allowing functional expression of recombinant proteins in insect cells (Guengerich et al., 1997). Insect cells lack endogenous CYP and uridine diphosphoglucuronosyl transferase activity. Microsomes from insect cells therefore

can be transfected with cDNA for human CYP and UGT. This has proven to be a useful tool in *in vitro* biotransformation studies (Miller et al., 2011). This model of baculovirus mediated microsomes of insect cells is sometimes referred to as baculosomes or supersomes (Gentest).

Unlike in HLM where all CYPs and UGTs are present, specifically expressed human CYP and UGT in supersomes allows the assessment of individual metabolic enzymes and their contribution to the metabolic pathway of the test compound (Lavigne et al., 2002). Currently, all common human CYPs and UGTs co-expressed with NADPH-cytochrome P450 reductase are commercially available (Miller et al., 2011). A control experiment - incubation with nontransfected supersomes, must always be conducted. A NADPA-regenerating system (which consists of β-NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase) or NADPH is required to supply the energy demand of the CYPs. For the UGT activity, uridine diphosphoglucuronic acid (UDPGA) has to be added as a cofactor (Invitrogen Life Technology, 2004).

After the determination of the metabolic pathway using the HLM, the use of cDNA-expressed CYPs can be an excellent way to confirm results (Kurkela et al., 2003). Although studies with supersomes may lack quantitative *in vivo* relevance, they address very particular questions with respect to the role of specific CYP isozymes and their roles in the metabolic pathways of xenobiotics. Supersomes can be used to study not only isozyme-specific drug biotransformation but also drug-drug interactions (Wang et al., 2010). Because of the availability of different genotypes of the CYP isozymes (eg CYP2C9*1, CYP2C9*2 and CYP2C9*3), the influence of different polymorphisms on the drug biotransformation can also be studied employing the supersomes (Schwarz et al., 2001). However, in UGT supersomes, the UGT active site is shielded behind a hydrophobic barrier resulting in latency of glucuronidations. This disadvantage can be overcome by using a pore-forming agent like alamethicin (Brandon et al., 2003). The various technologies are compared in Table 9.

Table 9: A relative comparison of the features of the various liver-based *in vitro* drug metabolism technologies

	In vivo correlation	Throughput	Technical simplicity	Commercial availability	Enzyme-specific probe	Viability period	Reproducibility
Perfused Liver	+++	+	+	-	+	+	+
Liver slices	+++	+	+	-	+	+	+
Primary Hepatocytes	++	++	++	-	++	+	++
Cultured Hepatocytes	++	++	++	-	++	+	++
Cryopreserved Hepatocytes	++	++	++	++	++	++	++
Liver cell lines	+	+++	+++	+++	+++	+++	++
Liver cytosolic fractions	+	+++	+++	+++	+++	+++	++
Liver microsomes	+	+++	+++	+++	+++	+++	+++
Recombinant CYP/UGT enzymes	+	+++	+++	+++	+++	+++	+++

3.4. Discussion of methods employed in this study

In this review, the available liver-based *in vitro* methods for drug biotransformation studies have been discussed. These techniques have made major contributions towards the development of various therapeutic agents and are still the preferable tools for future studies. It has been pointed out that the methods vary with their ease of use and the conclusions that can be inferred from them. They also present with unique advantages and disadvantages. The choice of any of the methods will depend on the research question to be answered. However, in certain instances, a combination of different *in vitro* tools may be required for a reliable assessment.

In this study, HLM was employed to investigate the HDI potential of selected medicinal herbs. This is because of its ease to use, commercial availability, long-time viability, ethical and technical simplicity and the ability for high throughput. HLM is the most often used in similar studies. Positive results from HLM can be confirmed through the use of hepatocytes or *in vivo* human studies. By standard practice, if there is no enzyme inhibition in HLM *in*

vitro, further studies are often not necessary. The disadvantage of the use of HLM as mentioned earlier, is poor quantitative extrapolation to human.

In addition, cryopreserved hepatocytes were utilized to establish CYP3A4 inhibition by *Lessertia frutescens*. This is because hepatocytes represent a closer system to human liver than HLM, but require more technical and complex procedures in the sourcing and usage.

The activities of the selected herbs in this study on drug transporters were investigated employing cell lines stably expressing the specific transporters.

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CHAPTER FOUR

SELECTED COMMONLY USED SOUTH AFRICA MEDICINAL HERBS – SELECTION AND PREPARATION FOR *IN VITRO* STUDIES

4.0. Summary

While consumers may purchase herbal products over-the-counter, many patients consult traditional health practitioners (THPs) who prescribe and dispense to them, various herbal products in accordance with their presenting complaints. This part of the study was conducted to identify medicinal herbs utilized by traditional health practitioners to manage various conditions. It involved the collection of samples and the administration of semi-structured interview. In all, 15 plant species belonging to 14 families were collected and identified scientifically. The parts used, methods of preparation and administration, together with specific indications were documented. An overview of published studies on the identified was provided. The absence of information on HDI potential of the medicinal herbs was identified.

4.1. Introduction

Traditional medicine especially the use of medicinal herbs is popular among South Africans where THPs are the first point of call for health services in most rural areas. There are three loose and often intertwined classifications of THP in South African. The *inyanga* is a traditional doctor or herbalist, typically male, who often uses medicinal herbs to treat various diseases. The *isangoma* is usually a woman who combines divination and herbalism, operating as a medium between the ancestors and the clients within religious/supernatural context. The faith healer integrates religious ritual and traditional practices (Freeman and Motsei, 1992). The reason for the persistent popularity of traditional healers across South Africa is rooted in traditional and cultural acceptance, relative affordability and accessibility. It is also believed that THPs adopt a more holistic health management approach appropriate for psychological and social components of chronic diseases including HIV/AIDS and tuberculosis in the various communities (Babb et al., 2007). There is therefore, an increasing acceptance of the need to incorporate THP into the mainstream health care (Mills et al., 2005a). This is in agreement with WHO recommendations where the popularity and acceptance of THP can be employed in their communities to mobilize people for voluntary counselling and testing programs, acceptance and adherence to drug regimens, cooperating with orthodox health practitioners to improving the well-being and quality of life of people living with chronic diseases especially HIV/AIDS (WHO, 2005). This advocacy has grown despite the absence of sufficient data on the safety and efficacy of herbal medicines.

Although the use of medicinal herbs predates the emergence of HIV/AIDS, the utilization of herbs as immune boosters and for symptomatic management of AIDS has been popularized in South Africa. The high burden of HIV/AIDS has attracted various policy designs to accommodate a holistic approach to manage the scourge. While the crux of the various HIV/AIDS policies has been the introduction of free antiretroviral drugs (ARV), it is believed that the past controversy over HIV/AIDS and the official promotion of herbal medicine for its management have contributed to the persistently high rate of consumption of medicinal herbs by people living with HIV/AIDS despite the availability and/or consumption of ARVs (Morris, 2002; MacPhail et al., 2002; Mills et al., 2005a; Malangu, 2005).

Studies have shown that many South Africans consult THPs especially for the treatment of Sexually Transmitted Infections (STIs) such as gonorrhoea, syphilis, and assumed HIV/AIDS Peltzer, 2001; 2003). While some HIV/AIDS patients consume medicinal herbs *ab initio*, studies have shown that a number of them resort to herbal medicine in response to adverse reactions to ARVs (Peltzer and Mngqundaniso, 2008). The number of HIV/AIDS patients who consume herbal medicine alone or concomitantly with ARVs is often under-estimated.

This is because, although the South African National ARV Treatment Guidelines require patients to disclose intake of any other medication including traditional medicines, most patients do not (Department of Health, South Africa, 2004). In a cross-sectional study carried out by Peltzer and colleagues (2008), up to 90% of respondents who were taking herbal therapies for HIV did not disclose this to their health care providers. This therefore calls for more research to establish the identities, therapeutic benefit and safety of the use of medicinal herbs in HIV/AIDS. This becomes very important because of the many documented cases of herbal toxicity, herb-drug interaction and therapy failure consequent upon concomitant use of herbs and prescription medicines.

The long history of the use of medicinal herbs has impacted on the socio-cultural identities of indigenous Africans. There are research efforts globally to document the ethno-medical data of medicinal plants used in various communities. This becomes necessary because most THPs, who are seen as the custodian of the herbal knowledge, seldom keep records of their practice. Medical claims for the medicinal herbs are not well proven scientifically.

4.2. Aim and Objectives

The aim of this chapter was to identify and select medicinal plants for HDI studies. Further objective was to conduct a review of published studies on the identified plants, including data on HDI potential.

4.3. Method

The medicinal herbs to be investigated for HDI potential were sourced in collaboration with HOPE Cape Town who engages THPs for community mobilization for HIV/AIDS awareness and management programmes. Through this collaboration, two THPs, who practice in Mfuleni and Delft, two major Black[3] communities in Cape Town, Western Cape Province, were recruited. They were registered with the traditional medicine council, had full-time practice in their communities, and had attended various HIV/AIDS training workshops. They were the major THP with patronage from neighbouring townships of Nyanga, Khayelitsha and Philippi. HIV/AIDS is endemic in these poor townships. Previous activity of HOPE Cape

[3] The race classification was based on the national census categories and described as Black (Africans), Coloured, Indian and White

Town had exposed the THPs to health education including the necessity of encouraging and referring their patients suspected of HIV/AIDS for voluntary testing, counselling and ART.

Mfuleni is a predominantly Black township about 40 kilometres from Cape Town, South Africa with a population of about 10,000 (according to 2001 census). Residents, like most Blacks in the Western Cape, claim origin from the Eastern Cape Province. The trado-cultural practices including traditional medicine are thus a reflection of the traditions of the Eastern Cape. Delft is a much bigger township about 34 kilometres North-East of Cape Town with a mixed population of Black and Coloured residents estimated in the year 2001 to be 92,000.

The sourcing of the medicinal herbs involved three stages. Plant Material Transfer Agreements mutually agreeable to the THPs and the University of Stellenbosch were signed (Appendix E). Samples of herbal materials used for various chronic ailments were obtained from them. The herbal samples in their natural habitat were also obtained in Goso, near Ngcobo, in Eastern Cape Province (about 1400 kilometres away), and identified by their local (IsiXhosa) names with the assistance of the THPs.

The collected plants were identified with the help of botanical experts in the Compton Herbarium, Cape Town and Voucher specimens were prepared and housed at the Division of Pharmacology, University of Stellenbosch. Information on the mode of use, dosage and indications were obtained and documented through semi-structured interview/questionnaire (Appendix F).

Further, a literature search was conducted through the databases of Pubmed, Embase and Google Scholar to published studies on the identified plants. The scientific and local names of the respective plants were used as search terms.

Ethic approval was obtained from the University of Stellenbosch Health Research Ethics Committee. Approval was obtained from the University of Stellenbosch legal department for the transfer of the plant materials.

4.3.1. Administration of the semi-structured interview/questionnaire to the THPs

The two THPs recruited for the purpose of sourcing the medicinal herbs used in this study were administered with a questionnaire (Appendix F). The content of the questionnaire was translated from English to IsiXhosa, the first language of the THPs, with the help of a fluent speaker of both languages. The THPs, having made to understand their roles in the research, consented by appending their signatures in the presence of witnesses. The major

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roles of the THPs were the supply of the medicinal herbs for this study, and the provision of information on the traditional indications and mode of use. The intellectual property of the THPs was protected by the strict adherence to the national guidelines governing the interactions with THPs and other indigenous knowledge custodians. This included the notification of the department of Environmental Affairs of the Republic of South Africa of the intention to perform this research, and the filling of the Bioprospecting and Discovery Notification Form. These were done in collaboration with the Legal and Patent units of Stellenbosch University.

4.3.2. Material transfer agreement

In addition to the administered semi-structured questionnaires, a Material transfer Agreement (MTA) was mutually agreed upon, prepared and signed by both the researchers and the THPs. This was witnessed by the Legal and Patent units of Stellenbosch University. The content of the MTA ensured that the plant materials were the property of the THPs who had voluntarily made them available as a service to the research community. It also restricted the recipients to use the materials for research purposes only in which case, the materials are not transferable to any other person. The THPs allows the researchers to publish any findings therefrom and to acknowledge them. Further detail of the MTA is provided in Appendix G.

4.4. Medicinal herbs collected

This study identified 15 medicinal herbs commonly prescribed for patients by practicing THPs in Mfuleni, Delft and the adjoining Black communities. The identified plants belong to 14 different families. The THPs visit the Eastern Cape regularly and seasonally to source for these plants. All the plants are administered as oral and/or topical infusions or decoctions. Various disorders managed with these herbal remedies include gastrointestinal, respiratory, skin and infectious diseases. The identities and use of the plants are summarized in Table 10 and sample plant pictures are provided in Appendix G. Both THPs agreed on the information provided on the supplied medicinal herbs.

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Table 10: Treatment practices and plant species used by THPs for HIV/AIDS patients

Scientific name	Family	Vernacular[4] names	Part used/ extracted	Preparation	Uses
Acacia karroo	Fabaceae	Sweet thorn (E), UmNga	Bark	Crush and boil in water	For cold, skin and eye infections, diarrhoea
Alepidea amatymbica Eckl & Zeyh.	Apiaceae	Iqwili	Rhizome	Chewed or boiled, allowed to cool, and the infusion taken	Respiratory problems - flu, coughs and chest complaints.
Bowiea volubilis Harv. Ex Hook. F	Hyacinthaceae	Umagaqana	Bulb	Slice and boil in water, take cold	Purgative, oedema, syphilis
Capparis sepiaria L. var citrifolia (Lam) Toelken	Capparidaceae	Isihlo esimbovu	Root	Crush and boil in water	Respiratory disorders, rheumatism, gout, earache, allergy
Chenopodium album L.	Chenopodiaceae	Fat hen (E), imbikicane embomvu	Leaves	Crush in hot water to make infusion	Anorexia, dysentery, diarrhoea, cough
Emex australis	Polygonaceae	Devil's thorn (E), inkunzane	Leaves	Boil in water to make decoction	Constipation, stomach cramps, appetite stimulation
Hypoxis hemerocallidea	Hypoxidaceae	African Potato (E) Afrika patat (A), Inkomfe (Z), ilabatheka	Corms	Boil bulb, eat whole or take aqueous decoction	Immune booster, skin rash
Kedrostis africana L. Cogn.	Cucurbitaceae	uTuvana	Leaves	Crush and boil in water for oral use and topical wash	Skin rash
Pachycarpus concolor E. May.	Asclepiadea	Itshongwe	Root	Infusion	Stomach ache, gastritis
Pentanisia prunelloides	Rubiaceae	Isicimamlilo,	Root	Crush and boil in water	Bacterial and viral

[4]Local names are in IsiXhosa except otherwise stated; A=Afrikaans; E=English; Z=Zulu

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Scientific name	Family	Vernacular[4] names	Part used/ extracted	Preparation	Uses
		Itshamlilo			infections, inflammation, skin rash
Ranunculus multifidus Forssk	Ranunculaceae	Igangashane	Root	Decoction (oral and enemas)	STIs, genital sores, warts, haemorrhoid
Spirostachys africana Sond.	Euphorbiaceae	Mthombothi	Stem bark	Crush and boil in water, take infusion while cold	Diarhoea, dysentery
Lessertia frutescens (formerly *Sutherlandia frutescens)*	Fabaceae	Cancer bush (E), Umnwele	Leaves	Boil in water to make infusions, allow to cool; whole	Rheumatism, stomach and liver problems, diarrhoea, cancer, immune boosting
Tulbaghia violacea (Harv)	Amaryllidaceae	Wild garlic (E), wildenoflok (A), Isivumba,	Bulb	Crush, boil in water to make infusion	Cold infusion to douche children; hot infusion for coughs, fever; as purgative
Zantedeschia aethiopica L. Spreng.	Araceae	Inyibiba	Leaves	Warm leaves as plasters, infusion for oral use	Dressing for wounds, boils, sores and minor burns. Oral use to aid wound healing

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4.5. Published studies on the selected plants

A large number of studies have been conducted on the identified plants investigating their potential for therapeutic effects. Of the 15 plants identified in this study, *Hypoxis hemerocallidea*, *Lessertia frutescens* and *Tulbaghia violacea* are popular in the literature for the management of HIV/AIDS-related disorders.

Hypoxoside, sterols and sterolins are important components of *Hypoxis hemerocallidea* that have been identified and thought to be responsible for pharmacological benefits (Albrecht et al., 1995). A geophyte with bright yellow flowers giving it its common name 'yellow stars', *H. hemerocallidea* has found use traditionally in the treatment of benign prostatic hyperplasia in South Africa (Buck, 1996; Lowe and Ku, 1996; Marandola et al., 1997; Owira and Ojewole, 2009); as purgative and delirium remedy among the Zulus of South Africa (Msonthi and Magombo, 1983); used to treat abdominal pain, fever, anorexia and vomiting by the Karanga people of Southern Africa (Watt and Breyer-Brandwijk, 1962; Mogatle et al., 2008); for uterine cancer in Swaziland (Amusan et al., 1995); and for sexual-related disorders in Mozambique (Banderia et al., 2001). Its major phytochemicals, hypoxoside and rooperol have been investigated for cancer therapy (Smit et al., 1995). Various other studies have suggested more therapeutic effects derivable from the corm African potato (Table 11).

Table 11: Published studies of the therapeutic effects of *H. hemerocallidea*

Effects investigated	**Methodology**	**Reference**
Anti-inflammatory effects	Aqueous and methanolic extracts of the corm in rat model	Ojewole, 2002
Hypoglycaemic activity	Aqueous extract of the corm in rat model	Mahomed and Ojewole, 2003
Antinociceptive, antidiabetic and anti-inflammatory effects	Aqueous extract of the corm in mice and rat models	Ojewole, 2006
Hypotensive and cardiodepressant activity	Corm extract isolated guinea pig cardiac muscle (*in vitro*) and on hypertensive rats (*in vivo*)	Ojewole et al., 2006
Antioxidant activity	*In vitro* investigation of hypoxoside, rooperol (both phytochemical constituent) and aqueous preparation on rat liver tissue	Nair et al., 2007; Laporta et al., 2007
Anticonvulsant activity	Aqueous extract of corm in rat model	Ojewole, 2008
Uterolytic effect	Aqueous extract of corm on uterine horns isolated from rats and guinea pigs	Nyinawumuntu et al., 2008
Antidiarrheal activity	Aqueous extract of corm in mice	Ojewole, 2009
Bronchorelaxant effect	Aqueous extract of corm on tracheal smooth muscle isolated from guinea pig	Ojewole et al., 2009
Prevention and treatment of inflammatory GIT disorders	Methanolic extract in mice	Liu et al., 2010
Antimicrobial activity	Aqueous, ethanolic and petroleum ether extract of leaf and corm	Ncube et al., 2012
Effective in the treatment of benign prostatic hyperplasia	Multicentric, placebo-controlled, double-blind clinical trials using beta-sitosterol, a bioactive component	Berges et al., 1995; Klippel et al., 1997
Anti-cancer activity	Human studies using hypoxoside	Albrecht et al., 1995

Widely known as cancer bush or kankerbos (Afrikaans) because of its traditional use in the management of cancer, *Lessertia frutescens* is one of the most reputable herbal remedies widely used among patients with HIV/AIDS to boost immunity (Prevoo et al., 2004; 2008). Its decoction is employed in the treatment of open wounds, fever, chicken pox, gastrointestinal cramps, rheumatism, heartburn, haemorrhoids, diabetes, inflammation and eye infections among indigenous South Africans (Van Wyk and Albrecht, 2008). It has been investigated for many of the anecdotal claims. Studies have suggested some possible anti-retroviral (Harnett

et al., 2005; Minocha et al., 2011), anti-cancer (Stander et al., 2007; 2009; Skerman et al., 2011), anti-diabetic (Chadwick et al., 2006; Mackenzie et al., 2009; 2012; anti-inflammatory (Ojewole, 2004), anti-infective (Katerere and Eloff, 2005) anti-stress (Prevoo et al., 2004) and anti-convulsant effects (Ojewole, 2008).

Tulbaghia violacea is traditionally used for the treatment of respiratory disorders including tuberculosis, asthma; gastrointestinal ailments, oesophageal cancer, fungal infection, fever and colds (Van den Heever et al., 2008). The close association between HIV/AIDS and tuberculosis has made it popular among people living with HIV/AIDS in South Africa. It contains a C–S lyase, cysteine and tetrathiaoctane derivatives (Kubeca et al., 2002). It inhibits angiotensin converting enzyme (ACE) supporting its use in hypertension (Duncan et al., 1999). Its extracts inhibited the growth of cancer cells and induced apoptosis in an *in vitro* study (Bungu et al., 2006). Other studies have suggested its anthelmintic (McGaw et al., 2000) and antifungal activity especially against *Candida albicans*, a common cause of infection in HIV/AIDS patients (Motsei et al., 2003; Thamburan et al., 2006).

Spirostachys africana is widely distributed in the vegetations of Southern Africa. A number of phytochemicals including stachenone, diosphenol and diterpenoid derivatives have been isolated and identified as active constituents (Duri et al., 1992). The latex is traditionally used in South Africa and Zimbabwe as purgative, to stimulate emesis, and manage infective diarrhoea and dysentery (Gelfand et al., 1995); as antimalarial in Tanzania and Mozambique and as topical anti-infective in South Africa (Munkombwe et al., 1997; 1998). It is used in South Africa, especially among the IsiXhosa-speaking people for the topical treatment of infantile cradle cap known locally as *ishimca* (Beach et al., 2010). *In vitro* studies have shown antimicrobial activity of isolated compounds against *Escherichia coli, Salmonella typhi, Shigella dysentery, Staphylococcus aureus* and *Vibrio cholera* (Mathabe et al., 2006; 2008). A clinical study of the antimalarial preparation from *S. africana* conducted in Mozambique reported significant reduction in parasitaemia (Jurg et al., 1991).

Bowiea volubilis is known variously as climbing onion, ugibisisila or iguleni (isiZulu), umgaqana (IsiXhosa), gibizisila (IsiSwati) and Knolklimop (Afrikaans). It is widely sought after by indigenous South Africans for numerous medical conditions including dermatological disorders, sore eyes, urinary complications, infertility, facilitation and induction of abortion/abortion (Steenkamp, 2003). Some studies have claimed that its extracts demonstrated moderate *in vitro* antibacterial and antifungal activity (Buwa et al., 2006; Van Vuuren and Naidoo, 2010).

The leaves of *Zantedeschia aethiopica* contain triterpenes, α-linolenic acid, galactolipids and sterols which possess activity against algae (Greca et al., 1998). Kee and co-workers (2008) reported that its extracts inhibited inhibition of thrombin-induced clotting in an *in vitro* study.

Chemopodium album is a popular edible herb known locally as Imbikilicane (IsiZulu). It is consumed nutritionally and for gastrointestinal complaints. *In vitro* antioxidant and anti-inflammatory activity of its extracts has been reported (Lindsey et al., 2002).

Alepidea amatymbica is one of the popular medicinal plants used for the treatment of abdominal disorders, constipation, respiratory tract infections and cold. Mulaudzi and co-workers (2009) reported that its extracts demonstrated *in vitro* antibacterial and anti-inflammatory activity.

The leaves and bark of *Acacia karroo* contain proanthocyanidin and flavonols which possess antioxidant analgesic and membrane-stabilizing properties (Malan and Swartz, 1995; Adedapo et al., 2008).

Emex australis is a common weed in South Africa used traditionally for gastrointestinal disorders. It possesses anti-inflammatory properties (Akula and Odhav, 2008).

Capparis sepiaria contains α, β-amyrin, erythrodiol, taraxasterol, triterpin alcohol, betulin, β-sitosterol, n-octacosanol, glycosides, alkaloids and amino acids (Rajesh et al., 2010). It is used in the treatment of oral ulcers (Hebbar et al., 2004). A study in animals has reported glucose-lowering activity in extracts from its leaves (Selvamani et al., 2008).

Pentanisia prunelloides is used traditionally among the Zulus to facilitate labour. It has been shown to exhibit direct smooth muscle activity on rat uterus and ileum preparations (Kaido et al., 1997). Its extract was reported to inhibit the activitity of cyclooxygenasein an *in vitro* study; demonstrate antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*; and inhibit viral replication of influenza A virus (Yff et al., 2002).

4.6. Discussion

Apart from the identified medicinal herbs commonly used by the THP, the traditional health practices of the THPs demonstrated persistence of culture and tradition despite changes in location. This is because the THPs source their medicinal herbs from Eastern Cape on

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regular and seasonal basis. Thus strong popularity of traditional health practices in Eastern Cape is carried through to these Black communities.

H. hemerocallidea, S. frutescens and *T. violacea* are generally associated with the traditional management of HIV/AIDS in South Africa. Besides these three, most of the other plants identified in this study are often administered for other conditions. Due to the presence of co-morbidities in HIV/AIDS patients, especially opportunistic infections, people living with HIV/AIDS consume them due to the various anecdotal reports of therapeutic benefit. In traditional health practice, precise instruction for medicinal herb use is therefore important. Phytochemical studies usually show significant variation in the chemical constituents of the different plant parts. This may explain the anecdotal belief in different indications for different parts of the same plants.

Infusions and decoctions are the most common herbal preparations used by the THPs. Infusions are made usually from leaves in hot water, left or shaken for about 30 minutes to few hours while decoction involves boiling usually harder plant parts like roots, bark and stem. All preparations are taken orally. In addition, decoction from *Kedrostis africana* is applied as topical wash for skin rash while extracts of *Ranunculus multifidus* are used as enemas. The involvement of multiple dosage routes for the same dosage formulation is a demonstration of the flexibility and sophistication of traditional medicine. All the traditional indications for the identified plants have well-established conventional drug treatment. However, there are insufficient data from relevant clinical studies to verify the effectiveness of the herbal preparations.

The literature search has demonstrated the extent of studies conducted on these plants. Apart from *Kedrostis Africana*, the search for which yielded no relevant study to the therapeutic claims, the identified plants in this study have been studied to some extent. Such studies are limited to *in vitro* and limited animal studies. Safety data on the use of the medicinal herbs are still not available.

Most importantly, the effect of the identified medicinal herbs on metabolic enzymes and drug transporters is unknown. In the event of concomitant use conventional standard drug therapy of the respective indications, pharmacokinetic herb-drug interaction is a source of clinical concern (see Chapter 2). This has been the basis for the selection of these medicinal herbs for HDI assessment in this study.

CHAPTER FIVE

INVESTIGATION OF THE INHIBITORY EFFECT OF SELECTED MEDICINAL HERBS ON CYP1A2, CYP2C9, CYP2C19 AND CYP3A4

5.0. Summary

The potential of the crude extracts of the 15 selected medicinal herbs to inhibit CYP1A2, CYP2C9, CYP2C19 and CYP3A4 was assessed using phenacetin *O*-dealkylation diclofenac 4'-hydroxylation, S-mephenytoin 4'-hydroxylation and testosterone 6β-hydroxylation respectively as probe reactions. The inhibitory effect, where observed, was quantified and expressed in IC_{50} (the concentration of extracts that caused 50% inhibition of CYP activity) as presented in Table 12.

Table 12: Summary of the inhibitory effects of the medicinal herbs on CYP1A2, CYP2C9, CYP2C19 and CYP3A4

Medicinal plant	IC_{50} value (µg/mL)			
	CYP1A2	CYP2C9	CYP2C19	CYP3A4
Lessertia frutescens (methanolic)	35.3 ± 3.6	24.4 ± 2.2	42.1 ± 3.2	26.4 ± 2.0
Lessertia frutescens (aqueous)	4.3 ± 0.3	5.9 ± 2.3	10.0 ± 0.8	32.6 ± 3.5
Hypoxis hemerocallidea	29.0 ± 6.0	-	220.4 ± 15.2	20.4 ± 1.6
Spirostachys africana	14.3 ± 0.6	-	-	47.4 ± 2.4
Bowiea volubilis	92.3 ± 5.5	-	>1000	8.1 ± 0.6
Zantedeschia aethiopica	>1000	327 ± 34.1	87.8 ± 10.1	11.7 ± 2.2
Chenopodium album	84.8 ± 7.5	-	51.4 ± 4.3	230 ± 5.1
Kedrostis africana	86.6 ± 2.6	16.6 ± 4.6	157.0 ± 8.8	1.3 ± 0.2
Alepidea amatymbica	712.9 ± 71.3	96.3 ± 9.5	>1000	-
Acacia karroo	120 ± 17	115 ± 11.3	32.9 ± 3.2	1.9 ± 0.3
Emex australis	-	97.4 ± 8.1	>1000	7.2 ± 0.6
Pachycarpus concolor	-	27.8 ± 5.7	35.6 ± 5.7	8.8 ± 1.1
Ranunculus multifidus	226.4 ± 42.3	255 ± 21.6	20.3 ± 2.3	10.1 ± 1.8
Capparis sepiaria	378 ± 45	19.3 ± 4.6	7.3 ± 1.4	3.1 ± 0.1
Pentanisia prunelloides	-	6.8 ± 1.1	>1000	31.6 ± 2.8
Tulbaghia violacea	767.4 ± 10.8	921 ± 15.3	>1000	>1000

(-) indicates the absence of inhibition

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5.1. Introduction

5.1.1. Cytochrome P450 1A2

The CYP1 family is made up of three subfamilies CYP1A1, CYP1A2 and CYP1B1. All three are transcriptionally controlled by the aryl hydrocarbon receptor-aryl hydrocarbon receptor nuclear translocator (AHR-ARNT) and inducible by polycyclic aromatic hydrocarbons (PAH) and smoking (Schmidt and Bradfield, 1996; Zevin and Benowitz, 1999). The expression of CYP1A1 in humans is generally low, with extrahepatic expressions in the lungs, lymphocytes, mammary gland, and placenta (Raunio et al., 1999). There is very little involvement of CYP1A1 in drug metabolism. It is however significant in its roles as activators of procarcinogens, thus playing important role in chemically-induced carcinogenesis (Ji et al., 2012). Like CYP1A1, the expression of CYP1B1 is mainly extrahepatic mainly in kidney, prostate, mammary gland, and ovary with little involvement in the metabolism of xenobiotics (Tang et al., 1999).

CYP1A2 has been shown to be exclusively expressed in the liver constituting about 13-15% of the total hepatic CYP content (Shimada et al., 1994; Martignoni et al., 2006). It is the primary enzyme responsible for the phase I metabolism of a number of drugs including tacrine, theophylline, and clozapine. Inducers of CYP1A2 include cigarette smoke, indole-3-carbinol-containing vegetables, charbroiled meat, rifampicin and phenytoin.

Phenacetin, as well as caffeine and theophylline are the most widely used *in vitro* and *in vivo* probe substrates for evaluating the activity of CYP1A2 (Faber et al., 2005). Phenacetin is a prodrug and was a popular analgesic drug in the 70s and 80s (Sanerkin, 1971; Clissold, 1986). Following oral absorption, phenacetin is completely absorbed but undergoes pre-systemic metabolism in the liver. Its first metabolic step is CYP1A2-catalyzed *O*-deethylation to form the active acetaminophen (paracetamol). This makes phenacetin a suitable probe substrate for CYP1A2 metabolic activity. Further metabolism of phenacetin usually includes phase two reactions of metabolite conjugation with glucuronide, sulfate or glutathione to form soluble complexes that are ultimately excreted in the urine.

5.1.2. Cytochrome P450 2C9

The human CYP2 family, made of A, B, C, D, E, F and J subfamilies is a heterogeneous group of enzymes. Those of important interest in the metabolism of xenobiotics are CYP2A6/7, CYP2B6, 2C9, 2C19, CYP2D6 and CYP2E1. CYP2C (mainly 8, 9, 18 and 19)

accounts for about 20% of the total human hepatic CYP (Gerbal-Chaloin et al., 2001; Martignoni et al., 2006).

The subfamily 2C is the second most abundant CYP after 3A representing over 20% of the total CYP present in the human liver. It comprises three active members: 2C8, 2C9 and 2C19 all of which are involved in the metabolism of some endogenous compounds including retinol and retinoic acid (Lewis, 2004)

CYP2C9 is the most abundantly expressed CYP2C isozyme (Rettie and Jones, 2005). Losartan, phenytoin, tolbutamide, and S-warfarin are some of the drugs metabolized primarily by CYP2C9. It is induced by rifampicin while amiodarone, fluconazole, and sulphaphenazole are known inhibitors.

The gene coding for the CYP2C9 enzyme has been shown to be polymorphic displaying significant functional variants. Thus, poor metabolizers, as a result of low CYP2C9 expression/activity are at risk of adverse drug reaction due to the accumulation of CYP2C9 substrates especially those with narrow therapeutic index such as phenytoin, S-warfarin, tolbutamide and glipizide (Pirmohamed and Park, 2003; Ninomiya et al., 2000). Other drugs metabolized by CYP2C9 are Irbesartan, losartan, cyclophosphamide, tamoxifen, fluvastatin, celecoxib, diclofenac, ibuprofen, lornoxicam, meloxicam, naproxen, glibenclamide, glimepiride, sulfonylurea, glipizide and tolbutamide, proton pump inhibitors (omeprazole, lansoprazole, pantoprazole, and rabeprazole), tricyclic antidepressants (imipramine, amitriptyline, and nortriptyline), selective serotonin reuptake inhibitors (citalopram, fluoxetine, and sertraline, moclobemide (an antidepressant), benzodiazepines (diazepam).

One of the most well established in vitro CYP2C9-specific probe substrate is diclofenac (Konecný et al., 2007; Tai et al., 2008). CYP2C9 is responsible for the 4'-hydroxylation of diclofenac. The generation of 4'-hydroxydiclofenac in an in vitro CYP2C9 medium from diclofenac is a typical CYP2C9 probe.

5.1.3. Cytochrome P450 2C19

CYP2C19 is one of the important CYP in drug biotransformation. It catalyzed the phase I metabolism of approximately 10% of commonly used drugs (Gardiner and Begg, 2006). Important drugs metabolized by CYP2C19 include proton pump inhibitors (omeprazole, lansoprazole, pantoprazole, and rabeprazole), tricyclic antidepressants (amitriptyline, imipramine, and nortriptyline) and selective serotonin reuptake inhibitors (citalopram,

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fluoxetine, and sertraline). Others are benzodiazepines (diazepam, flunitrazepam, quazepam, clobazam), barbiturates (hexobarbital, mephobarbital, phenobarbital), moclobemide, phenytoin, S-mephenytoin, bortezomib, voriconazole, selegiline, proguanil and nelfinavir (Zhou et al., 2009).

S-mephenytoin has been established, and used extensively as an *in vitro* probe substrate for CYP2C19 metabolic activity (Goldstein et al., 1994; Lee and Kim, 2011).

5.1.4. Cytochrome P450 3A4

CYP3A is the only subfamily of the human CYP3 family and is made of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 isoforms. CYP3A4 is expressed in the liver and in extrahepatic tissues. CYP3A4/5 accounts for about 80% of intestinal CYP where it is responsible for presystemic drug metabolism while CYP3A7 is found in fetal liver (Paine et al., 2006).

The CYP3A4 enzyme is the most important drug-metabolizing CYP accounting for about 40% of the total hepatic CYP (although the levels may vary 40-fold among individuals), and 50% of all CYP-mediated drug metabolism (Rendic, 2002; Ferguson and Tyndale, 2011; Singh et al., 2011). The inhibition of both intestinal and liver CYP3A4 has been shown to contribute to drug-drug interaction (Galetin et al., 2007). Examples of CYP3A4 substrates include the viral protease inhibitors, calcium channel blockers, statins, quinidine, erythromycin, cyclosporin, midazolam and triazolam. It is also responsible for the metabolism of endogenous substances such as testosterone, androstenedione and progesterone. .

About 40-fold inter-individual variation in the hepatic expression and metabolic function of human hepatic CYP3A4 has been reported (Lamba et al., 2002). It is therefore important to anticipate variation in the influence of the inhibition of CYP3A4 on the pharmacokinetics of administered substrates in different population groups.

Midazolam, testosterone, erythromycin, nifedipine, and felodipine have been used as probes for CYP3A4 activity with overlapping substrate specificity with CYP3A5 (Table 13). Rifampicin, dexamethasone and phenobarbital are known inducers of CYP3A4 while ketoconazole is a typical inhibitor. Due to the importance of CYP3A4 in drug metabolism and the genetic polymorphism associated with its expression, it is a recommended practice to investigate it with two different substrates (Bjornsson et al., 2003).

Table 13: Some validated probes substrates of CYP3A4/5 (adapted and updated from Lakhman et al., 2009)

Probe substrates	**Metabolized by**	
	CYP3A4	**CYP3A5**
Alfentanil	Yes	Yes
Alprazolam	No	Yes
Erythromycin	Yes	No
Cortisol	Yes	Yes
Midazolam	Yes	Yes
Quinidine	Yes	No
Tacrolimus	No	Yes
Testosterone	Yes	Yes
Triazolam	Yes	Yes
Vincristine	No	Yes

5.2. Aims and objectives

The aim of the present study was to assess the inhibitory activity of the crude extracts of the selected medicinal plants identified in Chapter Four on CYP1A2, CYP2C9, CYP2C19 and CYP3A4.

Specific objectives were to

1. optimize *in vitro* CYP-catalyzed reaction in HLM and using phenacetin and diclofenac as probe substrates
2. determine the enzyme kinetic parameters from such optimized technique in 1 above
3. apply the optimized methods to investigate the influence of the herbal extracts on CYP1A2, CYP2C9, CYP2C19 and CYP3A4 activity compared to furafylline, sulfaphenazole, ticlopidine and ketoconazole, the respective standard inhibitors of CYP1A2, CYP2C9, CYP2C19 and CYP3A4
4. assess time-dependent inhibition of CYP3A4-catalyzed metabolism of midazolam by extracts of *Lessertia frutescens*.

5.3. Materials and Methods

5.3.1. Materials

5.3.1.1. Medicinal herbs

Medicinal herbs employed for this study are those identified in Chapter 4 (Table 10).

5.3.1.2. Chemical compounds

The necessary chemical compounds were obtained as indicated: 1'-hydroxybufuralol maleate, 1'-hydroxymidazolam, 4'-hydroxymephenytoin, 6β-hydroxytestosterone, bufuralol hydrochloride and midazolam from Ultrafine Chemicals (Pty) Ltd (Manchester, UK); 6-hydroxychlorzoxazone, 7-hydroxycoumarin, acetaminophen, bupropion, chlorzoxazone, coumarin, furafylline, NADPH, estradiol-17β-D-glucuronide (sodium salt), paclitaxel, tranylcypromine, troleandomycin (TAO), warfarin, rifamycin, thioconazole, digoxin, cyclosporin A and testosterone from Sigma-Aldrich (Pty) Ltd (St. Louis, USA); dimethylsulfoxide (DMSO), di-potassium hydrogen phosphate (K_2HPO_4), acetonitrile, formic acid and methanol from Merck Chemicals (Pty) Ltd (Darmstadt, Germany); [$^{13}C_6$] 4' hydroxydiclofenac, [$^{13}C_6$] 7 hydroxycoumarin, [$^{2}H_3$] 4'-hydroxymephenytoin, diclofenac and paroxetine from Novartis Pharma AG (Basel, Switzerland); hydroxybupropion, [$^{2}H_6$] hydroxybupropion and [$^{2}H_9$] 1'-hydroxybufuralol from BD Biosciences (Pty) Ltd (San Jose, USA); 6α-hydroxypaclitaxel from Gentest BD Biosciences (Woburn, USA); [$^{2}H_3$] 6β-hydroxytestosterone, [$^{2}H_4$] 1'-hydroxymidazolam from Cerilliant Chemicals (Pty) Ltd (Texas, USA); phenacetin, magnesium chloride hexahydrate, ticlopidine hydrochloride and potassium dihydrogen phosphate (KH_2PO_4) from Fluka Chemicals (Pty) Ltd (Buchs, Switzerland); 4'-hydroxydiclofenac from Calbiochem (Pty) Ltd (La Jolla, USA), (3S,6S,12aS)-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1',2':1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester (Ko143) from Tocris Bioscience (Pty) Ltd (Ellisville, MI); 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-2 (PhIP) and its radio-labeled from (1.85 MBq/nmol, radiochemical purity >99%) from Toronto Research Chemicals (Pty) Ltd (North York, Canada); radio-labeled [^{3}H]Digoxin (1.103 MBq/nmol, radiochemical purity >97%) and [^{3}H]estradiol-17β-D-glucuronide (1.72 MBq/nmol, radiochemical purity >97% from Perkin Elmer Radiochemicals (Pty) Ltd (Waltham, MA, USA); zosuquidar trihydrochloride (LY335979) from Chembiotek (Pty) Ltd (Kolkata, India). Other chemicals and reagents employed were of analytical grade.

5.3.1.3. Assay enzymes and cells

Pooled mixed gender HLM prepared from 50 individual donors with total CYP and cytochrome b_5 content of 290 pmol/mg protein and 790 pmol/mg protein respectively was obtained from Gentest BD Biosciences (Woburn, USA). The Catalytic activities of enzymes were provided by the manufacturer (Table 14).

Table 14: Enzyme activities of human liver microsomes

Enzyme	Assay	Activity (pmol/(mg protein • min))
CYP1A2	phenacetin *O*-deethylase	580
CYP2A6	coumarin 7-hydroxylase	1100
CYP2B6	(S)-mephenytoin N-demethylase	90
CYP2C8	paclitaxel 6α-hydroxylase	69
CYP2D6	bufuralol 1'-hydroxylase	68
CYP2E1	chlorzoxazone 6-hydroxylase	2500
CYP3A4/5	testosterone 6β-hydroxylase	5800
CYP4A11	lauric acid 12-hydroxylase	1700
FMO	methyl p-tolyl sulfide oxidase	1000
UGT1A1	estradiol 3-glucuronidation	1200
UGT1A4	trifluoperazine glucuronidation	740
UGT1A9	propofol glucuronidation	5200
P450 oxidoreductase	cytochrome c reductase	340

5.3.2. Methods

5.3.2.1. Extraction of herbal products

Based on the obtained information on method of use of the medicinal herbs identified in Chapter 4 (Table 10), the dried herbal materials were powdered and extracted with water. In addition, *Lessertia* was extracted with 60% methanol.

Dried samples (leaves, corm, root, back or stem, as appropriate; Table 10) were powdered, weighed, and stirred in distilled water (50 mg/mL, 100ºC, 2 hr) and left to extract for 24 hr. The extracts were centrifuged, filtered through a 0.45 µm membrane filter system and freeze dried. Stock solutions of 20 mg/mL of dried extracts were made in water and stored at -20°C.

Lessertia is consumed both as aqueous decoction and often as whole leave (available in commercial capsule preparations of dried leaves). The air-dried and powdered leaves of *Lessertia* were extracted with water/methanol (40/60, 20 g/mL) in round bottom flask. After the initial constant stirring for 2 hr, the mixture was allowed to extract for 24 hr, decanted, and centrifuged (20,000 *g*, 5min). The supernatant was filtered (0.45 µL; Whatman International LTD, Maidstone, England) and dried using a vacuum rotary evaporator and freeze drying. The dried extract was reconstituted in methanol and stored at -20ºC for further use.

5.3.2.2. Preparation of CYP substrates and microsomal dilutions for incubation

Solutions of phenacetin, diclofenac, *S*-mephenytoin, testosterone and midazolam were prepared in 50% methanol over a range of 0.1-100 mM (0.1, 0.5, 1, 2, 4, 7.5 and 10 mM). Frozen (-80°C) HLM (20 mg/mL) was thawed in water at room temperature (25°C) and appropriate volume was measured with a micropipette, diluted in 0.5 M phosphate buffer (pH 7.4) to produce a final protein concentration of 0.4 mg/mL. This solution was kept on ice until further use.

5.3.2.3. Optimization of in vitro metabolism

Dilution of the test substance (phenacetin and diclofenac) stock solutions was done in methanol. Incubations were conducted in a 96-well plate format at 37°C using an IS89 96-well plate incubator (Wesbart, Leimuiden, The Netherlands). The final methanol concentration in the incubations was <1% ($^{v}/_{v}$). The wells were filled with 250µL of the prepared HLM mixture. 5µL of each concentration of prepared phenacetin and diclofenac solutions was pre-incubated with 250µL HLM mixture. This was followed by the initiation of metabolic reaction through the addition of 245µL NADPH solution. The total volume of the incubation was 500µL with the final substrate concentration of 1, 5, 10, 20, 40, 75 and 100 µM. Samples were incubated for 20 min at 37°C. Metabolic reactions were terminated by the addition of 10 µL 50% formic acid. By general standard, samples were tested in duplicates and repeated at a different day. The intra-plate, and inter-day variations were always less that 10% (which is the acceptable standard (Bjornsson et al., 2003). The composition and conditions of the incubation mixtures are summarized in Table 15.

Table 15: Microsomal incubation conditions for CYP1A2, CYP2C9, CYP2C19 and CYP3A4-catalyzed reactions

Parameters	Values
Concentration of potassium phosphate buffer, pH 7.4 in incubation mixture	50 mM
Concentration of magnesium chloride in incubation mixture	5 mM
Incubation pH	7.4
Final graded concentration of herbal extracts	0 – 100 µg/mL
Concentration of DMSO/methanol in incubation mixture	0.5%($^{v}/_{v}$)
Final concentration of NADPH in the incubation mixture	1 mM
Incubation time	20 min

CYP1A2

CYP1A2-specific metabolite	Acetaminophen
HLM Concentration	0.2mg protein/mL
Positive control	Furafylline
Initial phenacetin concentration	10 µM
Literature K_m values	14[a], 31[b], 54[c]

CYP2C9

CYP2C9-specific metabolite	4'-hydroxydiclofenac
HLM Concentration	0.1mg protein/mL
Positive control	Sulfaphenazole
Initial diclofenac concentration	5 µM
Literature K_m values	9.0 µM[d]

CYP2C19

CYP2C19-specific metabolite	4'-hydroxy-mephenytoin
HLM Concentration	0.5 mg protein/mL
Positive control	Ticlopidine
Initial *S*-mephenytoinl concentration	30 µM
Literature K_m values	51[e], 42[f], 31[g]

CYP3A4

CYP3A4-specific metabolite	6β-hydroxy-testosterone, 1′-hydroxymidazolam
HLM Concentration (mg protein/mL)	0.2 (testosterone), 0.1 (midazolam)
Positive control	Ketoconazole
Initial substrate concentration (µM)	30 (testosterone), 1 (midazolam)
Literature K_m values	50-60[h], 51[i] (testosterone), 3.3[j], 2.5-5.6[k] (midazolam)

[a]Brøsen et al., 1993; [b]Venkatakrishnan et al., 1998a; [c]Rodrigues et al., 1997; [d]Bort et al., 1999; [e]Coller et al., 1999; [f]Venkatakrishnan et al., 1998b; [g]Schmider et al., 1996; [h]Draper et al., 1998; [i]Kenworthy et al., 2001; [j]von Moltke et al., 1996; [k]Ghosal et al., 1996

5.3.2.4. Incubations with herbal extracts for IC_{50} determination

Graded concentrations of the herbal extracts were prepared in water (or methanol, as appropriate) such that the addition of 5 µL to the 500 µL incubation mixtures yielded a final

crude extract concentration ranging from 1 – 100 µg/mL respectively. CYP substrate solution was added and the mixture pre-incubated at 37°C for 10 minutes using an IS89 96-well plate incubator (Wesbart, Leimuiden, The Netherlands). Metabolic reactions were initiated by adding NADPH solution and terminated after 20 minutes through the addition of formic acid (10 µL; 50%). All incubations were performed in duplicate. Positive control incubations (with CYP-specific inhibitors (as specified in Table 15) were performed alongside. Negative control incubations were conducted without inhibitors or herbal extracts (0 µg/mL extract concentration). Reactions terminated at time 0 (T0; terminated before the addition of NADP solution) were used as independent controls to ascertain the absence of metabolism before initiation. Herbal samples were tested in duplicates and repeated at a different day. The intra-plate, and inter-day variations were always less that 10% (which is the acceptable standard (see Bjornsson et al., 2003). The final methanol concentration in the incubations was <1% ($^{v}/_{v}$). The concentrations of CYP substrates used were less than the determined or published K_m values (Table 15).

5.3.2.5. Post Incubation and quantitative analysis

LC-MS methods were developed to analyze CYP substrates/metabolites. Total separation and elution of the analytes were achieved within 10 minutes run time. Reproducibility of the quantitative analysis was assessed through repeat injections at different times. Intra-day and inter-day variations were insignificant (less than 5%). LC/MS conditions are summarized in Table 16.

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Table 16: Summary of the LC/MS analytical conditions for the quantitative determination of the metabolites

<table>
<tr><th>Conditions</th><th>Acetaminophen</th><th>4’-hydroxy-diclofenac</th><th>4'-hydroxy-mephenytoin</th><th>6β-hydroxy-testosterone</th><th>1′-hydroxy-midazolam</th></tr>
<tr><td>Mobile phase (plus 0.1% formic acid)</td><td colspan="5">A: Water
B: Acetonitrile</td></tr>
<tr><td>Gradient of mobile phase:</td>
<td><table><tr><th>Time range</th><th>A (%)</th><th>B (%)</th></tr><tr><td>0.0-1.0</td><td>65</td><td>35</td></tr><tr><td>1.0-1.1</td><td>1.0</td><td>99</td></tr><tr><td>1.1-5.5</td><td>1.0</td><td>99</td></tr><tr><td>5.5-5.6</td><td>65</td><td>35</td></tr><tr><td>5.6-10</td><td>65</td><td>35</td></tr></table></td>
<td><table><tr><th>Time range</th><th>A (%)</th><th>B (%)</th></tr><tr><td>0.0-1.0</td><td>50</td><td>50</td></tr><tr><td>1.0-2.0</td><td>5</td><td>95</td></tr><tr><td>2.0-8.0</td><td>5</td><td>95</td></tr><tr><td>8.0-8.1</td><td>50</td><td>50</td></tr><tr><td>8.1-11.</td><td>50</td><td>50</td></tr></table></td>
<td><table><tr><th>Time range</th><th>A (%)</th><th>B (%)</th></tr><tr><td>0.0 - 0.5</td><td>85</td><td>15</td></tr><tr><td>0.5 - 2.0</td><td>55</td><td>15</td></tr><tr><td>2.0 - 4.0</td><td>5</td><td>95</td></tr><tr><td>4.0 - 4.2</td><td>5</td><td>95</td></tr><tr><td>4.2 - 8.0</td><td>85</td><td>15</td></tr><tr><td>8.0</td><td>85</td><td>15</td></tr></table></td>
<td colspan="2">Isocratic 70% A and 30% B</td></tr>
<tr><td>Injection volume (μL)</td><td>2.5</td><td colspan="4">2</td></tr>
<tr><td>Flow speed (μL / min)</td><td colspan="2">300</td><td colspan="3">60</td></tr>
<tr><td>Column type</td><td colspan="5">Luna Phenyl-Hexyl 3 μm, inner dimensions 50 x 1 mm (Phenomenex, Torrance, USA)</td></tr>
<tr><td>Column temperature</td><td colspan="2">40°C</td><td colspan="3">30°C</td></tr>
<tr><td>Nebulizing temperature</td><td colspan="5">375°C</td></tr>
<tr><td>Mode</td><td>Positive</td><td colspan="2">Negative</td><td colspan="2">Positive</td></tr>
<tr><td>Quantifier transition (m/z)</td><td>180.2 / 110.1</td><td>293.88/249.00</td><td>169.4/102.3</td><td>289.2/96.8</td><td>326/291</td></tr>
<tr><td>Qualifier transition(s) (m/z)</td><td>180.2 / 138.1</td><td colspan="3">No qualifier with a suitable intensity</td><td>342/203</td></tr>
<tr><td>Dwell time (msec)</td><td>400</td><td colspan="2">300</td><td colspan="2">200</td></tr>
<tr><td>Retention time (min)</td><td>3.35</td><td>6.65</td><td>6.65</td><td colspan="2">3.2</td></tr>
<tr><td>Total run time (min)</td><td>10</td><td>11</td><td>8</td><td colspan="2">5</td></tr>
<tr><td>Lower limit of detection</td><td>0.1 μM</td><td>0.1 μM</td><td>0.1 μM</td><td>0.05 μM</td><td>0.01 μM</td></tr>
<tr><td>Internal standard</td><td colspan="4">Thiacetazone (Retention time- 3.4 min; Quantifier transitions - 293.88/249.00)</td><td>$[^{2}H_{4}]$ 1′-hydroxymidazolam</td></tr>
</table>

Before chromatographic analysis, samples were subjected to solid-phase extraction using OASIS HLB 96-well plate 30 µm (30 mg) Elution plate (Waters, Milford, USA). This was performed by sequential washing with 1 mL each of water and water-methanol (95/5; v/v) followed by two elutions with 1 mL methanol. The elutes were dried employing the 96-well Micro-DS96 evaporator (Porvair Sciences Ltd., Shepperton, UK) at 37°C and reconstituted in 100 µL of 10% acetonitrile containing 0.1% formic acid for LC-MS analysis.

Enzyme activity was measured in terms of substrate depletion/metabolite formation. The rate of substrate metabolism was profiled against substrate concentrations employing the SigmaPlot® Enzyme Kinetic Software (SigmaPlot: Enzyme Kinetic Module, Systat Software Inc., Chicago, IL, USA) from where the enzyme kinetic parameters (K_m and V_{max}) were determined.

5.3.2.6. Determination of IC_{50} of Extracts

All absolute activities were converted into relative activities by defining the enzymatic activity without addition of inhibitor as 100% and recalculating the other activities relative to this number. The enzyme inhibition parameter (IC_{50}) was calculated by employing the kinetic equation for sigmoid curves (Equation 5.1) where x = concentration; y = relative enzyme activity; and s = slope factor using the SigmaPlot® enzyme kinetic software.

$$y = \frac{100\%}{1 + \left(\frac{x}{IC50}\right)^{s}}$$

Equation 5.1

5.3.2.7. Incubations in HLM for the assessment of time-dependent inhibition

Following the inhibitory effects of some of the extracts on various CYP isozymes, methanolic extracts of *Lessertia frutescens* was assessed for time-dependent inhibition of CYP3A4 activity. For this procedure, all incubation steps were performed in a 96-well plate format at 37°C using a liquid handling workstation epMotion 5075VAC (Eppendorf, Hamburg, Germany). LT (3.13, 6.25, 12.5, 25, 50 and 100 µg/mL) and positive control (5 µM troleandomycin) were added to phosphate buffer. After the addition of HLM (0.5mg/mL protein) and 10 min thermal equilibration, the reactions were initiated by adding NADPH (1mM). The samples were preincubated for 0, 4, 8, 16, 32 and 48 min respectively. All

incubations were done in duplicate. After the preincubation period, 20 µL of the microsomal incubations (corresponding to 0.025 mg/mL protein) were diluted 20-fold with buffer containing the probe substrate and NADPH (in order to minimize competitive inhibition by the test compounds) and incubated with midazolam (20µM) for 6 minutes at 37°C. Potassium phosphate buffer (1mM) was used as negative control. Reactions were terminated by the addition of 20 µL formic acid (50%).

5.3.2.8. Calculation of kinetic parameters for time-dependent inhibition

The enzyme activity in the presence of each inhibitor concentration was determined relative to activity at time t = 0. The resulting data pairs of relative enzyme activities and preincubation times were fitted with an Excel template in a least square approach to the exponential function (Equation 5.2) where y = relative activity; x = preincubation time; a = optimal starting value for relative activity; b = optimal negative rate of inactivation for a given inhibitor concentration

$$y = a \cdot e^{-b \cdot x} \qquad \textit{Equation 5.2}$$

The rate of CYP3A4 inactivation in the absence of test substance was subtracted from all other inactivation rates. The resulting values along with the corresponding inhibitor concentrations were used in a second least square fit to calculate kinetic parameters by linear regression to the Michaelis-Menten–like equation (Equation 5.3) where y = inactivation rate constant; x = inhibitor concentration; KI = inhibitor concentration that produces half maximal inhibition; kinact = maximal inactivation rate constant.

$$y = \frac{kinact \cdot x}{KI + x} \qquad \textit{Equation 5.3}$$

5.3.2.9. Statistical analysis

The percentage inhibition observed for the test extracts is defined in absolute terms. The absolute values were obtained by comparing the metabolite production in the presence of test extracts, to metabolite production in the absence of any inhibitor (test extract or known inhibitors) (Bjornsson et al., 2003). The nonlinear-regression was generated by profiling the extract concentration against observed enzyme activity. The kinetic parameters were determined from the generated plot from the SigmaPlot® software with $R^2 > 0.9$ and $p < 0.05$ as the minimum level of significance.

Positive controls are used in *in vitro* drug metabolism studies for qualitative purposes only. The positive controls (known CYP inhibitors) only provide indications that the enzymes can be inhibited in the experimental set up. By general standard, the use of HLM is for qualitative determination of the ability/potential of chemical substances to inhibit CYP enzymes (Brandon et al., 2003). The results obtained may not be linearly related to *in vivo* situation.

5.4. Results

5.4.1. Extraction yield of the medicinal products

The product of the crude extraction as weighed was documented, and the yield calculated (Table 17)

Table 17: Extraction yield of the medicinal products

Scientific name	Yield (% $^{w}/_{w}$)
Hypoxis hemerocallidea	12.6
Lessertia frutescens	Methanolic: 28.5 aqueous: 20
Spirostachys africana	18.1
Bowiea volubilis	15.6
Zantedeschia aethiopica	15.2
Chenopodium album	17.7
Kedrostis africana	20.6
Alepidea amatymbica	14.5
Acacia karroo	15.3
Emex australis	19.0
Pachycarpus concolor	16.5
Ranunculus multifidus	14.1
Capparis sepiaria	17.3
Pentanisia prunelloides	19.2
Tulbaghia violacea	16.4

5.4.2. Optimization of CYP1A2 and CYP2C9 activity

The rate of phenacetin and diclofenac metabolism was profiled against the initial phenacetin and diclofenac concentrations using both the Michaelis-Menten and Hill enzyme kinetic plot of the SigmaPlot® EK software. The K_m and V_{max} of phenacetin metabolism was determined to be 34.3 µM and 4.9 nmol/min/mg respectively; while those of diclofenac were 7.5 µM and 1.3 nmol/min/mg respectively (Figures 1 – 4).

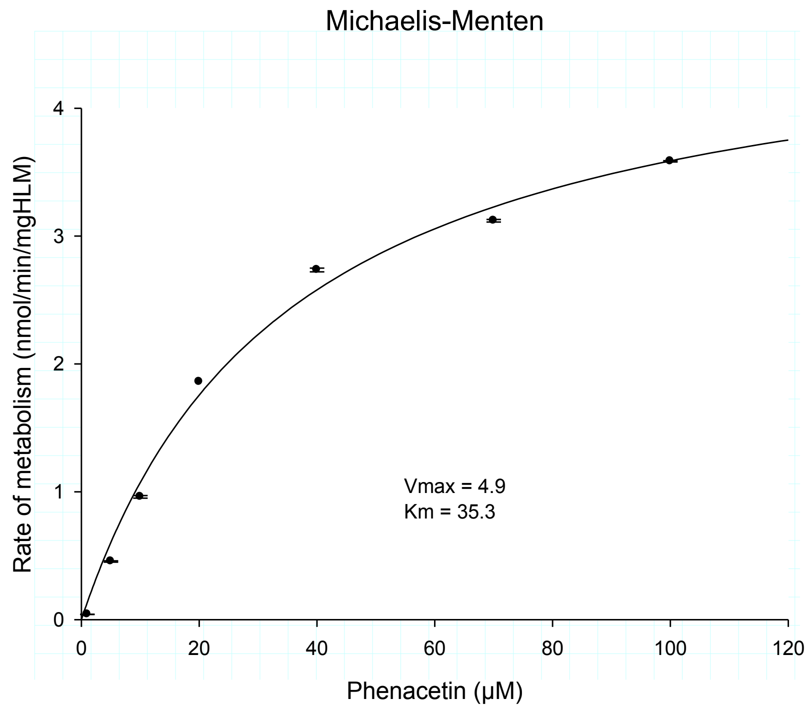

Figure 1: Michaelis-Menten plot of the CYP1A2-catalyzed phenacetin metabolism (R^2 = 0.99)

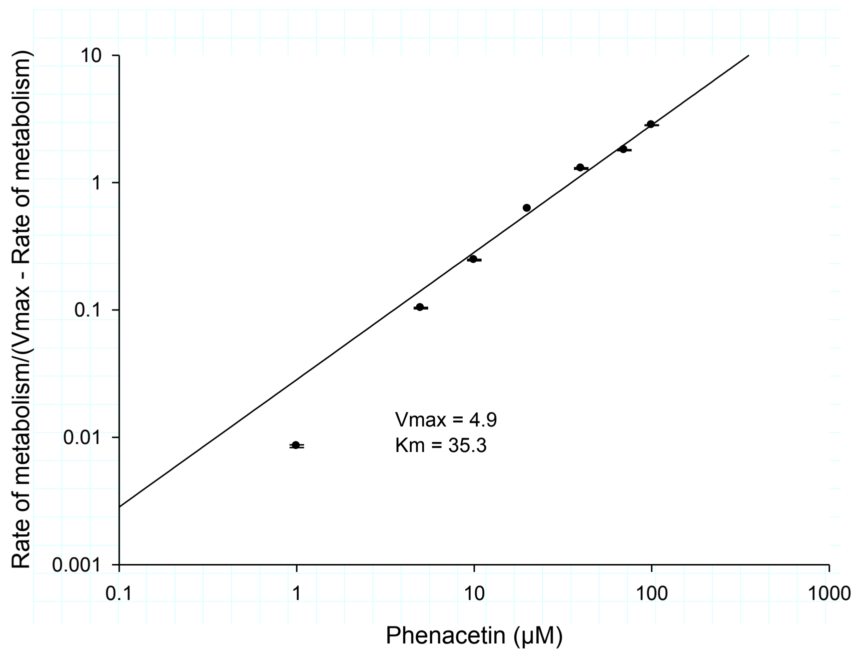

Figure 2: Hill plot of the CYP1A2-catalyzed phenacetin metabolism (R^2 = 0.99)

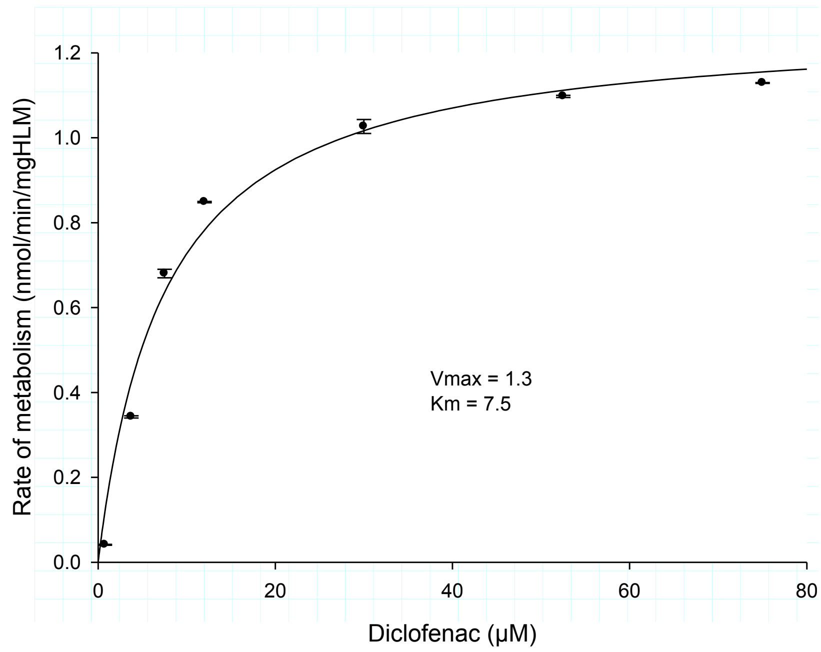

Figure 3: Michaelis-Menten plot of the CYP2C9-catalyzed diclofenac metabolism (R^2 = 0.99)

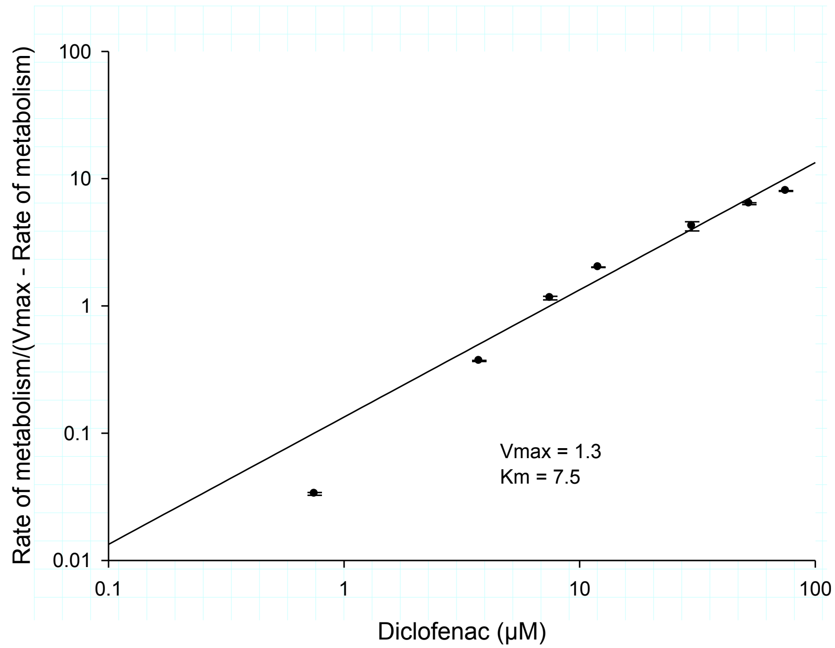

Figure 4: Hill plot of the CYP2C9-catalyzed diclofenac metabolism (R^2 = 0.99)

5.4.3. Influence of the herbal extracts on CYP activity.

The effect of the graded concentrations of the herbal extracts and the positive control (Post Ctrl) were estimated. Results represent the mean of intra-day and inter-day duplicates. Intra-day and inter-day variations were less than 10%. Figures 5 - 8 provide sample bar charts showing the reproducibility and the activity of control incubations.

All the results were profiled in each enzyme group employing the SigmaPlot® Enzyme Kinetic software based on Equation 5.1.from where the IC_{50} values were estimated (Figures 9 - 73).

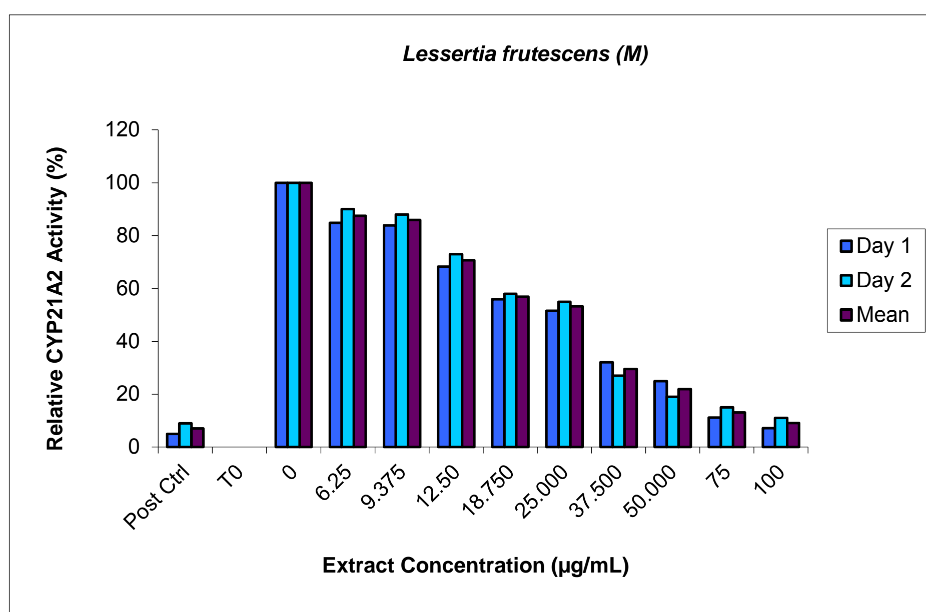

Figure 5: The influence of graded concentrations of crude methanolic extracts of *Lessertia frutescens* on the CYP1A2-catalyzed metabolism of phenacetin

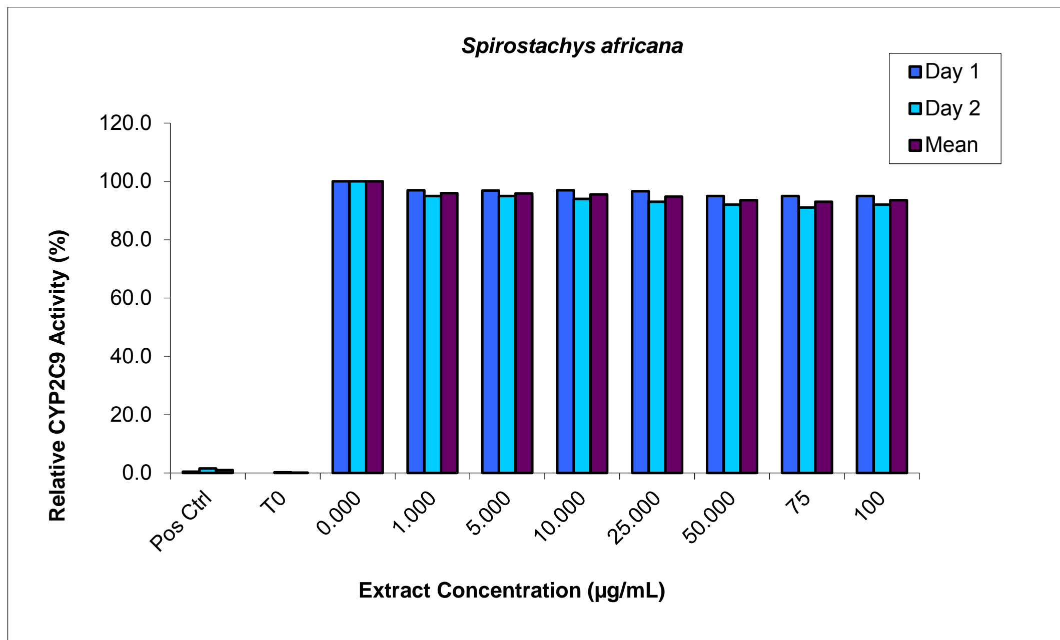

Figure 6: The influence of graded concentrations of crude aqueous extracts of *Spirostachys africana* on the CYP2C9-catalyzed metabolism of diclofenac

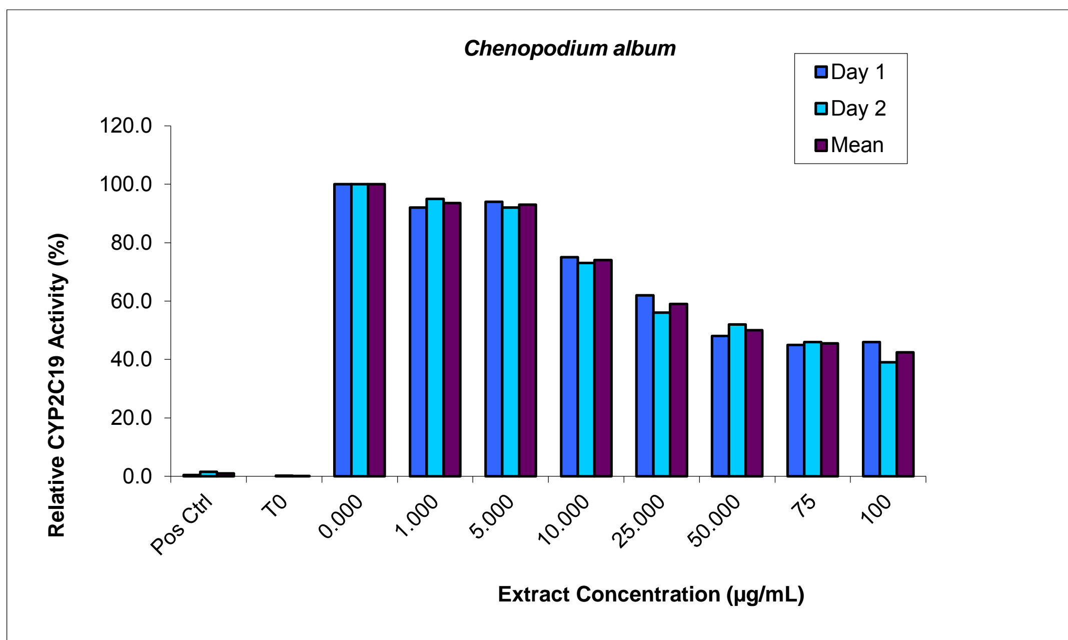

Figure 7: The influence of graded concentrations of crude aqueous extracts of *Chenopodium album* on the CYP2C19-catalyzed metabolism of *S*-mephenytoin

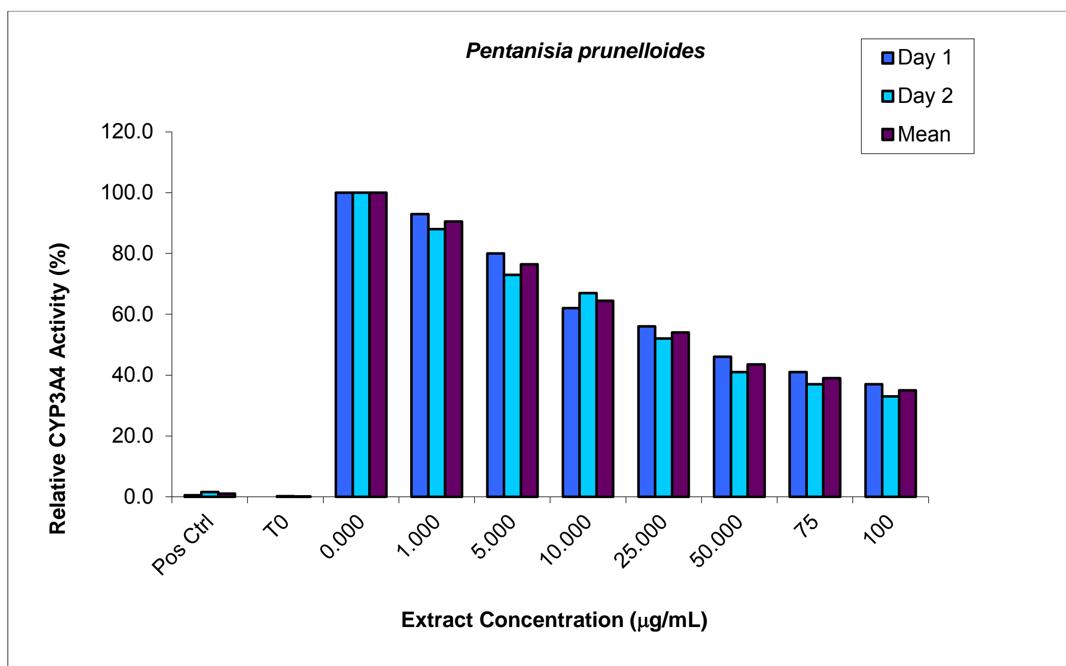

Figure 8: The influence of graded concentrations of crude aqueous extracts of *Pentanisia prunelloides* on the CYP3A4-catalyzed metabolism of testosterone

5.4.3.1. Influence of the herbal extracts on CYP1A2 activity.

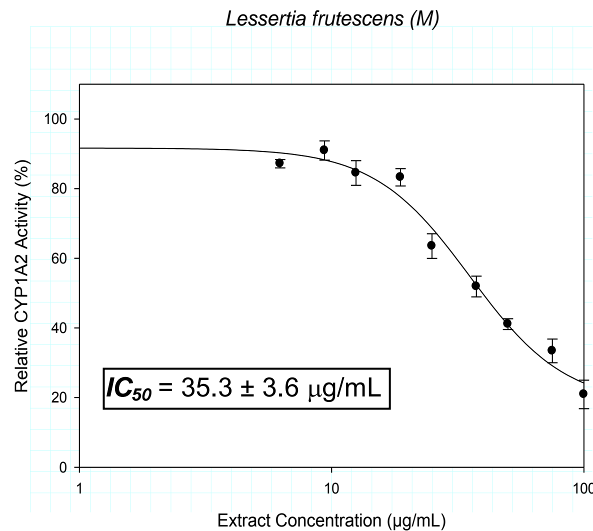

Figure 9: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude methanolic extracts of *Lessertia frutescens* (R^2 = 0.99)

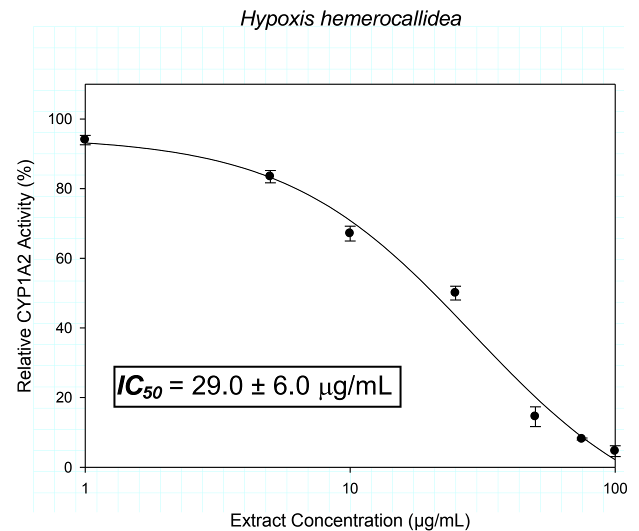

Figure 11: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Hypoxis hemerocallidea* (R^2 = 0.99)

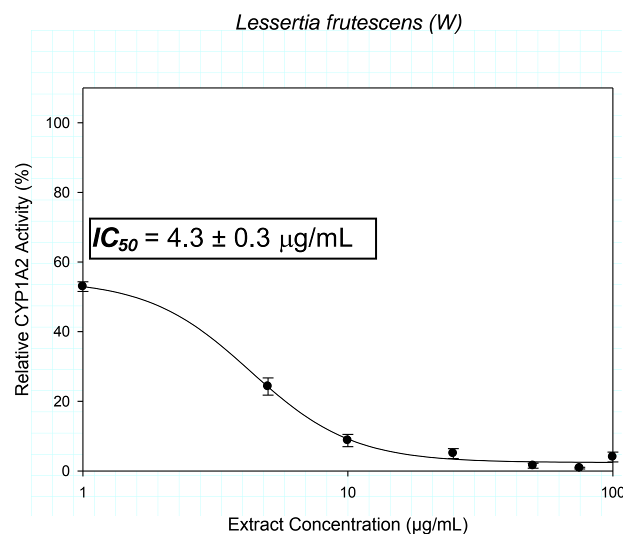

Figure 10: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Lessertia frutescens* (R^2 = 0.99)

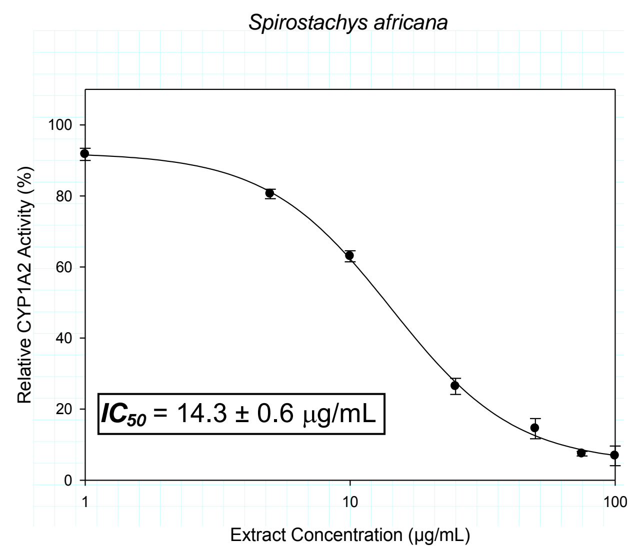

Figure 12: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Spirostachys africana* (R^2 = 0.99)

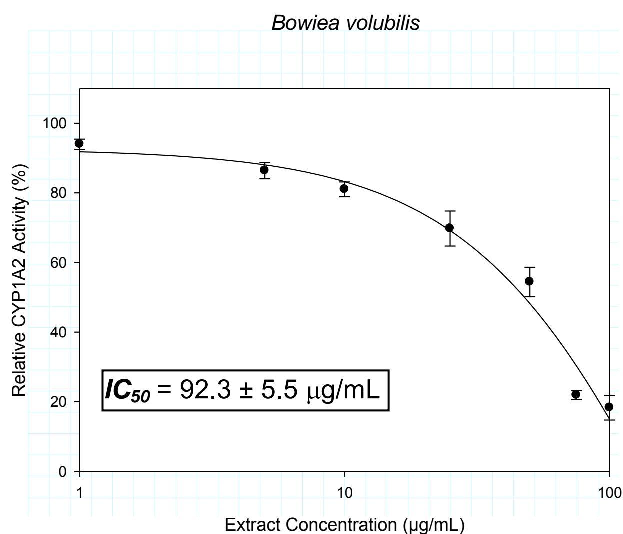

Figure 13: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Bowiea volubilis* (R^2 = 0.99)

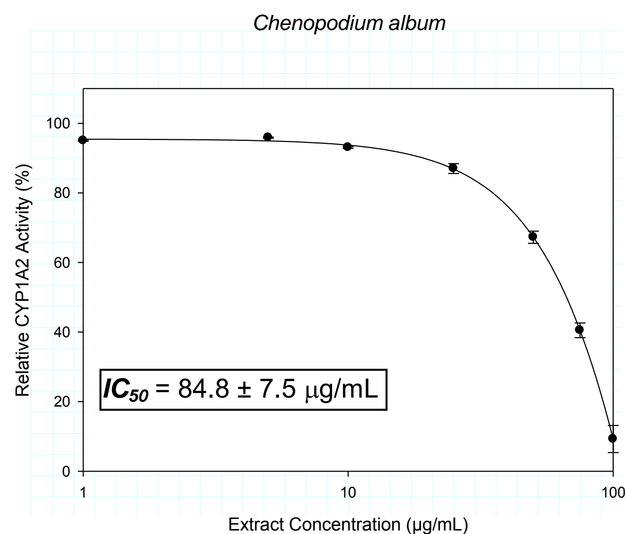

Figure 15: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Chenopodium album* (R^2 = 0.99)

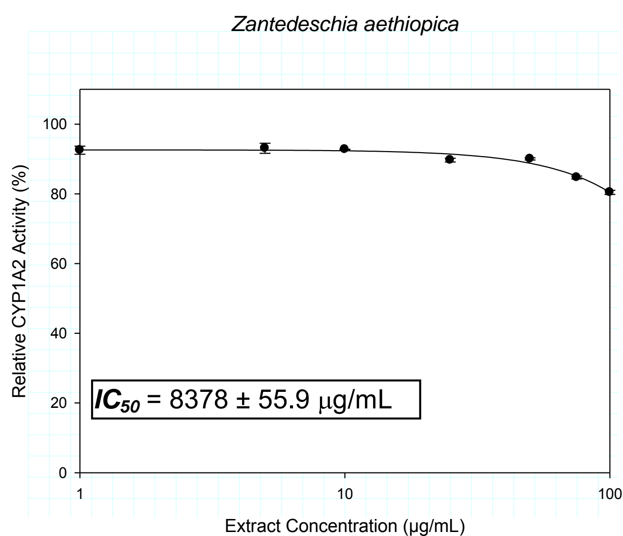

Figure 14: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Zantedeschia aethiopica* (R^2 = 0.99)

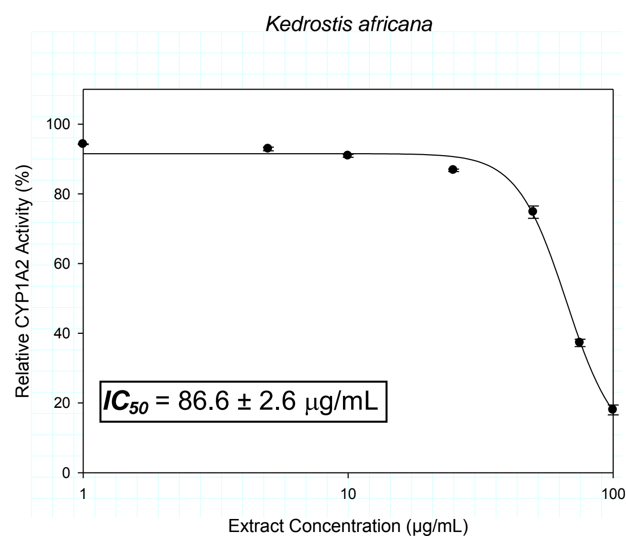

Figure 16: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Kedrostis africana* (R^2 = 0.99)

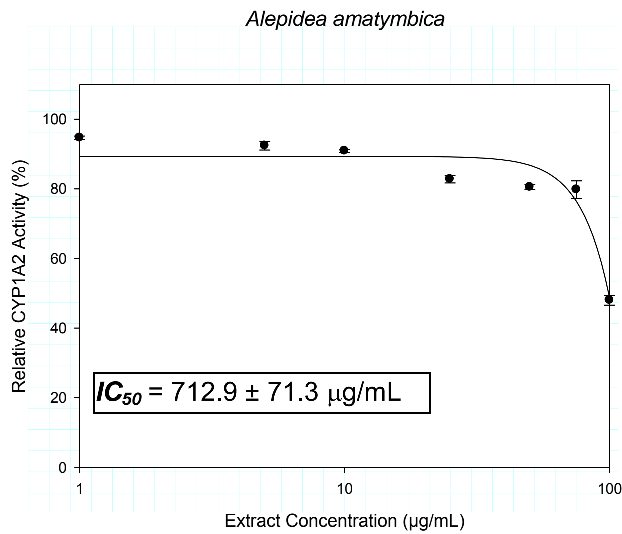

Figure 17: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Alepidea amatymbica* (R^2 = 0.99)

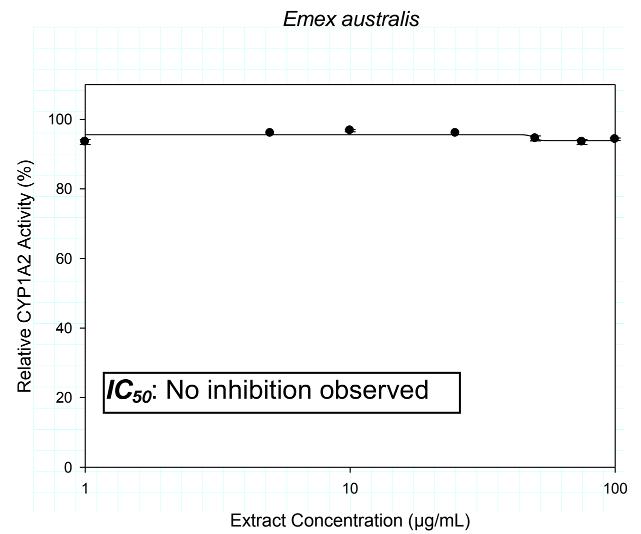

Figure 19: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Emex australis* (R^2 = 0.99)

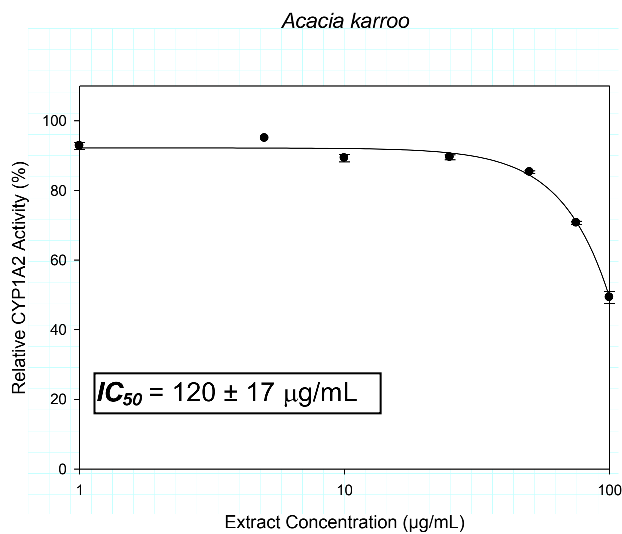

Figure 18: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Acacia karroo* (R^2 = 0.99)

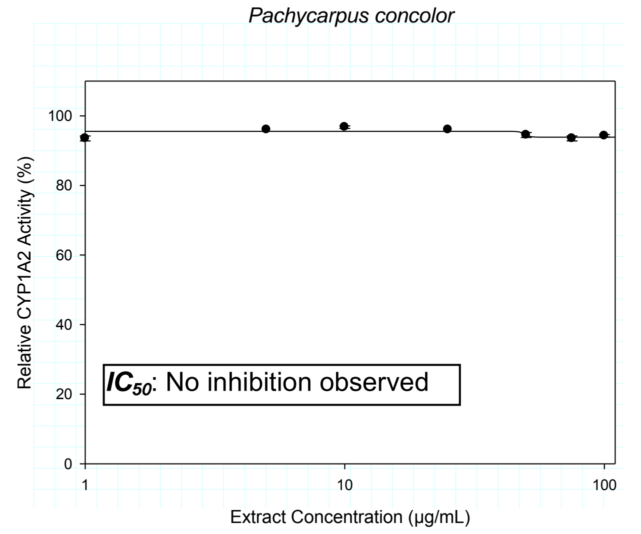

Figure 20: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Pachycarpus concolor* (R^2 = 0.99)

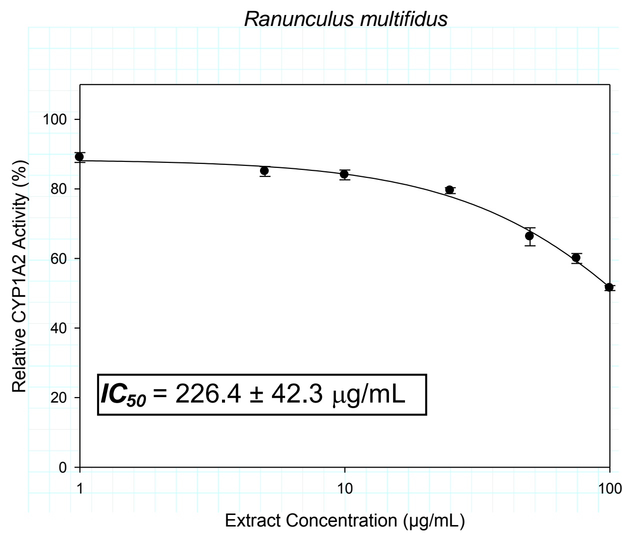

Figure 21: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Ranunculus multifidus* (R^2 = 0.99)

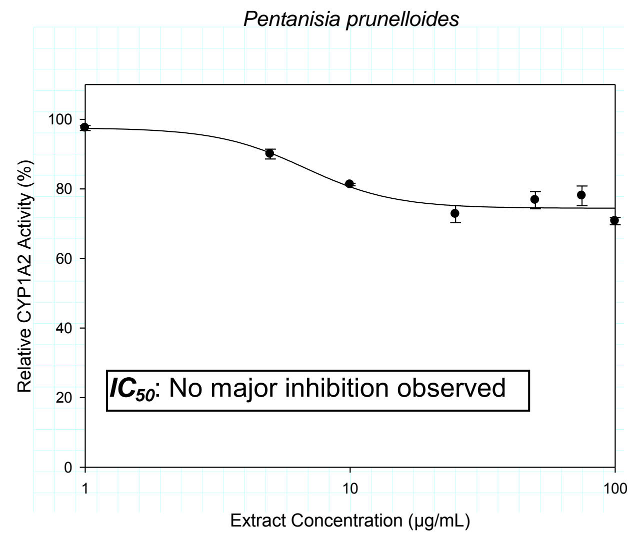

Figure 23: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Pentanisia prunelloides* (R^2 = 0.98)

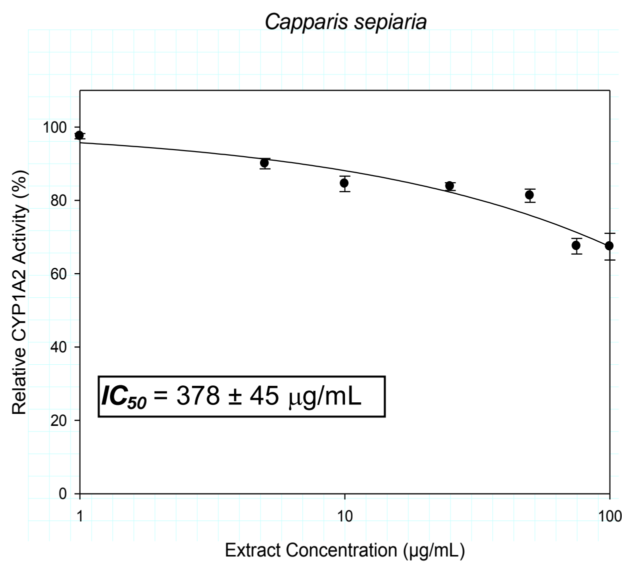

Figure 22: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Capparis sepiaria* (R^2 = 0.99)

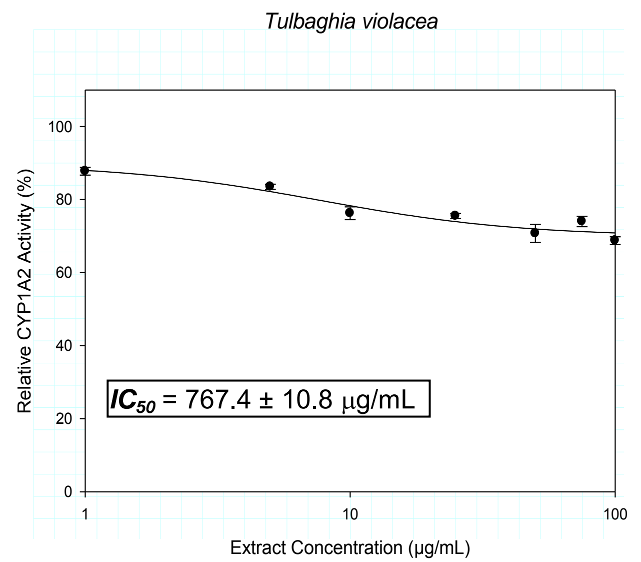

Figure 24: The profile of CYP1A2-catalyzed metabolism of phenacetin in the presence of graded concentrations of crude aqueous extracts of *Tulbaghia violacea* (R^2 = 0.99)

5.4.3.2. Influence of the herbal extracts on CYP2C9 activity.

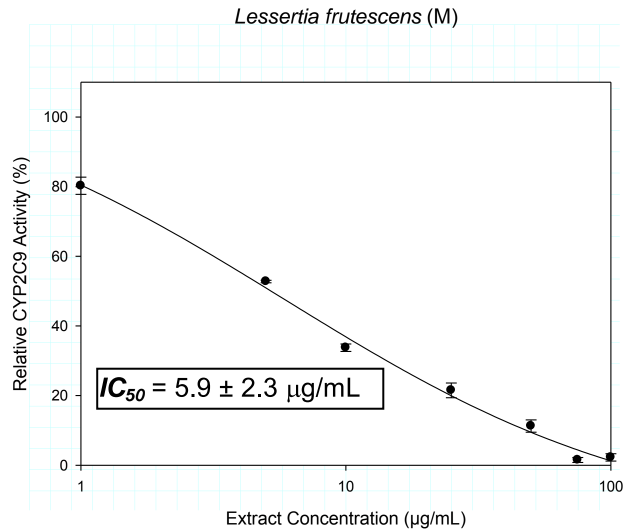

Figure 25: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude methanolic extracts of *Lessertia frutescens* (R^2 = 0.99)

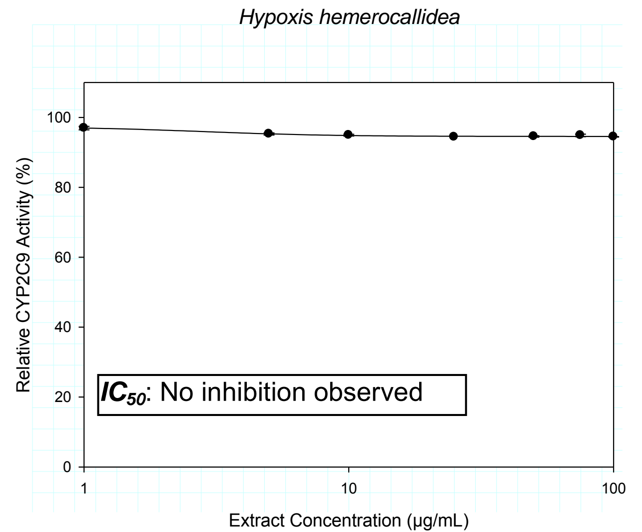

Figure 27: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Hypoxis hemerocallidea* (R^2 = 0.99)

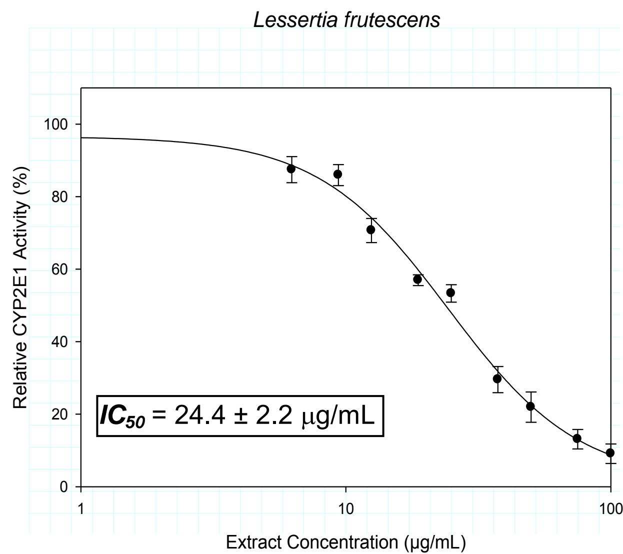

Figure 26: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Lessertia frutescens* (R^2 = 0.99)

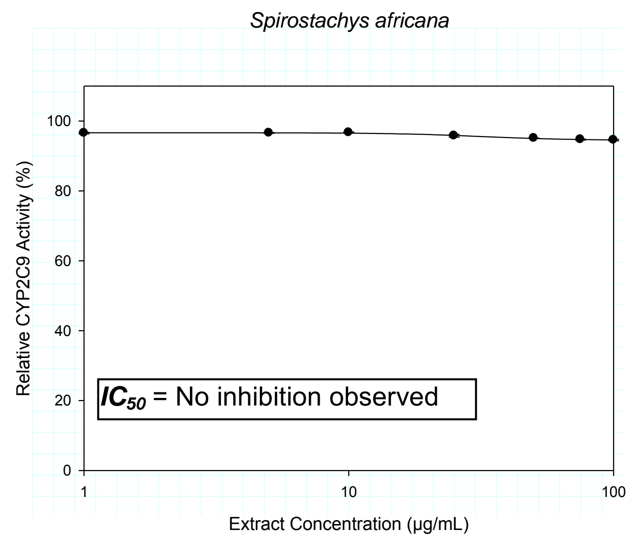

Figure 28: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Spirostachys africana* (R^2 = 0.99)

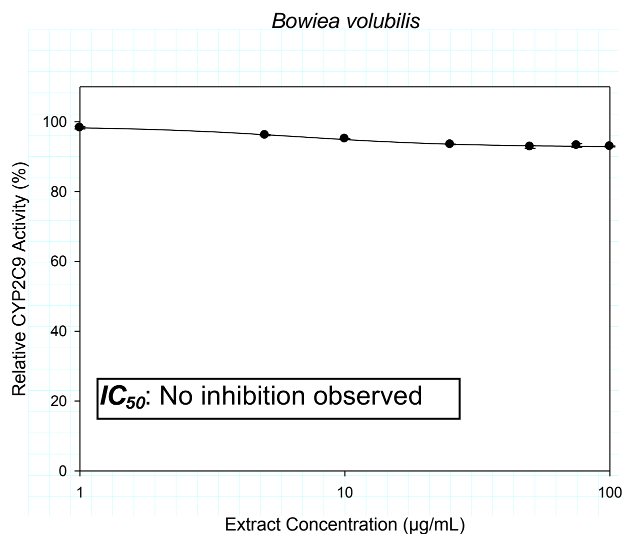

Figure 29: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Bowiea volubilis* (R^2 = 0.99)

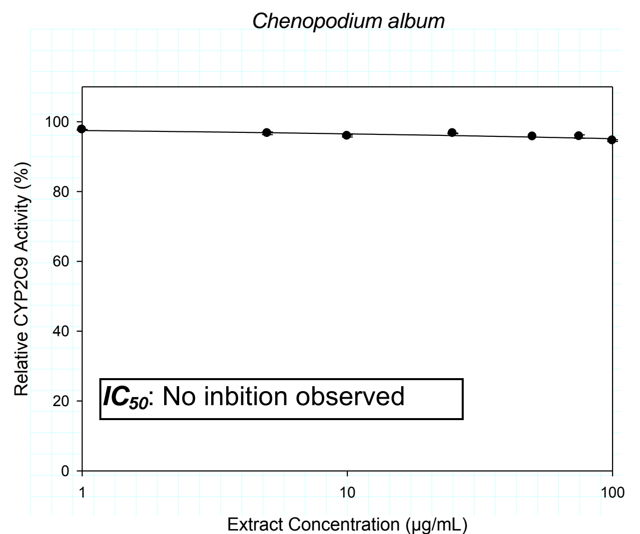

Figure 31: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Chenopodium album* (R^2 = 0.99)

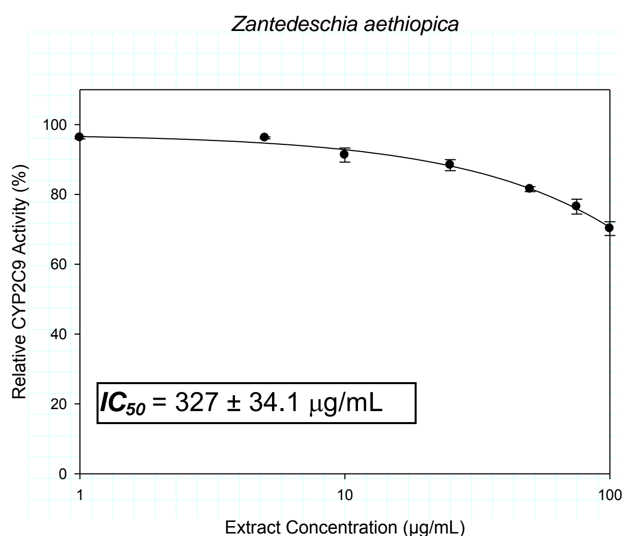

Figure 30: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Zantedeschia aethiopica* (R^2 = 0.99)

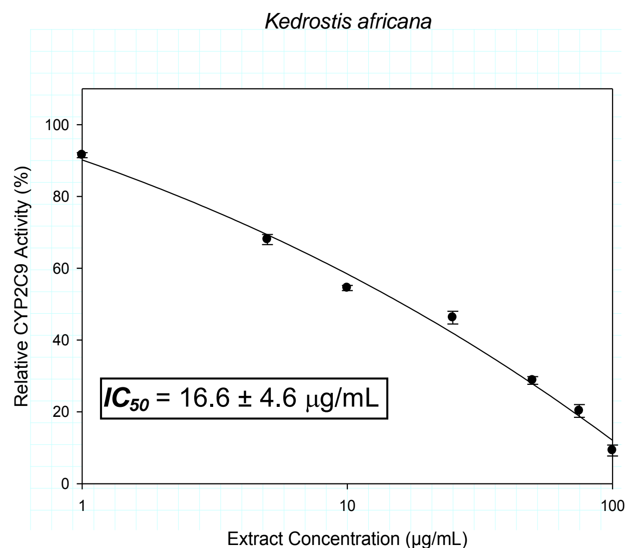

Figure 32: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Kedrostis africana* (R^2 = 0.99)

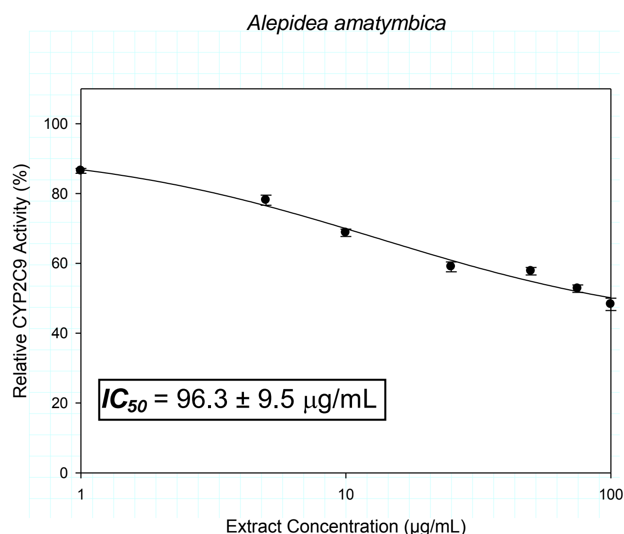

Figure 33: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Alepidea amatymbica* (R^2 = 0.99)

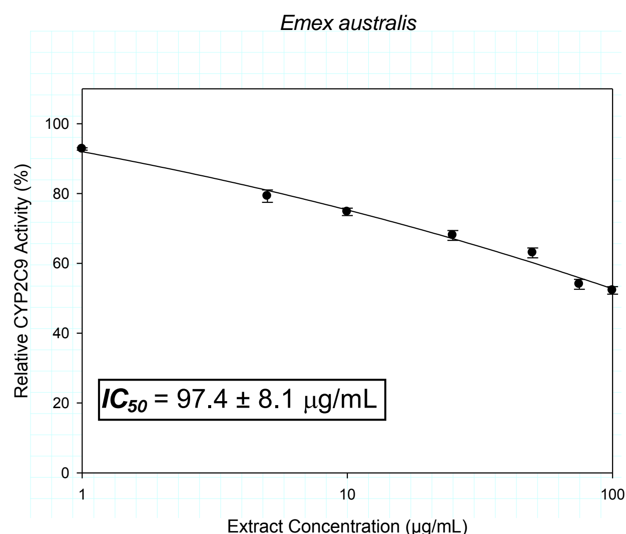

Figure 35: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Emex australis* (R^2 = 0.99)

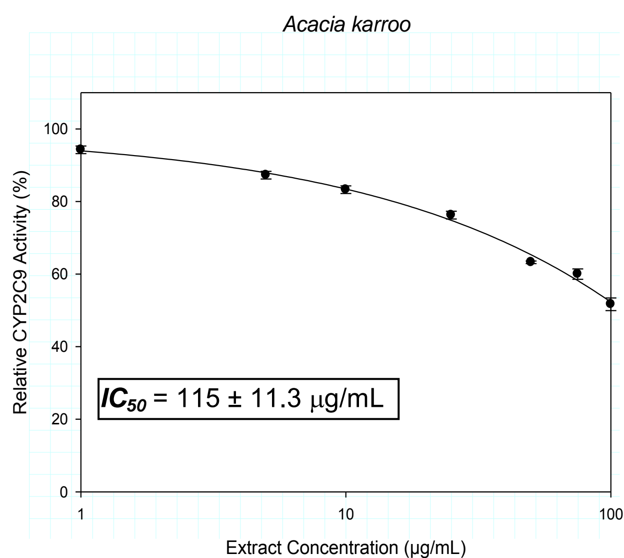

Figure 34: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Acacia karroo* (R^2 = 0.99)

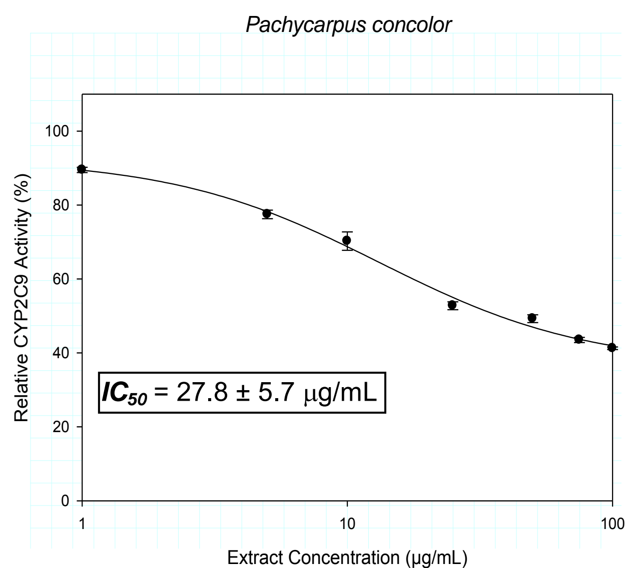

Figure 36: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Pachycarpus concolor* (R^2 = 0.99)

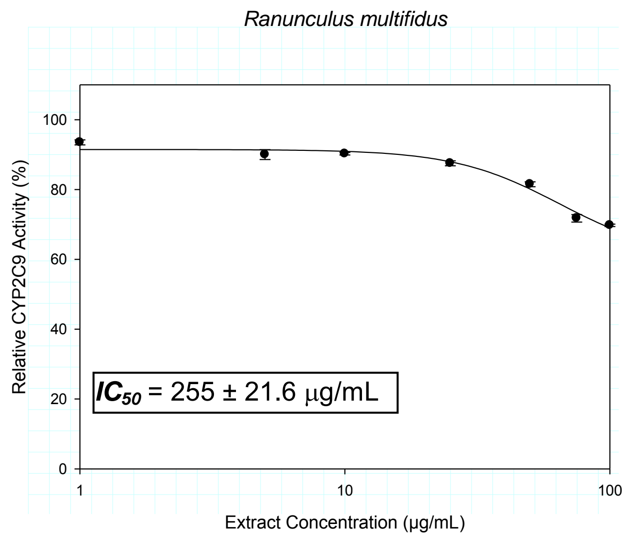

Figure 37: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Ranunculus multifidus* (R^2 = 0.99)

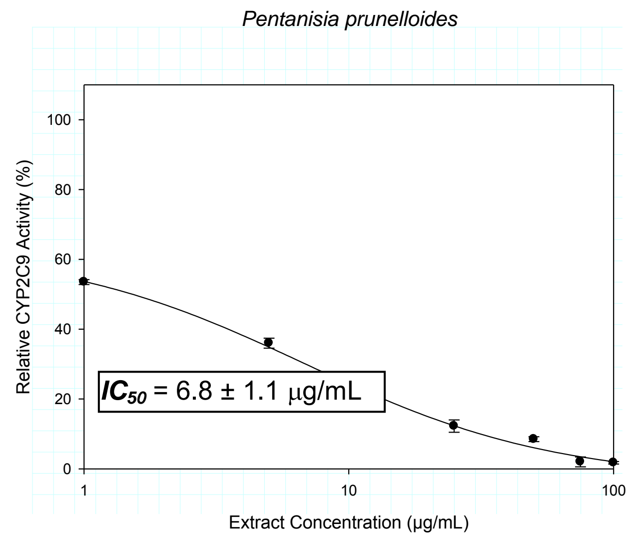

Figure 39: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Pentanisia prunelloides* (R^2 = 0.99)

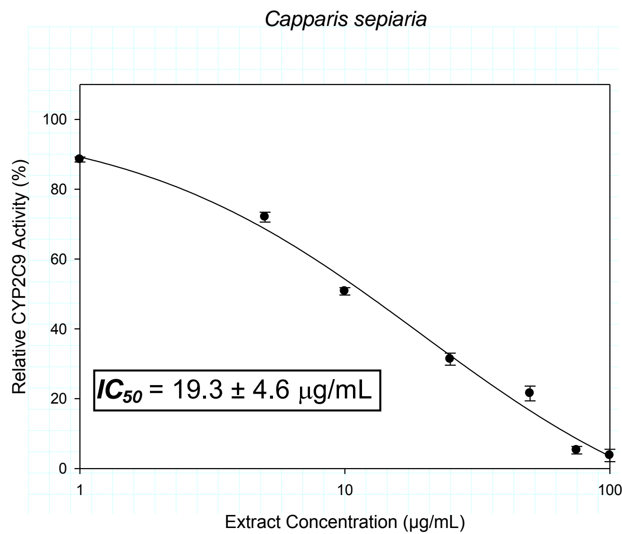

Figure 38: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Capparis sepiaria* (R^2 = 0.99)

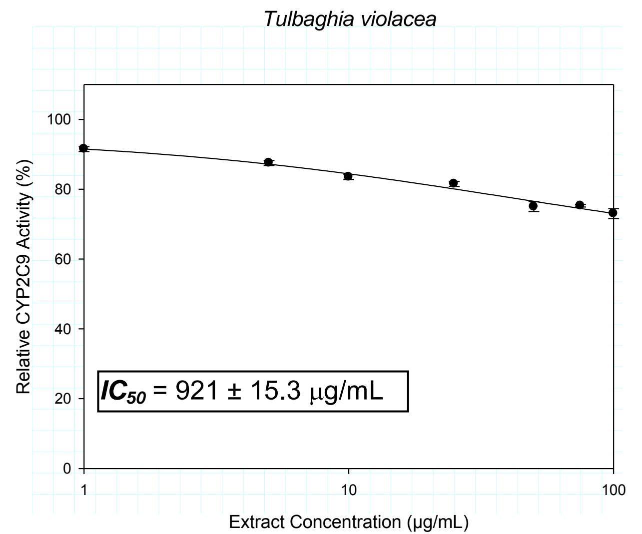

Figure 40: The profile of CYP2C9-catalyzed metabolism of diclofenac in the presence of graded concentrations of crude aqueous extracts of *Tulbaghia violacea* (R^2 = 0.99)

5.4.3.3. Influence of the herbal extracts on CYP2C19 activity

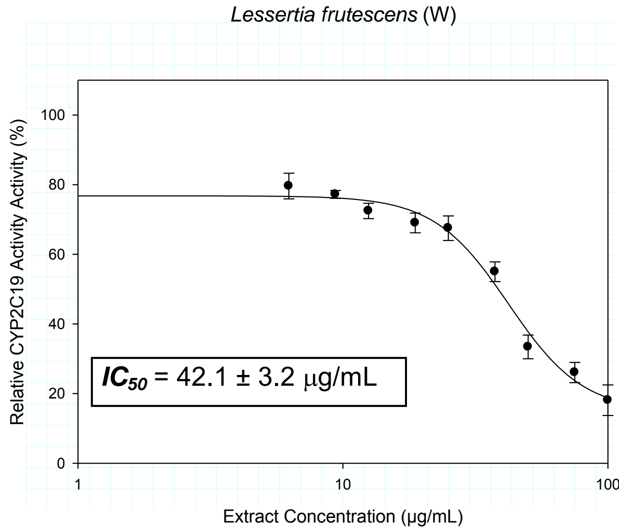

Figure 41: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Lessertia frutescens* (R^2 = 0.99)

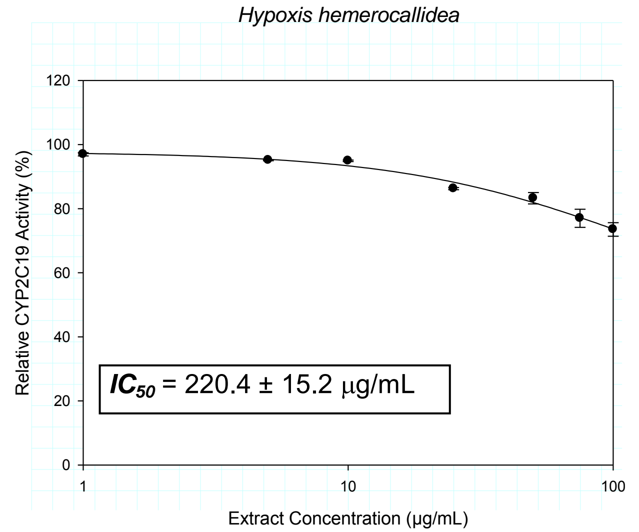

Figure 43: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Hypoxis hemerocallidea* (R^2 = 0.99)

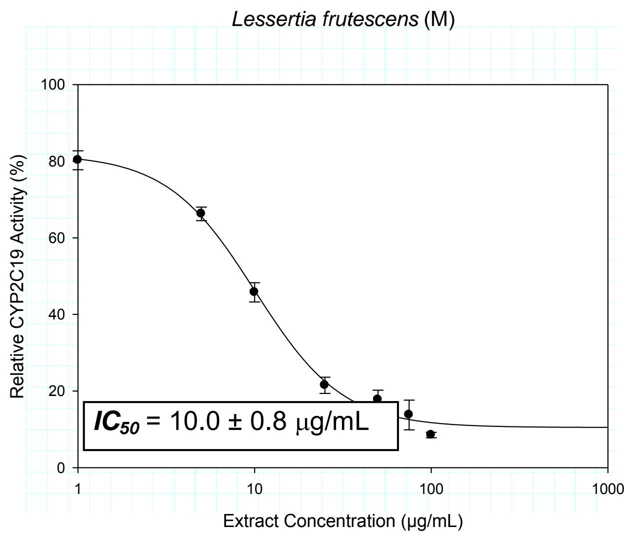

Figure 42: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude methanolic extracts of *Lessertia frutescens* (R^2 = 0.99)

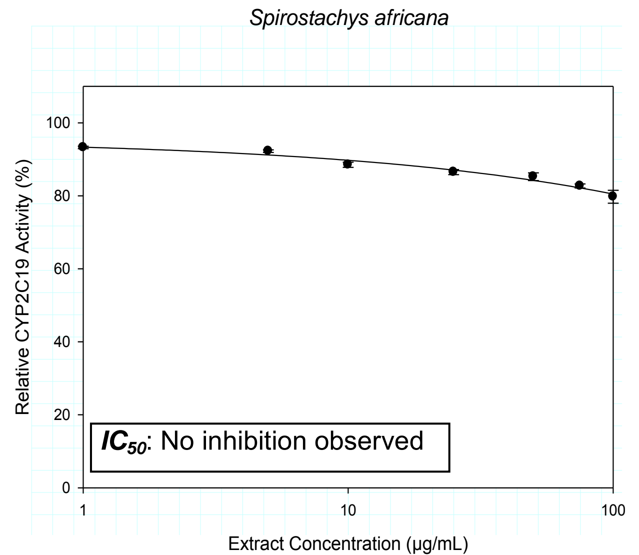

Figure 44: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Spirostachys africana* (R^2 = 0.99)

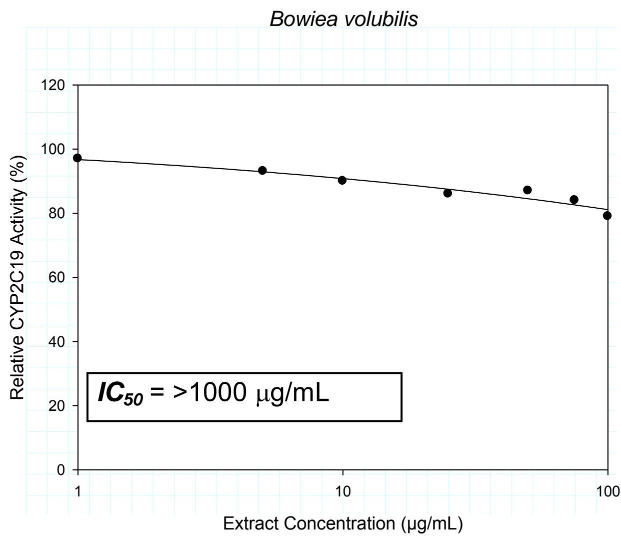

Figure 45: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Bowiea volubilis* (R^2 = 0.99)

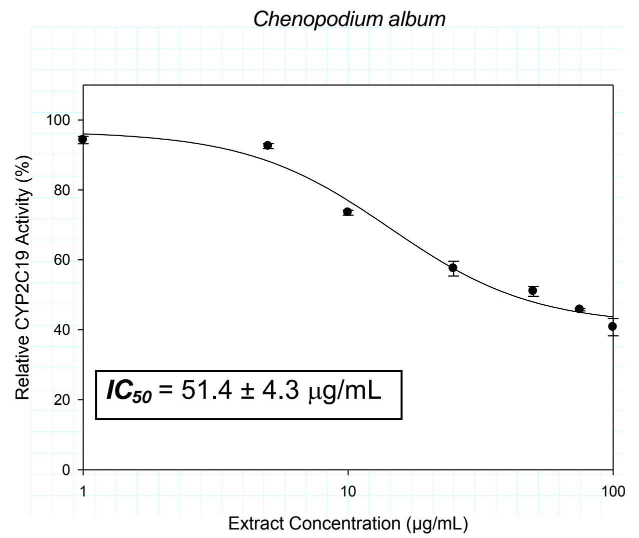

Figure 47: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Chenopodium album* (R^2 = 0.99)

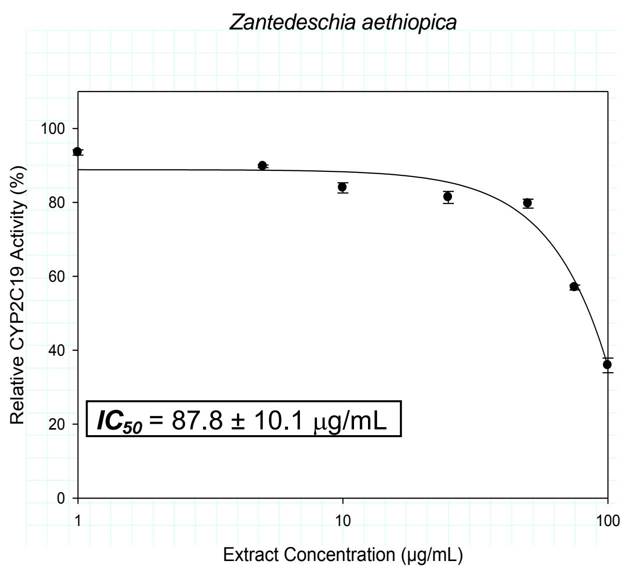

Figure 46: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Zantedeschia aethiopica* (R^2 = 0.99)

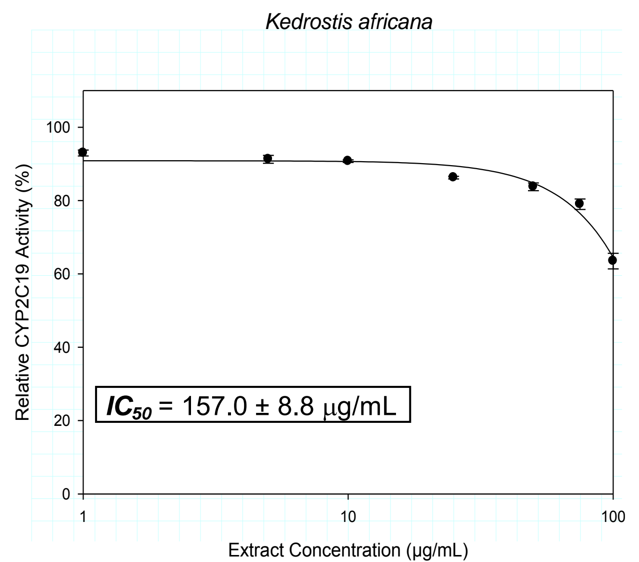

Figure 48: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Kedrostis africana* (R^2 = 0.99)

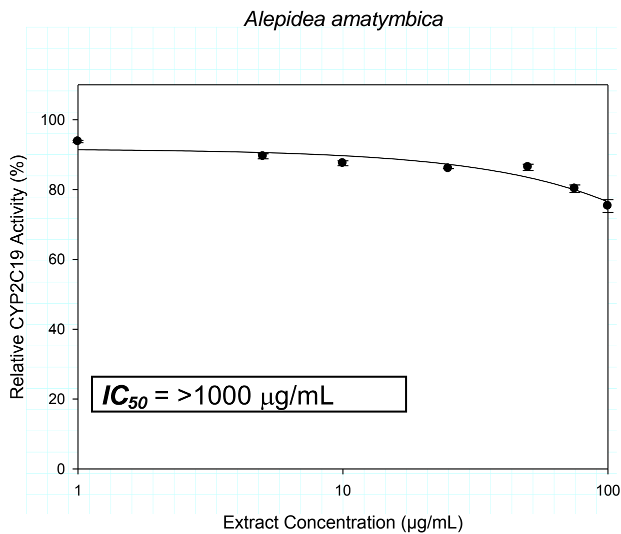

Figure 49: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Alepidea amatymbica* (R^2 = 0.99)

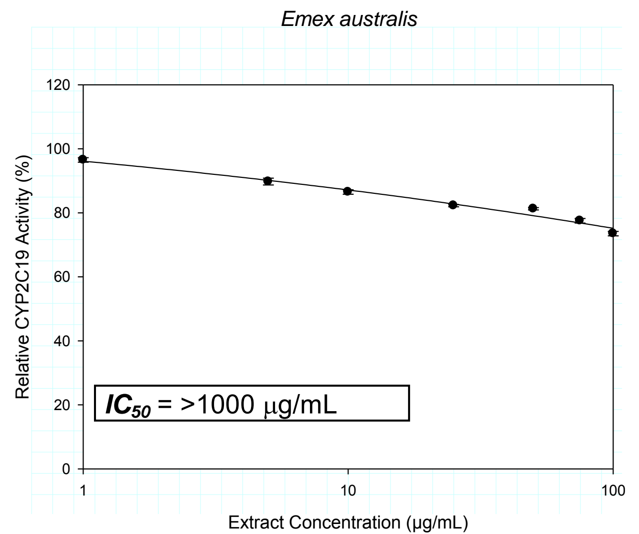

Figure 51: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Emex australis* (R^2 = 0.99)

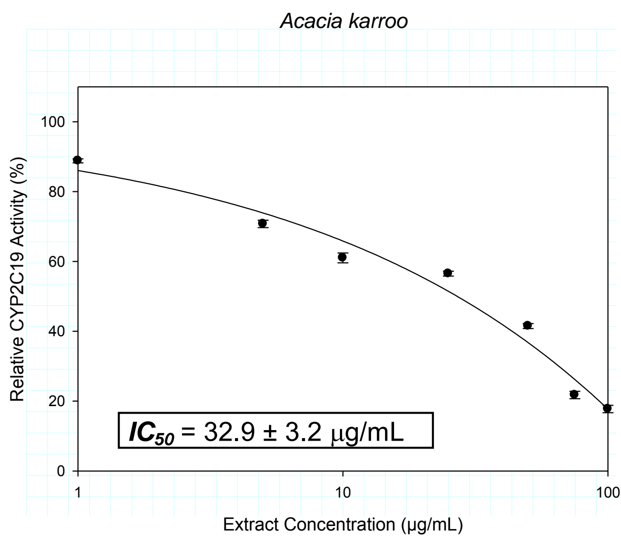

Figure 50: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Acacia karroo* (R^2 = 0.99)

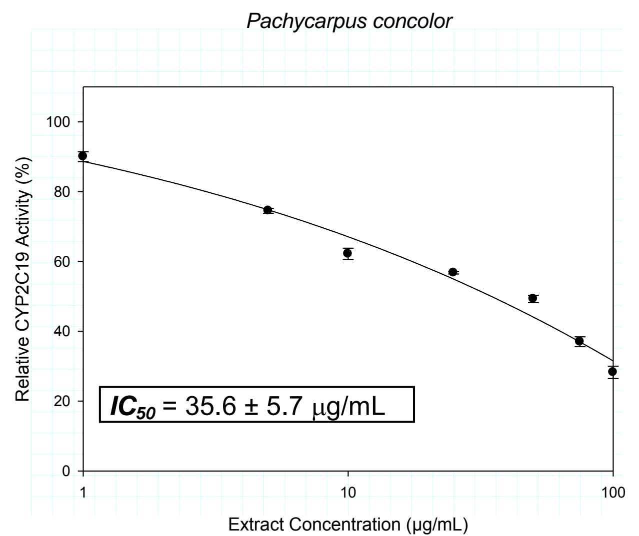

Figure 52: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Pachycarpus concolor* (R^2 = 0.99)

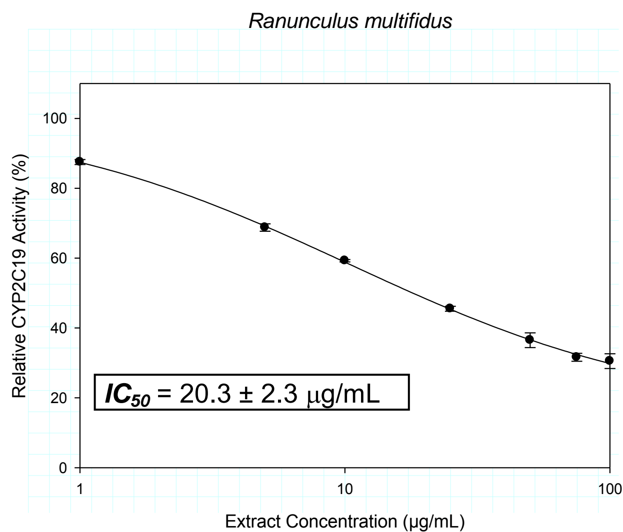

Figure 53: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Ranunculus multifidus* (R^2 = 0.99)

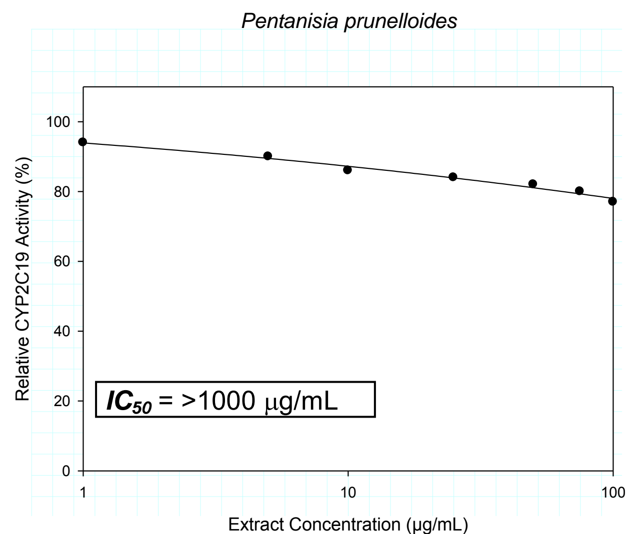

Figure 55: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Pentanisia prunelloides* (R^2 = 0.99)

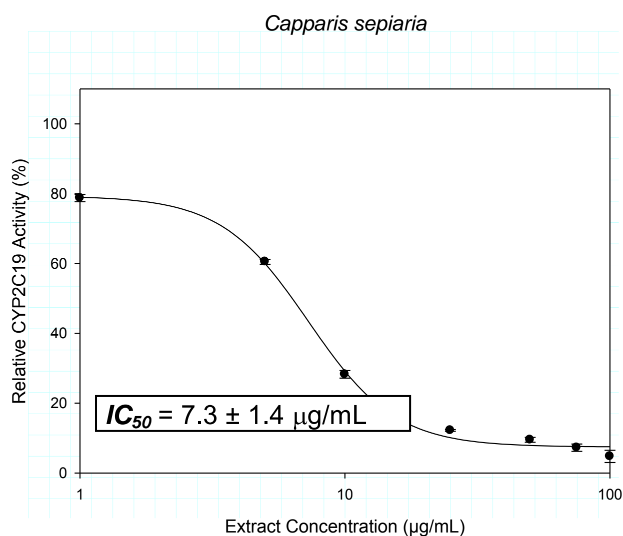

Figure 54: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Capparis sepiaria* (R^2 = 0.99)

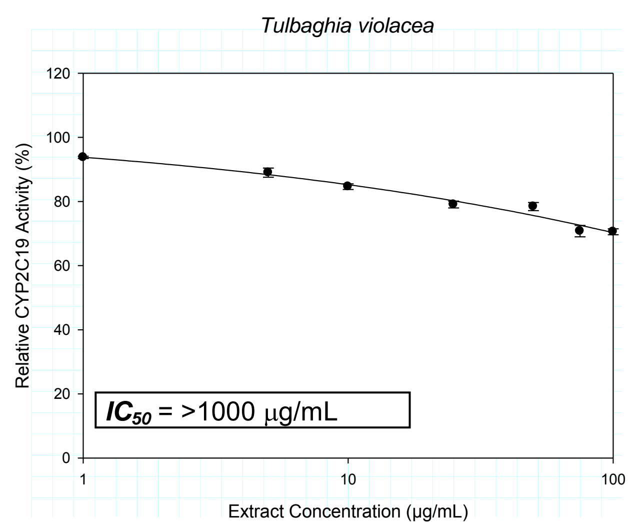

Figure 56: The profile of CYP2C19-catalyzed metabolism of *S*-mephenytoin in the presence of graded concentrations of crude aqueous extracts of *Tulbaghia violacea* (R^2 = 0.99)

5.4.3.4. Influence of the herbal extracts on CYP3A4 activity

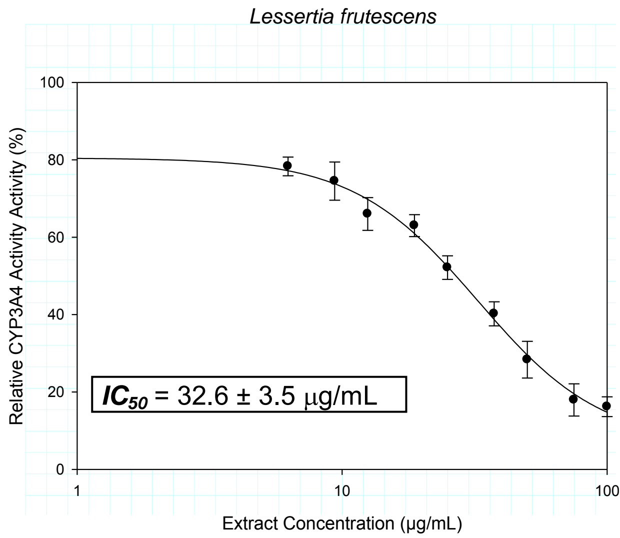

Figure 57: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude methanolic extracts of *Lessertia frutescens* (R^2 = 0.99)

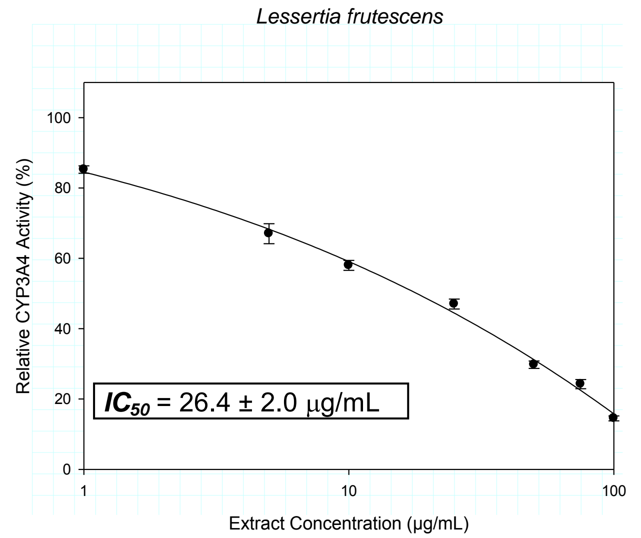

Figure 59: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Lessertia frutescens* (R^2 = 0.99)

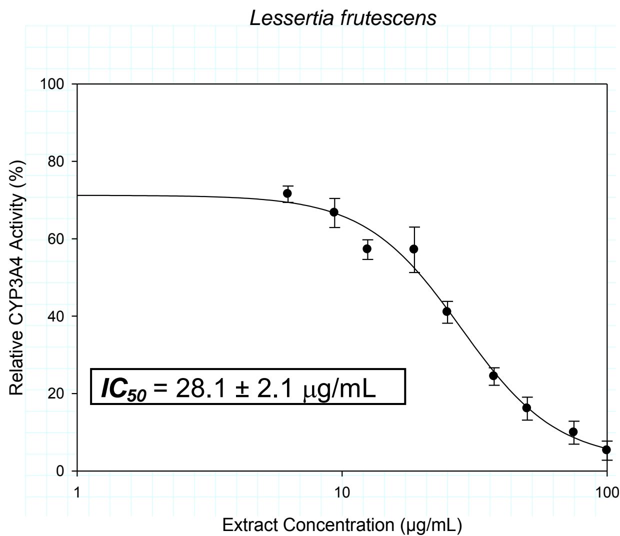

Figure 58: The profile of CYP3A4-catalyzed metabolism of midazolam in the presence of graded concentrations of crude methanolic extracts of *Lessertia frutescens* (R^2 = 0.99)

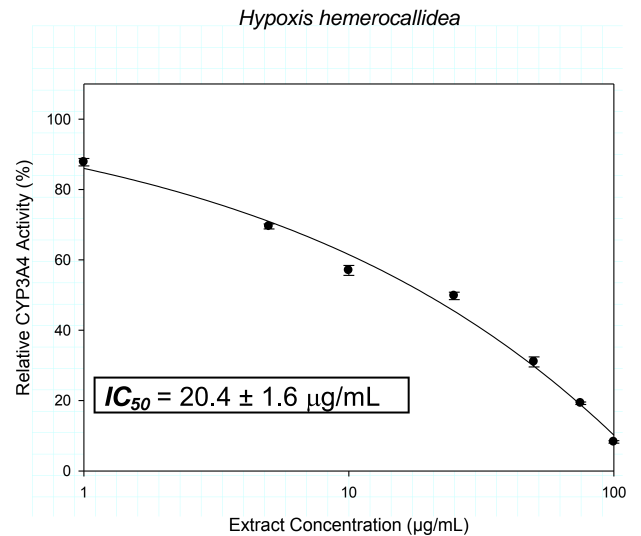

Figure 60: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Hypoxis hemerocallidea* (R^2 = 0.99)

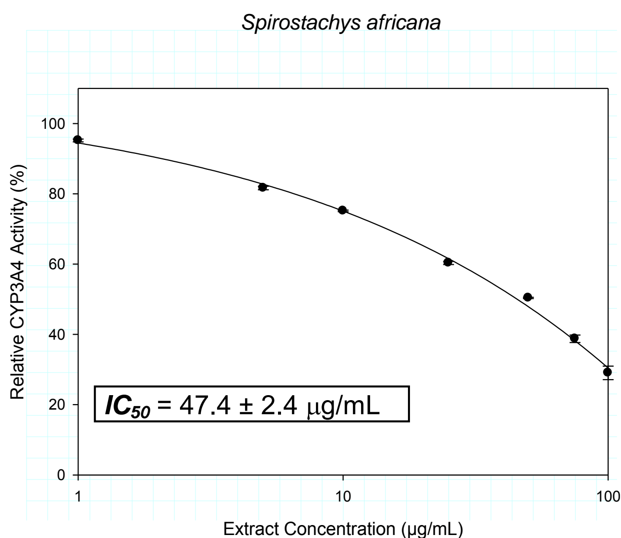

Figure 61: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Spirostachys africana* (R^2 = 0.99)

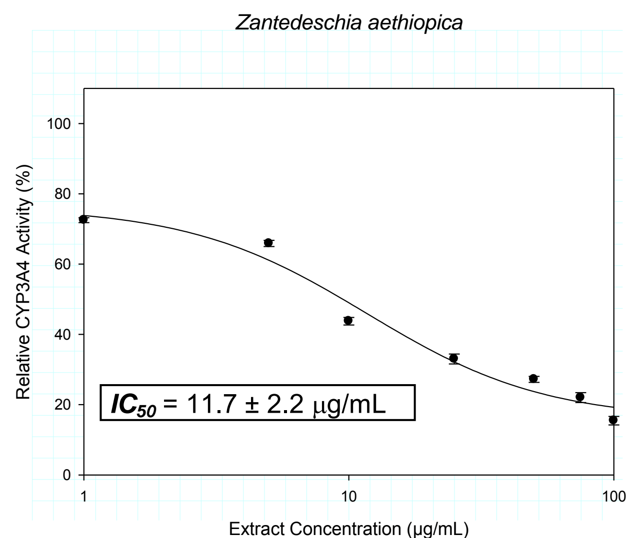

Figure 63: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Zantedeschia aethiopica* (R^2 = 0.99)

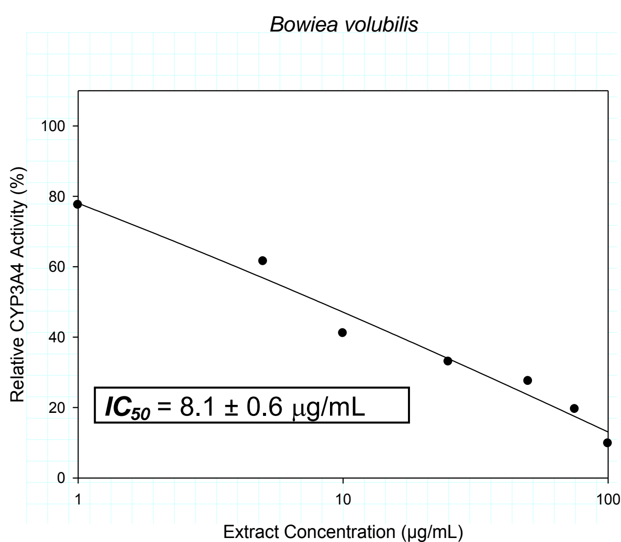

Figure 62: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Bowiea volubilis* (R^2 = 0.99)

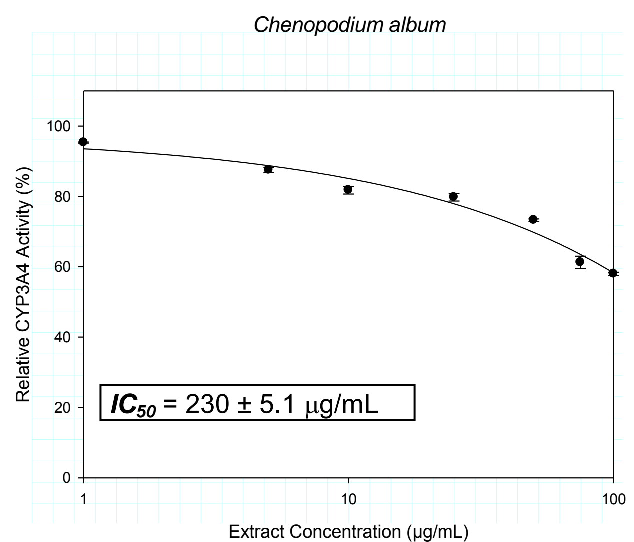

Figure 64: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Chenopodium album* (R^2 = 0.99)

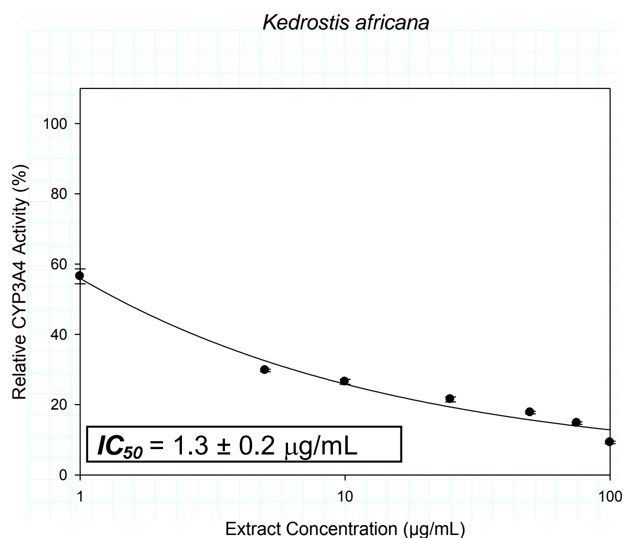

Figure 65: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Kedrostis africana* (R^2 = 0.99)

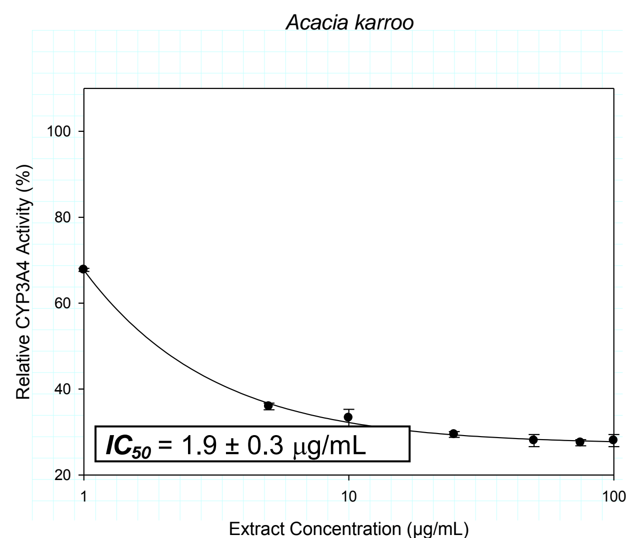

Figure 67: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Acacia karroo* (R^2 = 0.99)

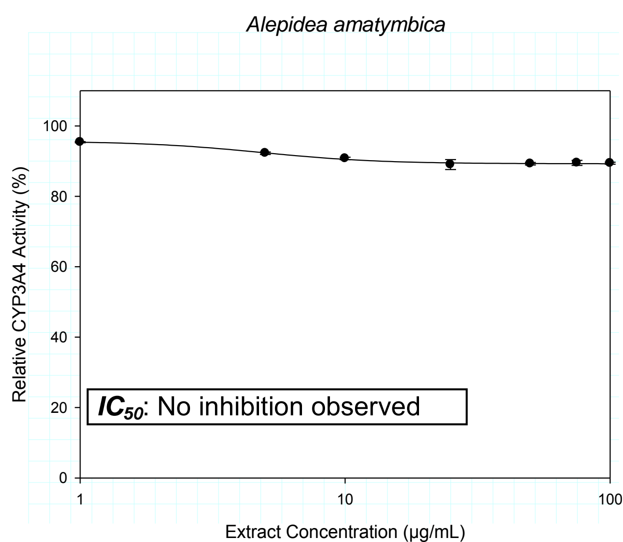

Figure 66: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Alepidea amatymbica* (R^2 = 0.99)

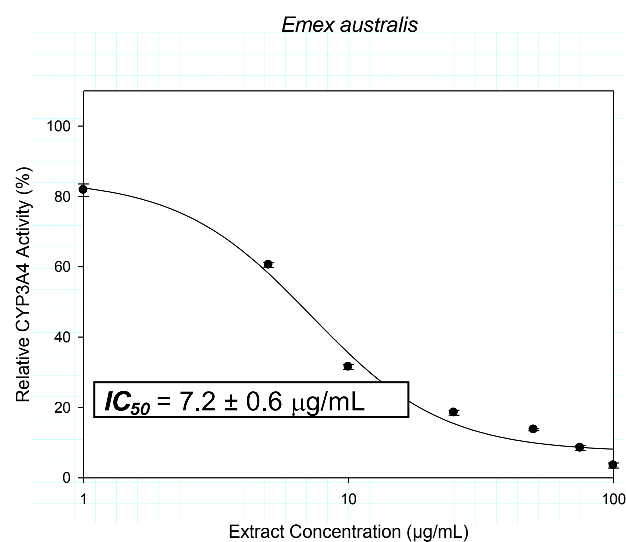

Figure 68: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Emex australis* (R^2 = 0.99)

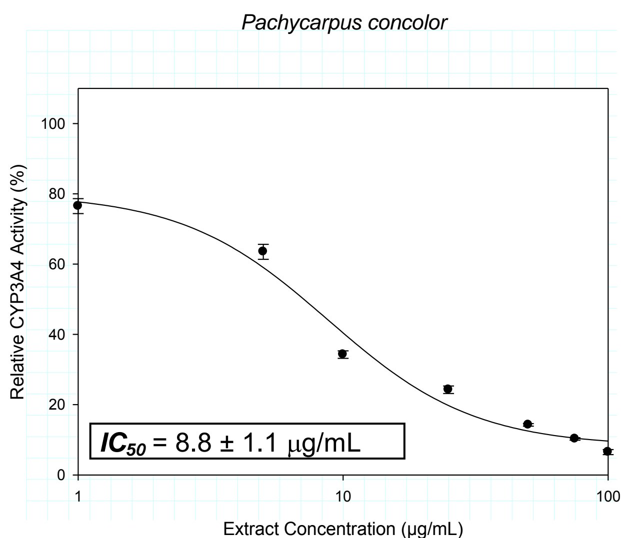

Figure 69: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Pachycarpus concolor* (R^2 = 0.99)

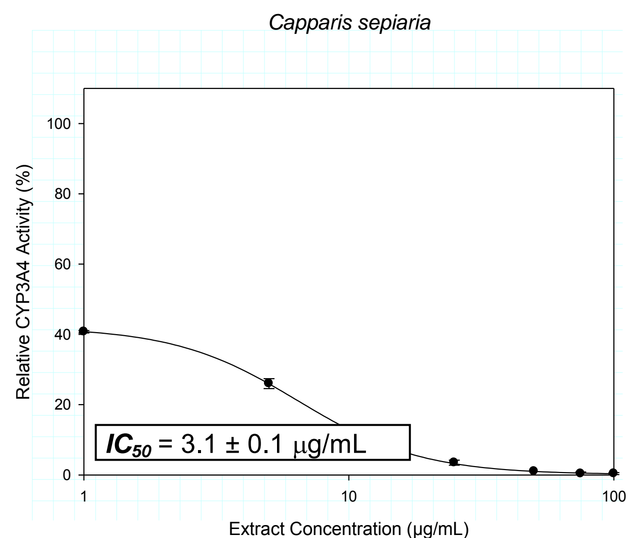

Figure 71: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Capparis sepiaria* (R^2 = 0.99)

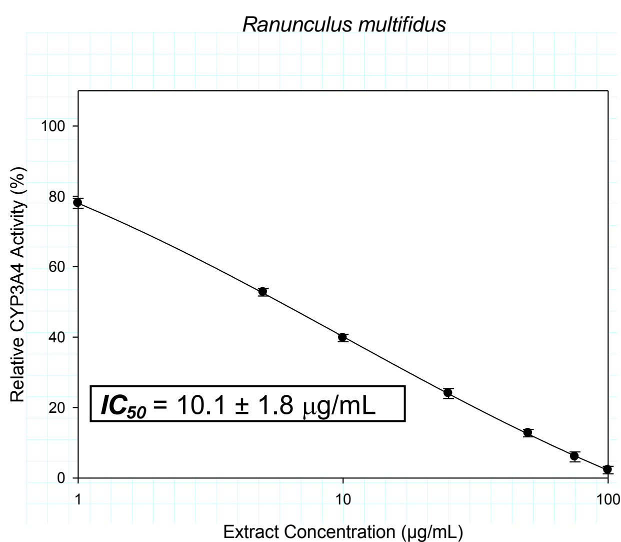

Figure 70: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Ranunculus multifidus* (R^2 = 0.99)

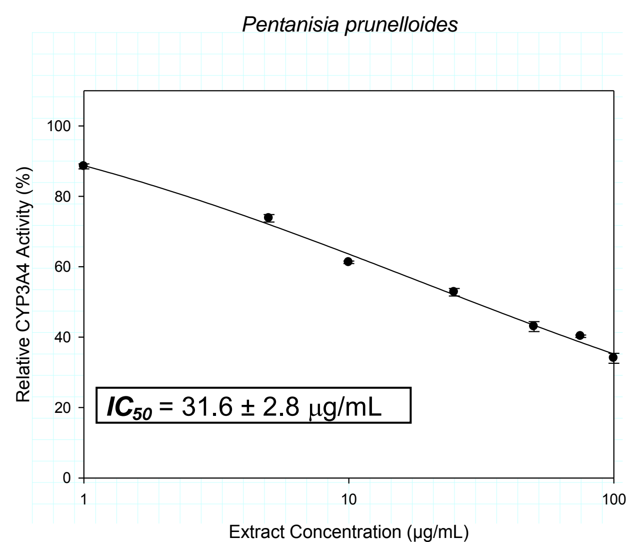

Figure 72: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Pentanisia prunelloides* (R^2 = 0.99)

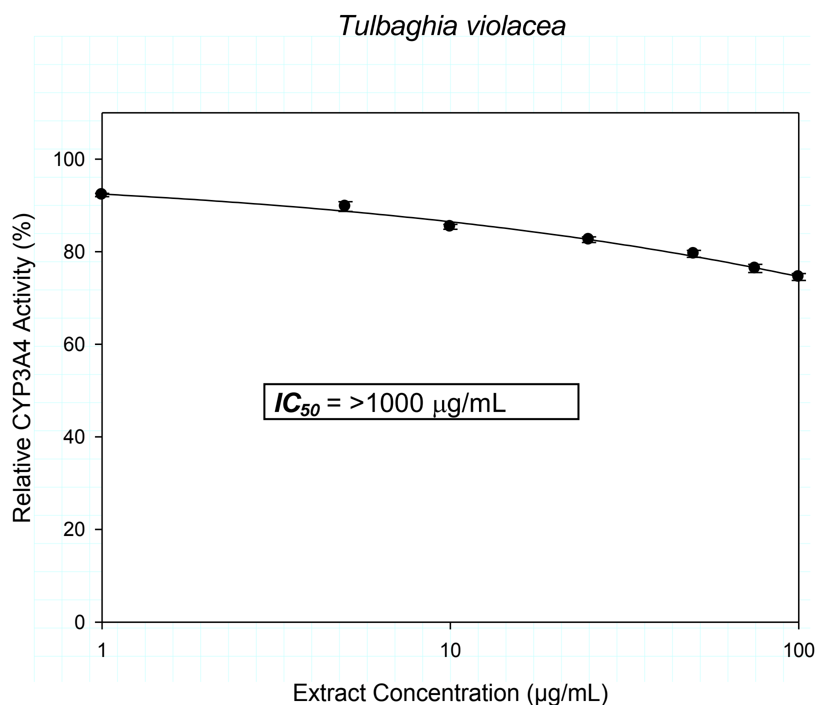

Figure 73: The profile of CYP3A4-catalyzed metabolism of testosterone in the presence of graded concentrations of crude aqueous extracts of *Tulbaghia violacea* (R^2 = 0.99)

5.4.4. Lessertia-induced time-dependent inhibition of CYP3A4

Time-dependent (irreversible) inhibition of CYP3A4/5 by graded concentration of *Lessertia frutescens* (LT) was observed with a KI = 296 µg/mL and kinact = 0.063 min^{-1}) under the conditions of this study. The influence of preincubation time, LT concentration and the positive control troleandomycin (TAO) on the enzymatic activity of CYP3A4/5 is illustrated in Figure 74.

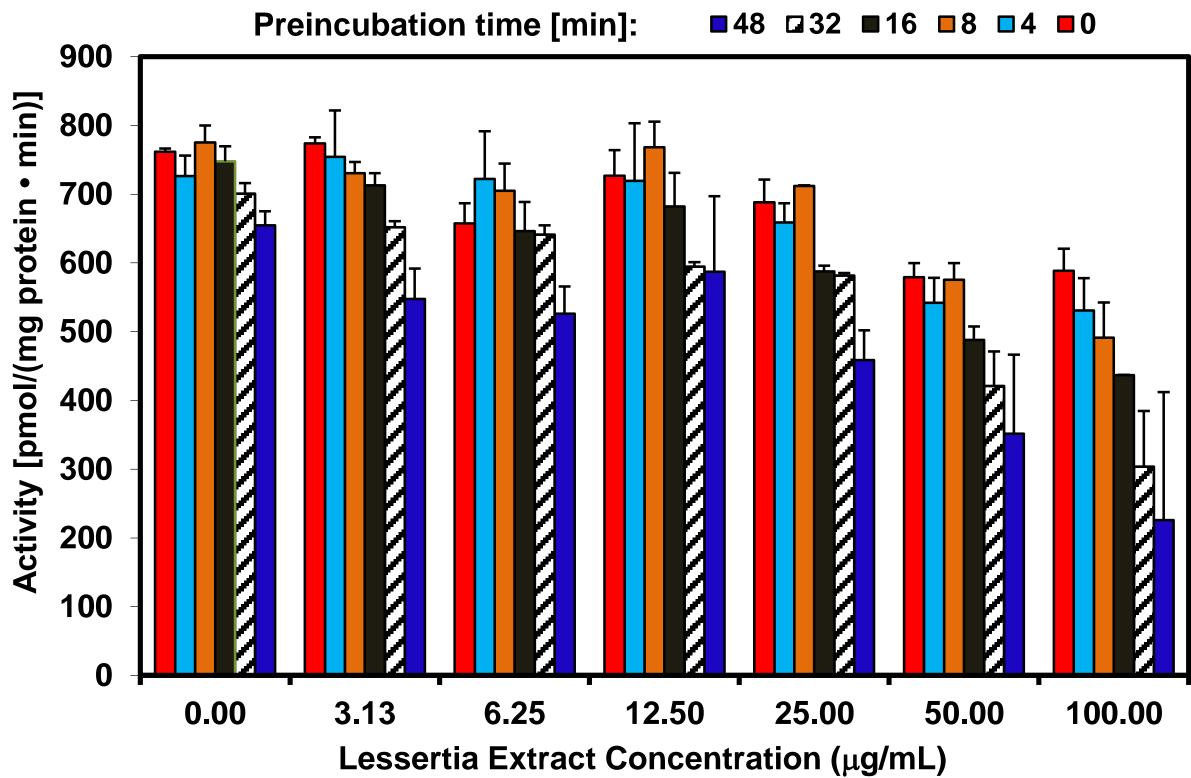

Figure 74: Effect of preincubation time and Lessertia extracts on the enzymatic activity of CYP3A4.

5.5. Discussion

From the information gathered from the THPs, all the medicinal herbs are water-extracted for traditional use. Thus, in order to make the findings of this HDI assessment more relevant to immediate needs, water extraction was performed, and the yields were noted. Modern consumption of popular herbal products such as St John's wort, ginseng and ginkgo has taken the form of compressed tablets or whole-part capsules. This exposes consumers to more phytochemical constituents which are extracted in the GIT. Studies on such herbs tend to use organic extracts - toluene, methanol, ethanol etc – in order to obtain as much phytoconstituents as possible. In this study, in order to reflect what the consumers are exposed to, the choice of water-extraction was considered more appropriate. The advantage of this choice is that, only water-solube constituents as consumed by the patients are investigated, providing a truer reflection. On the other hand, the method of extraction including the filtration process which may exclude certain phytoconstituents, may provide variations between what is taken by consumers and what is available for HDI investigation after extraction.

Formulations of the dried leaves of *Lessertia frutescens* are now commercially available in South Africa. It is also known that whole leaves are consumed. This exposes consumers to most of its constituents. In order to reflect this, a water-methanol (40:60) extraction of *Lessertia frutescens* was performed.

The enzyme kinetic parameters especially the K_m obtained from the optimization procedure for CYP1A2 (34.3 μM) and CYP2C9 (7.5 μM) compare satisfactorily with literature values (Table 15).

There was a potent inhibition of CYP1A2 activity by a number of the herbal extracts. Apart from *Emex australis, Pachycarpus concolor* and *Pentanisia prunelloides* which demonstrated no inhibitory effects, the other herbal extracts inhibited CYP1A2 to varying degrees. A relative comparison (using an arbitrary IC_{50} value of 100 μg/mL) (see Budzinski et al., 2000; Obach, 2000; Liu et al., 2006) showed that *Alepidea amatymbica, Acacia karroo, Capparis sepiaria, Ranunculus multifidus, Tulbaghia violacea* and *Zantedeschia aethiopica* are very weak inhibitors of CYP1A2 (IC_{50} > 100 μg/mL).

On the other hand, *Bowiea volubilis, Kedrostis Africana, Chenopodium album, Lessertia frutescens* (methanolic), *Hypoxis hemerocallidea, Spirostachys africana* and *Lessertia frutescens* (aqueous), in ascending order of potency demonstrated strong inhibition of

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CYP1A2 activity (IC_{50} = 1-100 μg/mL). It is also interesting to note the variation in the inhibitory activity of aqueous and methanolic extracts of *Lessertia*. This is an indication of the differences in the phytochemical composition of the two extracts which reflects the variation in solubility. It suggests that the inhibitory phytochemical would be available *in vivo* to patients who consume either of whole leaves capsules or aqueous decoctions, as is the practice.

CYP1A2 is responsible for the primary metabolism of a wide range of clinical drugs (Table 18). For similar *in vivo* inhibitory effects on CYP1A2 activity, similar concentrations of phytoconstituents must be achieved after herbal intake. While information on the absorption of the herbal products is not available, the influence of the herbs on the drug metabolizing enzymes of the GIT may affect the absorption of concomitantly administered drugs. This may not be very important for CYP1A2 substrates because CYP1A2 is mainly expressed in the liver. If significant phytochemical constituents are absorbed however, with the potential of reaching blood levels comparable to the estimated *in vitro* IC_{50} values, a wide range of clinical drugs will be susceptible to herb-induced accumulation as a result of CYP1A2 inhibition.

Table 18: Some examples of CYP1A2 substrates (adapted from Zou et al., 2009)

Drug	Drug class	Metabolic pathway (s)	Estimated contribution by CYP1A2 (%)
R-Acenocoumarol	Oral anticoagulant	6-Hydroxylation	10-20
Acetaminophen	Analgesic	Oxidation	10-15
Albendazole	Benzimidazole anthelmintic	4-hydroxylation	5-10
Almotriptan	Selective 5-HT$_{1B/1D}$ agonist (for treating migraine)	N-demethylation	5-10
Alosetron	Selective 5-HT$_3$ antagonist (for irritable bowel syndrome)	N-dealkylation and hydroxylation	10-20
Amiodarone	Antiarrhythmic	N-deethylation	5-10
Aminoflavone	Anticancer	3-hydroxylation	10-20
Aminopyrine	Analgesic	N-demethylation	40-50
Amitriptyline	Tricyclic antidepressant	10-Hydroxylation	5-10
Antipyrine	Analgesic	N-demethylation and 3- and 4-hydroxylation	10-25
Azelastine	Antihistamine	N-demethylation	5-10
Bortezomib	Proteasome inhibitor (for treatment of multiple myeloma)	Deboronation and dehydrogenation	10-25
Bufuralol	β-Blocker	1′-Hydroxylation	5-10
Caffeine	CNS stimulant	N-demethylation	95
Carbamazepine 2- and	Anticonvulsant	3-Hydroxylation	5–10
Carvedilol	β-Blocker	8-Hydroxylation and O-demethylation	5–10
Cinnarizine	Antihistamine	N-dealkylation	5–10
Clomipramine	Tricyclic antidepressant	N-demethylation	5–10
Clozapine	Antipsychotic	N-demethylation and N-oxidation	40–55
Coumarin	Anticancer	7-Hydroxylation	5–10
Cyclobenzaprine	Tricyclic antidepressant	N-demethylation	5–10
5,6-Dimethylxanthenone-4 acetic acid	Anticancer	6-Methyl hydroxylation	10–20
Diphenhydramine	Antidepressant	N-demethylation	5–10
Duloxetine	Antihistamine	4-, 5-, and 6-Hydroxylation	30–40
Efavirenz	Non-nucleoside reverse transcriptase inhibitor	7- and 8-Hydroxylation	5–10
Erlotinib	Epidermal growth factor receptor tyrosine kinase inhibitor (for cancer treatment)	O-demethylation	5–10
Estradiol	Steroid in oral contraceptives	2- and 4-hydroxylation	5–10

Flunarizine	Antihistamine	N-dealkylation	5–10
Flutamide	Non-steroidal antiandrogen for prostate cancer treatment	2-Hydroxylation	10–25
Fluvoxamine	SSRI	O-demethylation	5–10
Furafylline	Antiasthmatic		10–20
Guanabenz	α_2-Adrenoreceptor agonist (antihypertensive)	N-hydroxylation	5–10
Haloperidol	Antipsychotic	N-dealkylation	5–10
Imatinib	Bcr-Abl tyrosine kinase inhibitor (as anticancer agent)	O-demethylation	
Indiplon	Hypnotic drug	N-demethylation	5–10
Imipramine	Tricyclic antidepressant	N-demethylation	10–20
KR-62980	Selective peroxisome proliferator-activated receptor-γ modulator	Hydroxylation	5–10
KR-63198	Selective peroxisome proliferator-activated receptor-γ modulator	Hydroxylation	5–15
Leflunomide	Disease-modifying anti-inflammatory agent	N—O bond cleavage	40–55
Lidocaine	Antiarrhythmic and local anesthetic drug	N-deethylation and 3-hydroxylation	10–20
Maprotiline	Antidepressant		5–10
Melatonin	Pineal hormone	6-Hydroxylation and O-demethylation	40–60
Mexiletine	Antiarrhythmic drug	Para- and 2-hydroxylation	10–20
Mianserin	Antidepressant	N-oxidation and N-demethylation	5–10
Mirtazapine	Antidepressant	N-oxidation, 8-hydroxylation, and N-demethylation	5–15
Naproxen	NSAID	O-demethylation	5–10
Nabumetone	NSAID	Aliphatic hydroxylation	30
Nicotine	Agent for treating nicotine dependence	N-hydroxylation and C-oxidation	5–10
Nortriptyline	Tricyclic antidepressant	10-Hydroxylation	5–10
Olanzapine	Atypical antipsychotic drug	N-demethylation, and 4- and 7-hydroxylation	30–40
Ondansetron	Selective serotonin 5-HT3 receptor antagonist	7- and 8-Hydroxylation	5–10

	(antiemetic)		
Paraxanthine	CNS stimulant	8-Hydroxylation	80
Pentoxifylline	A drug used to treat intermittent claudication	Xanthine 7-demethylation	10–20
Phenacetin	Analgesic drug	O-deethylation	86
Promazine	Antipsychotic drug	N-demethylation and 5-sulfoxidation	30–45
Propafenone	Antiarrhythmic drug	5-Hydroxylation and N-desalkylation	10–25
Propofol	Sedative agent	4-Hydroxylation	10–25
Propranolol	β-Blocker	N-desisopropylation	30–50
Riluzole	Antiglutamate agent for treatment of ALS	N-hydroxylation, and possibly 4-,	75–80
		5-hydroxylation and O-dealkylation	
Rofecoxib	Selective COX-2 inhibitor (withdrawn	5-Hydroxylation	5–10
	from the market)		
Ropinirole	Non-ergoline dopamine agonist	N-deethylation	30–40
	(for treatment of Parkinson's disease)		
Ropivacaine	Local anesthetic drug	3-Hydroxylation	50–65
Selegiline	Selective irreversible MAO-B inhibitor	N-demethylation	5–15
	(for treatment of Parkinson's disease)		
Tacrine	Centrally acting cholinesterase inhibitor	1-, 2-, 4-, and 7-hydroxylation	50–65
	(for treatment of Alzheimer's disease)		
Tegafur (tetrahydrofuranyl- 5-fluorouracil)	Anticancer drug (a prodrug)	N-dealkylation	10–20
Terbinafine	Antifungal agent	N-demethylation and side chain oxidation	10–25
Thalidomide	Antiangiogenic agent (for treatment	5′-, 5-, and 6-hydroxylation	5–10
	of multiple myeloma)		
Theophylline	Bronchodilator	N-demethylation	90–95
Thioridazine	Antipsychotic drug	5-Sulfoxidation and N-demethylation	35–45
Tizanidine	Muscle relaxant		80–95

Trazodone	Antidepressant	Hydroxylation, dealkylation, and N-oxidation	15–20
Triamterene	Potassium-sparing diuretic	4′-Hydroxylation	5–10
Verapamil	Calcium channel blocker	N-demethylation	20–30
R-Warfarin	Oral anticoagulant	6-, 8-, and 10-Hydroxylation	10–20
WHI-P131 (JANEX-1)	Janus kinase-3 inhibitor (an anti-inflammatory agent)	7-O-demethylation	5–15
Zileuton	Specific inhibitor of 5-lipoxygenase (for treatment of asthma)	Ring hydroxylation	5–15
Zolmitriptan	Selective agonists of 5-$HT_{1B/1D}$ receptor (for treatment of migraine)	N-demethylation and N-oxidation	30–40
Zolpidem	Non-benzodiazepine hypnotic drug	Ring hydroxylation	5–10
Zotepine	Atypical antipsychotic drug	2- and 3-Hydroxylation	5–10
Zoxazolamine	Muscle relaxant	6-Hydroxylation	5–10

Abbreviations: ALS - amyotrophic lateral sclerosis, CNS - central nervous system, COX - cyclooxygenase, MAO - monoamine oxidase, NSAID - non-steroid anti-inflammatory drug, SSRI - selective serotonin reuptake inhibitor

Hypoxis hemerocallidea, Spirostachys africana, Bowiea volubilis, and *Chenopodium album* did not inhibit the activity of CYP2C9 in the presence of up to 100 µg/mL of the extracts. It is reasonable to conclude that, *in vivo* inhibition of CYP2C9 by these herbs is unlikely especially within the tested concentration of the extracts.

With IC_{50} values greater than 100µg/mL, the inhibitory activity of *Acacia karroo* (IC_{50} = 115 ± 11.3 µg/mL), *Ranunculus multifidus* (IC_{50} = 255 ± 21.6 µg/mL), *Tulbaghia violacea* (IC_{50} = 921 ± 15.3 µg/mL) and *Zantedeschia aethiopica* (IC_{50} = 327 ± 34.1 µg/mL) on CYP2C9 can be said to be weak. Depending on the achievable phytochemical concentration *in vivo*, this inhibition may not be relevant clinically.

However, the following demonstrated strong inhibitory activity, in ascending order of potency, against CYP2C9 activity: *Emex australis* (IC_{50} = 97.4 ± 8.1 µg/mL), *Alepidea amatymbica* (IC_{50} = 96.3 ± 9.5 µg/mL), *Pachycarpus concolor* (IC_{50} = (27.8 ± 5.7 µg/mL), *Lessertia frutescens* (aqueous; IC_{50} = 24.4 ± 2.2 µg/mL), *Capparis sepiaria* (IC_{50} = 19.3 ± 4.6 µg/mL), *Kedrostis africana* (IC_{50} = 16.6 ± 4.6 µg/mL), *Pentanisia prunelloides* (IC_{50} = 6.8 ± 1.1 µg/mL) and *Lessertia frutescens* (methanolic; IC_{50} = 5.9 ± 2.3 µg/mL).

CYP2C9 is responsible for the primary metabolism of a number of clinically important drugs (Table 19). Such drugs are susceptible to HDI induced by these herbs, if sufficient phytochemical concentrations are achieved *in vivo*.

Table 19: Examples of clinically important drugs that are metabolized by CYP2C9

Drugs	Medical Uses	CYP2C9-mediated reaction	References
58C80	Antimalarial	t-butyl oxidation	Weaver et al., 1995
Amitriptyline	Antidepressant	Hydroxylation	Yu et al., 2012
Celecoxib	Analgesic	Hydroxylation	Gong et al., 2012
Diclofenac	Analgesic	4'-hydroxylation	Konecný et al., 2007
Fluoxetine	Antidepressant	Hydroxylation	Yu et al., 2012
Glimepiride	Oral hypoglycemic	Hydroxylation	Holstein et al., 2011
Glipizide	Oral hypoglycemic	Hydroxylation	Tan et al., 2010
Glyburide	Oral hypoglycemic	Hydroxylation	Holstein et al., 2011
Ibuprofen	Analgesic	Isopropyl hydroxylation	García-Martín et al., 2004
Irbesartan	Antihypertensive	Monohydroxylation	Bourrié et al., 1999
Lornoxicam	Analgesic	5'-hydroxylation	Bonnabry et al., 1996
Losartan	Hypnotic	Hydroxylmethyl oxidation	Bae et al., 2011
Mefenamic acid	Analgesic	3'-methyl hydroxylation	Bonnabry et al., 1996
Naproxen	Analgesic	O-demethylation	Bae et al., 2009
Phenytoin	Anticonvulsant	4-hydroxylation	Ninomiya et al., 2000
Piroxicam	Analgesic	5'-hydroxylation	Perini et al., 2006
Rosiglitazone	Oral hypoglycemic	Hydroxylation	Holstein et al., 2011
S-Warfarin	Anticoagulant	7-hydroxylation	Liu et al., 2012
Tamoxifen	Anti-cancer	Hydroxylation	Wiseman and Lewis, 1996
Tolbutamide	Oral hypoglycemic	4-methyl hydroxylation	Vestergren et al., 2012
Torasemide	Diuretic	Hydroxylation	Vormfelde et al., 2008

With CYP2C19, the following can be classified as relatively strong inhibitors: *Acacia karroo* (IC_{50} = 32.9 ± 3.2 µg/mL), *Capparis sepiaria* (IC_{50} = (7.3 ± 1.4 µg/mL), *Chenopodium album* (IC_{50} = 51.4 ± 4.3 µg/mL), *Pachycarpus concolor* (IC_{50} = 35.6 ± 5.7 µg/mL), *Ranunculus*

multifidus (IC_{50} = 20.3 ± 2.3 µg/mL), *Lessertia frutescens* (aqueous: IC_{50} = 142.1 ± 3.2 µg/mL; methanolic: IC_{50} = 10.0 ± 0.8 µg/mL) and *Zantedeschia aethiopica* (IC_{50} = 87.8 ± 10.1 µg/mL).

On the other hand, *Hypoxis hemerocallidea* and *Kedrostis africana* with IC_{50} values of 220.4 ± 15.2 and 157.0 ± 8.8 µg/mL respectively can be considered weak inhibitors of CYP2C19. A near-total absence of inhibition was observed with *Spirostachys africana* while *Bowiea volubilis, Alepidea amatymbica, Emex australis, Pentanisia prunelloides* and *Tulbaghia violacea* with IC_{50} values >1000 µg/mL demonstrated relatively less potent CYP2C19 inhibition.

The inhibition of CYP2C19 will be clinically dangerous for CYP2C19 substrates like phenytoin and R-warfarin which have narrow safety margins. Such drugs as omeprazole, proguanil, diazepam, moclobemide, imipramine and other substrates of CYP2C19 are susceptible to HDI in the presence of these herbal CYP2C19 inhibitors.

CYP3A4 is the most important CYP in drug metabolism. As noted earlier, the phase I metabolism of about half of all marketed drugs is CYP3A4-dependent. In the light of this fact, inhibition of CYP3A4 activity by herbal products portends a more likelihood for HDI with concurrently administered orthodox medicine.

The result from this study showed that only 2 (*Alepidea amatymbica* and *Tulbaghia violacea*) out of the 15 herbs exerted no inhibitory effects on CYP3A4 activity. Of the remaining 13, only *Chenopodium album* (IC_{50} = 230 ± 5.1 µg/mL) can be considered a relatively weak CYP3A4 inhibitor. All the other 12 herbs inhibited CYP3A4 activity potently with IC_{50} values less than 100 µg/mL: *Lessertia frutescens* (methanolic, IC_{50} = 26.4 ± 2.0 µg/mL; aqueous IC_{50} = 32.6 ± 3.5 µg/mL), *Hypoxis hemerocallidea* (IC_{50} = 20.4 ± 1.6 µg/mL), *Spirostachys Africana* (IC_{50} = 47.4 ± 2.4 µg/mL), *Bowiea volubilis* (IC_{50} = 8.1 ± 0.6 µg/mL), *Zantedeschia aethiopica* (IC_{50} = 11.7 ± 2.2 µg/mL), *Kedrostis Africana* (IC_{50} = 1.3 ± 0.2 µg/mL), *Acacia karroo* (IC_{50} = 1.9 ± 0.3), *Emex australis* (IC_{50} = 7.2 ± 0.6 µg/mL), *Pachycarpus concolor* (IC_{50} = 8.8 ± 1.1 µg/mL), *Ranunculus multifidus* (IC_{50} = 10.1 ± 1.8 µg/mL), *Capparis sepiaria* (IC_{50} = 3.1 ± 0.1 µg/mL) and *Pentanisia prunelloides* (IC_{50} = 31.6 ± 2.8 µg/mL).

There was no major difference in the inhibitory activity of *Lessertia* on CYP3A4 activity when testosterone (IC_{50} = 26.4 ± 2.0 µg/mL) was substituted with midazolam (IC_{50} = 28.1 ± 2.1 µg/mL) as probe substrate. Although the use of one CYP-specific substrate provides results that are generally acceptable for predictive purposes, the use of two substrates (especially with CYP3A4) can be beneficial to rule out variations in inhibitory effects due to substrate

choice (Bjornsson et al., 2003). Such results confirm cross-substrate effects, and a demonstration of the inhibitory effects to be on the enzyme and not on the substrate.

Both the aqueous and methanolic extracts of *Lessertia* inhibited CYP3A4. This suggests that the consumption of either of whole leaf products and aqueous decoctions by patients may predispose to HDI. It is also important to note that the level of inhibition of the aqueous (32.6 ± 3.5 µg/mL) and methanolic (26.4 ± 2.0 µg/mL) extracts of *Lessertia* is similar.

As shown with the time-dependent inhibition in Figure 74, inhibitory influence of *Lessertia* will be more potent on repeated consumption due to the suppression of enzyme viability/activity. In reality, herbal remedies are consumed repeatedly over a long period of time. With a possible continuous suppression of enzymatic activity, administered drugs may accumulate, and precipitate toxicity.

A futher *in vitro*-*in vivo* estimation of the effects of the herbs is presented in Chapter 10.

5.6. Conclusion

The effect of the crude extracts of the 15 selected medicinal herbs (Chapter 4) on CYP1A2, CYP2C9, CYP2C19 and CYP3A4 activity was investigated. Extracts of the following inhibited the activity of CYP1A2 to varying degrees: *Acacia karroo*, *Alepidea amatymbica*, *Bowiea volubilis*, *Capparis sepiaria*, *Chenopodium album*, *Kedrostis Africana*, *Hypoxis hemerocallidea*, *Ranunculus multifidus*, *Spirostachys Africana*, *Lessertia frutescens*, *Tulbaghia violacea* and *Zantedeschia aethiopica*. Extracts of *Emex australis*, *Alepidea amatymbica*, *Pachycarpus concolor*, *Lessertia frutescens*, *Capparis sepiaria*, *Kedrostis africana* and *Pentanisia prunelloides* potently inhibited CYP2C9 activity. The extracts of *Acacia karroo, Capparis sepiaria, Chenopodium album, Pachycarpus concolor, Ranunculus multifidus,* and *Lessertia frutescens* inhibited CYP2C19 activity to varying degrees. In excemption of *Alepidea amatymbica* and *Tulbaghia violacea*, the extracts of all the medicinal plants inhibited CYP3A4 activity to varying degrees. *Lessertia* demonstrated time-dependent inhibition of CYP3A4 activity suggesting a more potent inhibition on continous repetitive use. It is therefore concluded that there is the potential for HDI between these herbs and the substrates of the respective CYP, if sufficiently high *in vivo* concentrations of the phytoconstituents are achieved.

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CHAPTER SIX

THE ASSESSMENT OF THE INHIBITORY EFFECT OF *LESSERTIA FRUTESCENS* AND *HYPOXIS HEMEROCALLIDEA* ON CYP2A6, 2B6, 2C8, 2D6 AND 2E1

6.0. Summary

The influence of the crude extracts of *Lessertia frutescens* and *Hypoxis hemerocallidea* on the metabolic activity of CYP2A6, CYP2B6, CYP2C8, CYP2D6 and CYP2E1 was investigated using standard probe reactions in human liver microsomes. The inhibitory effects, where observed, was estimated in IC_{50} value terms and presented in Table 20.

Table 20: Summary of the inhibitory effects of *Lessertia frutescens* and *Hypoxis hemerocallidea* on CYP2A6, 2B6, 2C8, 2D6 and 2E1

	IC_{50} value (µg/mL) for CYP inhibition	
CYP	*Lessertia frutescens*	*Hypoxis hemerocallidea*
2A6	570 ± 28.8	210 ± 9.5
2B6	19.7 ± 2.1	98.5 ± 4.5
2C8	25.5 ± 1.9	125 ± 4.7
2D6	714 ± 44.3	-
2E1	>1000	-

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6.1. Introduction

This chapter presents the *in vitro* inhibitory effects of two of the most commonly used medicinal herbs among people living with HIV/AIDS (Mills et al., 2005b) in South Africa – *Lessertia frutescens* (*Lessertia*) and *Hypoxis hemerocallidea* (*Hypoxis*) on the other CYP isozymes - CYP2A6, 2B6, 2C8, 2D6 and 2E1. 50% methanolic extract of *Lessertia* was used to obtain most phytoconstituents. This reflects the usual consumption in which consumers take whole-leave *Lessertia* capsules where they are exposed to most phytoconstituents. Aqueous extract of *Hypoxis* was used to reflect the popular consumption of aqueous decoction in traditional use.

CYP2A6 is found in the liver constituting about 4% of total CYP content (Yun et al., 1991). There is also evidence suggesting extrahepatic expression in the nasal mucosal (Gu et al., 2000). The discovery of the involvement of CYP2A6 as the catalytic enzyme responsible for the metabolism of nicotine and other tobacco-related nitrosamines has increased interest in CYP2A6 (Hecht et al., 2000). CYP2A6 is responsible for the metabolism of a number of clinically important drugs (Table 21). Phenobarbital and rifampicin are known inducers of CYP2A6 activity. Coumarin 7-hydroxylation is a standard probe marker reaction for measuring the *in vitro* and *in vivo* activity of CYP2A6 (Kim et al., 2005; Schlicht et al., 2007). Coumarin is a naturally occuring compound in plants and is present in tobacco products, alcoholic beverages, soaps, detergents, and cosmetic preparations where it serves as sweetener, fixative, or stabilizer. It has been used for the management of lymphedema (Farinola and Piller, 2007). CYP2A6 is the major enzyme responsible for the metabolism (70-80%) of coumarin to 7-hydroxycoumarin in humans.

Table 21: Examples of drugs metabolized by CYP2A6-mediated reactions (Adapted and updated from Lewis, 2003)

Drugs	Medical Uses	CYP2A6-mediated reaction
Coumarin	Lymphedema	7-hydroxylation
SM-12502	Anticoagulant	S-oxidation
Nicotine	Stimulant	5'-oxidation
Losigamone	Antiseizure	3-hydroxylation
4-Nitroanisole	-	O-demethylation
Paracetamol	Analgesic	3-hydroxylation
Quinoline	Antimalarial	1-oxidation
Halothane	Anaesthetic	Reduction
Hexamethylphosphoramide	-	Demethylation
Valproic acid	Anticonvulsant	
Cyclophosphamide	Anticancer	4-hydroxylation
Ifosfamide	Anticancer	4-hydroxylation
Pilocarpine	Antiglaucoma	3-hydroxylation
Tegafur	Anticancer	5-fluorouracil formation
Cisapride	Anticancer	O-dealkylation
Disulfiram	Alcohol withdrawal	Hydroxylation
Methoxyflurane	Analgesic	Reduction

SM-12502 = (+)-cis-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one

CYP2B6 accounts for 1-2% of total hepatic CYP. Until recently, the human expression and metabolic role of CYP2B6 has been thought to be negligible (Niu et al., 2011). Current studies have however indicated that the average relative contribution of CYP2B6 to total hepatic CYP content range from 2-10% (Chen et al., 2012). Cyclophosphamide is one of the important substrates of CYP2B6. Some other clinically important substrates of CYP2B6 include ifosfamide, tamoxifen, efavirenz, nevirapine, bupropion, mephobarbital, valproic acid, mexiletine, procainamide, artemisinin, tazofelone, aminopyrine, antipyrine, propofol, ketamine, pentobarbital, ropivacaine, lidocain, sevoflurane, methadone, pethidine, selegiline, diazepam, temazepam, clotizazepam, midazolam, estrone, testosterone (Wang et al., 2008).

Like for other CYPs like CYP2D6, 2C9 and 2C19, studies have established genetic variations in CYP2B6 with potentially significant clinical significance (Gandhi et al., 2012; Huang et al., 2012). CYP2B6 is induced by phenobarbital and rifampicin. Bupropion hydroxylation is a standard *in vitro* and *in vivo* probe reaction for CYP2B6. Some important substrates of CYP2B6 are listed in Table 22.

Table 22: Examples of CYP2B6-mediated drug metabolic reactions (Adapted and updated from Lewis, 2003)

Drugs	Pharmacological uses	CYP2B6-mediated reaction
4-Trifluoromethyl 7-ethoxycoumarin	CYP2B6 biomarker substrate	O-deethylation
7-Benzyloxyresorufin	CYP2B6 biomarker substrate	O-debenzylation
S-Mephenytoin	Anticonvulsant	N-demethylation
Benzphetamine	Weight loss	N-demethylation
Testosterone	Anabolic agent	16β-hydroxylation
Bupropion	Antidepressant	t-butyl oxidation
PNU 249173	CYP2B6 biomarker substrate	cyclopentyl hydroxylation
Diazepam	Sedative	N-demethylation
7-Ethoxycoumarin	Anticoagulant	O-deethylation
4-Chloromethyl 7-ethoxycoumarin	Anticoagulant	O-deethylation
3-Cyano-7-ethoxycoumarin	Anticoagulant	O-deethylation

CYP2C8 represents about 7% of the total hepatic CYP. Extrahepatic expression is found in the brain, kidney, adrenal gland, heart, ovary, uterus, mammary gland, and duodenum (Delozier et al., 2007). It is responsible for the primary metabolism of thiazolidinediones (rosiglitazone, pioglitazone and troglitazone); meglitinides (repaglinide); 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (cerivastatin); chemotherapeutic agents (paclitaxel and all-*trans*retinoic acid); antimalarials (amodiaquine and chloroquine); antiarrhythmics (amiodarone) and retinoid derivatives (tazarotenic acid) (Delozier et al., 2007). CYP2C8 plays some minor role in the metabolism of NSAIDs (ibuprofen, diclofenac and tenoxicam), fluvastatin, simvastatin acid, carbamazepine, cyclophosphamide, dapsone, diltiazem, ifosfamide, loperamide, methadone, morphine, torsemide, verapamil and zopiclone (Totah and Tettie, 2005). More important CYP2C8 substrates are shown in Table 23. 6α-hydroxylation of paclitaxel is a standard probe reaction for CYP3C8 activity (See Chapter 3).

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Table 23: Some CYP2C8 substrates and other CYP isoforms that contribute to their metabolism (adapted - with permission - and updated from Daily and Aquilante, 2009; see also Totah and Retie, 2005)

Specific drugs	Drug class	Treatment indication	Other CYP enzymes that play a role in metabolism
Major contribution of CYP2C8 to metabolism			
All-trans-retinoic acid	Vitamin A derivative	Promyelocytic leukemia	CYP2C9
Amiodarone	Class III antiarrhythmic	Supraventricular and ventricular arrhythmias	CYP3A4, CYP1A2, CYP2C19, CYP2D6
Amodiaquine	4-aminoquinoline	Malaria	CYP3A4, CYP1A1, CYP1B1
Cerivastatin*	HMG-CoA reductase inhibitor	Hyperlipidemia	CYP3A4
Chloroquine	4-aminoquinoline	Malaria	CYP3A4, CYP2D6
Paclitaxel	Taxane	Solid malignant tumors (e.g., breast, ovarian, lung)	CYP3A4, CYP3A5
Repaglinide	Meglitinide	Type 2 diabetes	CYP3A4
Rosiglitazone, pioglitazone	Thiazolidinedione	Type 2 diabetes	CYP2C9, CYP3A4, CYP1A1
Tazarotenic acid	Retinoid derivative	Acne and psoriasis	–
Troglitazone*	Thiazolidinedione	Type 2 diabetes	CYP3A4
Intermediate or minor contribution of CYP2C8 to metabolism			
Carbamazepine	Anticonvulsant	Epilepsy	CYP3A4 (major)
Cyclophosphamide	Nitrogen mustard alkylating agent	Lymphoma, leukemia, solid tumors	CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP3A
Dapsone	Antibacterial sulfone	Leprosy and pneumocystis pneumonia	CYP2C9 (major)
Diclofenac	NSAID	Pain and inflammation	CYP2C9 (major), CYP3A4, and CYP2C19
Diltiazem	Calcium channel blocker	Hypertension, arrhythmias, angina	CYP3A4, CYP2C9
Fluvastatin	HMG-CoA reductase inhibitor	Hyperlipidemia	CYP2C9 (major), CYP3A4
Ibuprofen	NSAID	Pain and inflammation	CYP2C9
Ifosfamide	Nitrogen mustard	Various cancers	CYP2A6, CYP2B6,

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Specific drugs	Drug class	Treatment indication	Other CYP enzymes that play a role in metabolism
	alkylating agent		CYP2C9, CYP2C19, CYP3A
Loperamide	μ-opioid receptor agonist	Antidiarrheal	CYP2B6, CYP2D6, CYP3A4
Methadone	Opioid agonist	Pain and addiction to opioids	CYP3A4, CYP2D6, CYP2B6
Morphine	Opioid agonist	Pain	CYP3A4
Simvastatin acid	HMG-CoA reductase inhibitor	Hyperlipidemia	CYP3A4 (major)
Tenoxicam	NSAID	Pain and inflammation	CYP2C9 (major)
Torasemide	Loop diuretic	Edema	CYP2C9 (major)
Verapamil	Calcium channel blocker	Hypertension, arrhythmias, angina	CYP3A4 (major), CYP3A5
*Withdrawn from the market.			

CYP2D6 is an important drug metabolizing enzyme responsible for the metabolism of haloperidol, metoprolol, propranolol, codeine, bufuralol, debrisoquine, imipramine, desipramine, nortriptyline, ondansetron and dextromethorphan. CYP2D6 is particularly important because of its display of genetic polymorphism leading to large inter-individual and inter-ethic/racial differences in CYP2D6-mediated drug metabolism. CYP2D6 is not inducible. Bufuralol 1'-hydroxylation is a probe reaction for CYP2D6 activity.

CYP2E1, which constitutes about 7% of the hepatic CYP, is well known as the catalyst for the metabolism of ethanol. It is also known to activate chemical carcinogens. Drugs metabolized by CYP2E1 include paracetamol, enflurane, chlorzoxazone, salicylic acid and halothane. It is inhibited by disulfiram and induced by acetone, ethanol, pyridine, pyrazole, and isoniazid. 6-hydroxylation of chlorzoxazone is a CYP2D6-specific reaction, used to probe its activity.

6.2. Aims and objectives

The aim of this chapter was to investigate the inhibitory influence of a 50% methanolic extract of *Lessertia*, and the aqueous extracts of *Hypoxis* on the metabolic activity of CYP2A6, 2B6, 2C8, 2D6 and 2E1. Further objective was to quantify the inhibitory effects, where observed, in IC_{50} terms.

6.3. Materials and Methods

6.3.1. Materials

The sourcing, selection and preparation of the medicinal herbs, the chemical compounds and the HLM used in this study are as described in earlier chapters (Chapter 4 and 5). In addition, 1'-hydroxybufuralol maleate, and bufuralol hydrochloride were purchased from Ultrafine Chemicals (Pty) Ltd (Manchester, UK); 6-hydroxychlorzoxazone, 7-hydroxycoumarin, bupropion, chlorzoxazone, coumarin, paclitaxel and [$^{13}C_6$] 7 hydroxycoumarin were obtained from Novartis Pharma AG (Basel, Switzerland); hydroxybupropion, [$^{2}H_6$] hydroxybupropion and [$^{2}H_9$] 1'-hydroxybufuralol were purchased from BD Biosciences (Pty) Ltd (San Jose, USA) and 6α-hydroxypaclitaxel from Gentest BD Biosciences (Woburn, USA). All other chemicals used were of analytical grade.

6.3.2. Methods

6.3.2.1. Incubation to determine the inhibitory effects of herbal extracts on the CYPs

Using similar methodology as in Chapter 5 (Section 5.3), test substrates were co-incubated with graded concentrations of herbal extracts in HLM mixture. The concentration of substrates used was less than the literature K_m value (Table 24). Isozyme-specific inhibitors were employed as positive controls. The results were analyzed similarly (see Chapter 5). A summary of the incubation conditions is provided in Table 24. Table 25 provides the summary of the LC/MS conditions for analysis.

Table 24: Microsomal incubation conditions for the CYP-catalyzed reactions

Parameters	Values
Concentration of potassium phosphate buffer, pH 7.4 in incubation mixture	50 mM
Concentration of magnesium chloride in incubation mixture	5 mM
Incubation pH	7.4
Final graded concentration of herbal extracts	0 – 100 µg/mL
Concentration of DMSO/methanol in incubation mixture	0.5%($^{v}/_{v}$)
Final concentration of NADPH in the incubation mixture	1 mM
Incubation time	20 min

CYP2A6

CYP2A6-specific metabolite	7-hydroxycoumarin
HLM Concentration	0.2mg protein/mL
Positive control	Sulfaphenazole
Initial coumarin concentration	2.5 µM
Literature K_m values	[a]0.5-3

CYP2B6

CYP2B6-specific metabolite	Hydroxybupropion
HLM Concentration	0.1mg protein/mL
Positive control	Sertraline
Initial bupropion concentration	25 µM
Literature K_m values	[b]76, [c]89, [d] 130

CYP2C8

CYP2C8-specific metabolite	6α-hydroxypaclitaxel
HLM Concentration	0.2mg protein/mL
Positive control	Sulfaphenazole
Initial paclitaxel concentration	10 µM
Literature K_m values	[e]4.0, [f]15

CYP2D6

CYP2D6-specific metabolite	1'-hydroxybufuralol
HLM Concentration	0.2mg protein/mL
Positive control	Quinidine
Initial bufuralol concentration	5 µM
Literature K_m values	[g]13, [h]44

CYP2E1

CYP2E1-specific metabolite	6-hydroxychlorzoxazone
HLM Concentration	0.5mg protein/mL
Positive control	Diethyl-dithiocarbamate
Initial chlorzoxazone concentration	10 µM
Literature K_m values	[i]22-49

[a] Shimada et al., 1996, Draper et al., 1997; [b] Faucette et al., 2000; [c] Hesse et al., 2000; [d] Li et al., 2003; [e] Rahman et al., 1994; [f] Cresteil et al., 1994; [g] Boobis et al., 1985; [h] Yamazaki et al., 1994; [i] Peter et al., 1990

Table 25: Summary the LC/MS analytical conditions for the quantitative determination of CYP metabolites

	7-hydroxycoumarin	**6-hydroxychlorzoxazone**	**Hydroxybupropion**	**6α-hydroxypaclitaxel**	**1'-hydroxybufuralol**
Instrument type	Agilent Chemstation 1100 HPLC system (Agilent Technologies, Palo Alto, USA) equipped with a capillary pump and a triple quadrupole Quattro Ultima mass spectrometer (Waters, Milford, USA)				
Mobile phase	A: Water (plus 0.1% formic acid) B: Acetonitrile(plus 0.1% formic acid)				
Mobile phase proportion	Isocratic with 30% solvent B		Time (min)	Solvent A (%)	Solvent B (%)
			0.0	85	15
			0.5	85	15
			2	5	95
			4	5	95
			4.2	85	15
			8	85	15
Flow speed	60 µl / min				
Retention time (min)	3.5	4.2	5.2	6.2	4.5
Column type	Luna Phenyl-Hexyl 3 µm, inner dimensions 50 x 1 mm (Phenomenex, Torrance, USA)				
Mode	Positive	Negative	Positive	Positive	Positive
Retention time	1.8 min	2.2 min	3.4 min	4.8 min	6.3 min
Total run time	3 min		8 min		
Internal standard	[$^{13}C_6$]7-hydroxy-coumarin	[$^{2}H_3$] 4'-hydroxy-mephenytoin	[$^{2}H_6$] hydroxybupropion	warfarin	[$^{2}H_9$] 1'-hydroxybufuralol
Column temperature	30°C				
Nebulizing temperature	375°C				
Mass- to-charge range	3000 *m/z*				
MRMs	10 MRM (multiple reaction monitoring) per analyte				
Reference	A solution mix of acetaminophen, caffeine, sulfaguanidine, sulfadimethoxine, Val,-Tyr-Val, verapamil, terfenadine, leucine-enkephalin and reserpine				
Dwell time (msec)	200 msec				
Injection volume	2 µl				

6.3.2.2. Analysis of the inhibitory effects on the CYPs

The CYP activity in the presence of the herbal extracts was profiled against the extract concentrations. IC_{50} values were estimated with the SigmaPlot® Enzyme Kinetic software based on Equation 6.1.

$$y = \frac{100\%}{1+\left(\frac{x}{IC50}\right)^{s}}$$

Equation 6.1

6.3.2.3. Statistical analysis

The percentage inhibition observed for the test extracts is defined in absolute terms. The absolute values were obtained by comparing the metabolite production in the presence of test extracts, to metabolite production in the absence of any inhibitor (test extract or known inhibitors) (Bjornsson et al., 2003). The nonlinear-regression was generated by profiling the extract concentration against observed enzyme activity. The kinetic parameters were determined from the generated plot from the SigmaPlot® software with $R^2 > 0.9$ and $p < 0.05$ as the minimum level of significance.

Positive controls are used in *in vitro* drug metabolism studies for qualitative purposes only. The positive controls (known CYP inhibitors) only provide indications that the enzymes can be inhibited in the experimental set up. By general standard, the use of HLM is for qualitative determination of the ability/potential of chemical substances to inhibit CYP enzymes (Brandon et al., 2003). The results obtained may not be linearly related to *in vivo* situation.

6.4. Results: Influence of the herbal extracts on CYP activity

Figures 75 - 84 show the results of the inhibitory activity of the herbal extracts on CYP2A6, CYP2B6, CYP2C8, CYP2D6 and CYP2E1 activity. Values represent the mean of intra-day and inter-day duplicates. Intra-day and Inter-day variations were less that 10%.

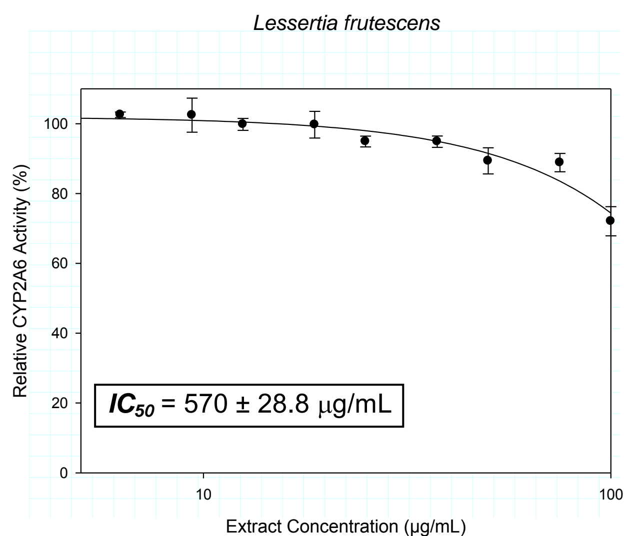

Figure 75: The profile of CYP2A6-catalyzed metabolism of coumarin in the presence of graded concentration of crude methanolic extracts of *Lessertia frutescens* (R^2 = 0.99)

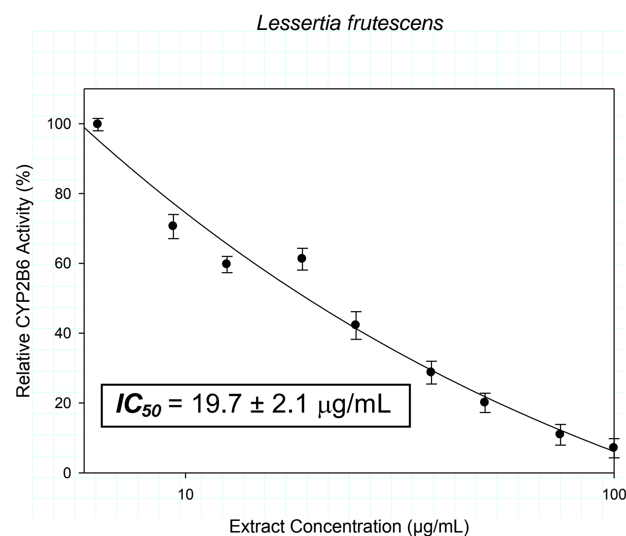

Figure 77: The profile of CYP2B6-catalyzed metabolism of bupropion in the presence of graded concentration of crude methanolic extracts of *Lessertia frutescens* (R^2 = 0.99)

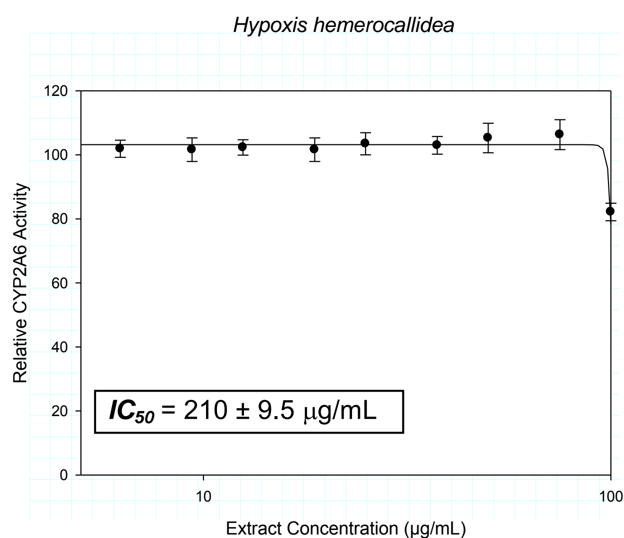

Figure 76: The profile of CYP2A6-catalyzed metabolism of coumarin in the presence of graded concentration of crude aqueous extracts of *Hypoxis hemerocallidea* (R^2 = 0.99)

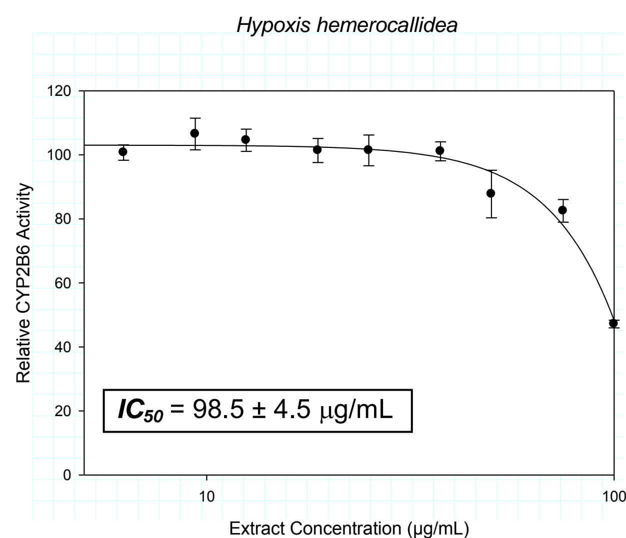

Figure 78: The profile of CYP2B6-catalyzed metabolism of bupropion in the presence of graded concentration of crude aqueous extracts of *Hypoxis hemerocallidea* (R^2 = 0.99)

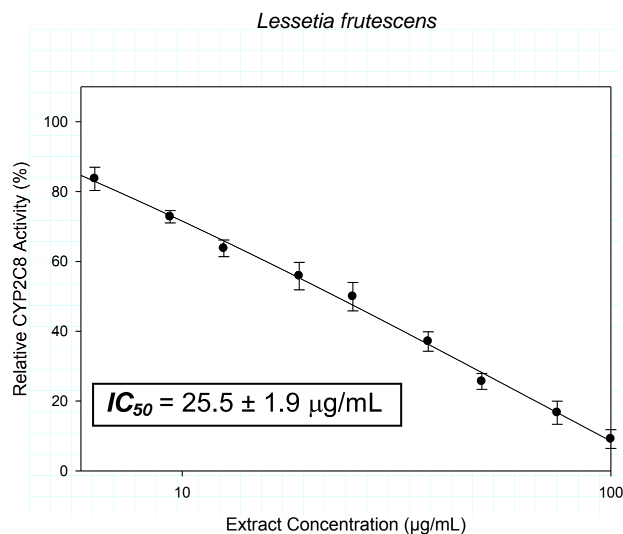

Figure 79: The profile of CYP2C8-catalyzed metabolism of paclitaxel in the presence of graded concentration of crude methanolic extracts of *Lessertia frutescens* (R^2 = 0.99)

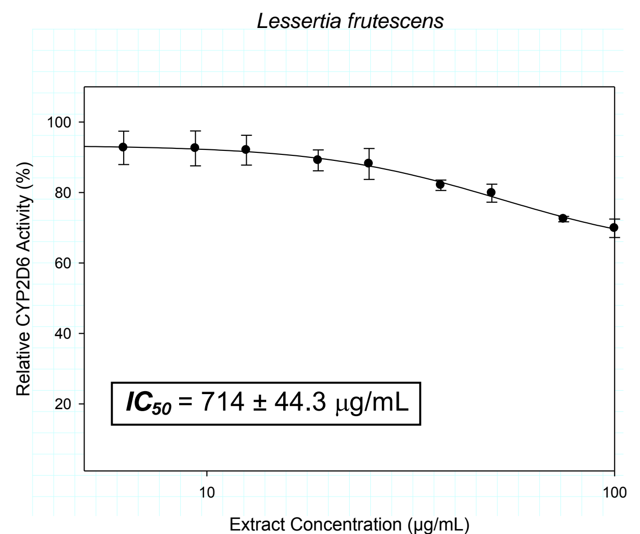

Figure 81: The profile of CYP2D6-catalyzed metabolism of bufuralol in the presence of graded concentration of crude methanolic extracts of *Lessertia frutescens* (R^2 = 0.99)

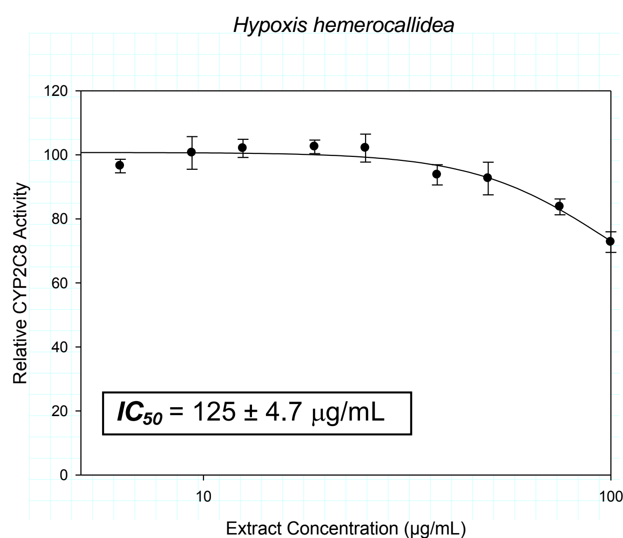

Figure 80: The profile of CYP2C8-catalyzed metabolism of paclitaxel in the presence of graded concentration of crude aqueous extracts of *Hypoxis hemerocallidea* (R^2 = 0.99)

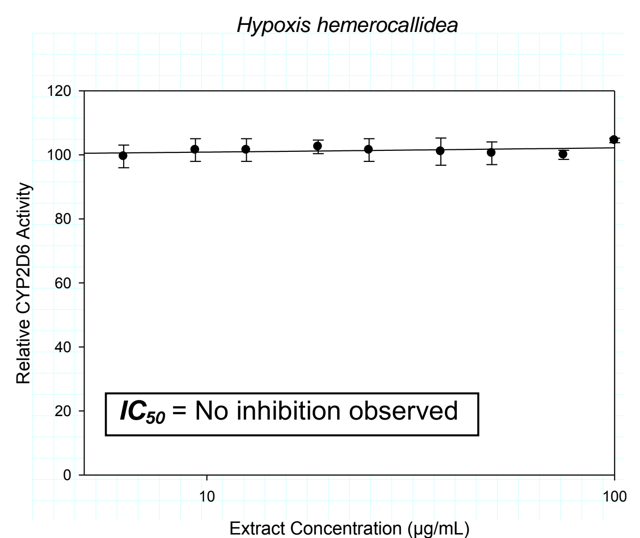

Figure 82: The profile of CYP2D6-catalyzed metabolism of bufuralol in the presence of graded concentration of crude aqueous extracts of *Hypoxis hemerocallidea* (R^2 = 0.99)

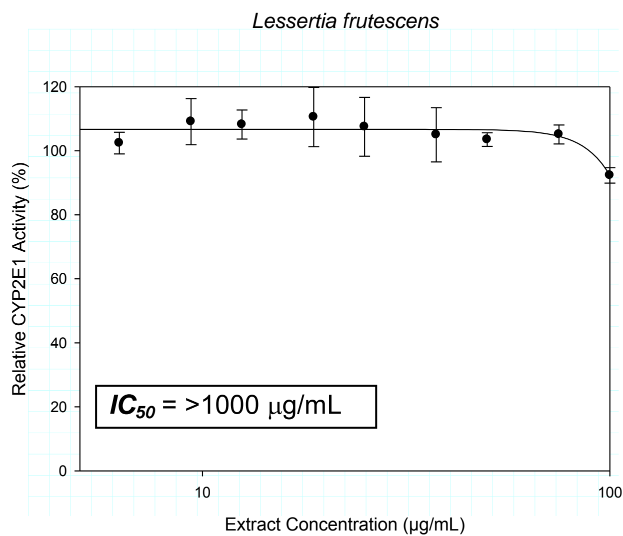

Figure 83: The profile of CYP2E1-catalyzed metabolism of chlorzoxazone in the presence of graded concentration of crude methanolic extracts of *Lessertia frutescens* (R^2 = 0.99)

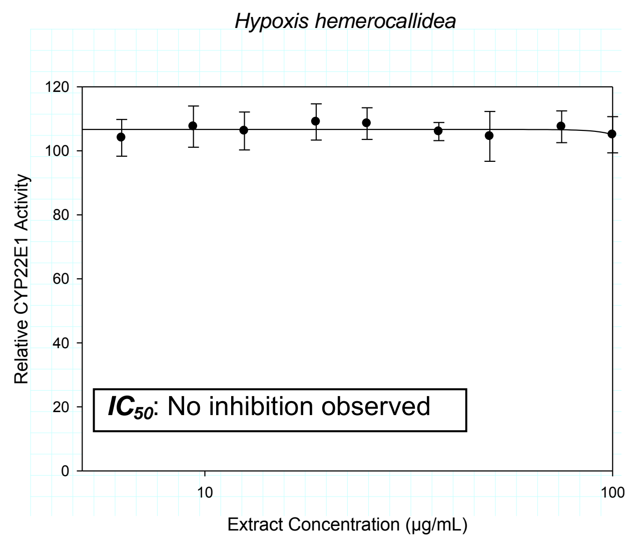

Figure 84: The profile of CYP2E1-catalyzed metabolism of chlorzoxazone in the presence of graded concentration of crude aqueous extracts of *Hypoxis hemerocallidea* (R^2 = 0.99)

6.5. Discussion

Lessertia demonstrated a very strong inhibition of the metabolic activity of CYP2B6 (IC_{50} = 19.7 ± 2.1 μg/mL) and CYP2C8 (IC_{50} = 25.5 ± 1.9 μg/mL), with a weak inhibition of CYP2A6 (IC_{50} = 570 ± 28.8 μg/mL) and CYP2D6 (IC_{50} = 714 ± 44.3 μg/mL) and a less potent inhibition of CYP2E1 (IC_{50} >1000 μg/mL). *Hypoxis* on the other hand only inhibited the activity of CYP2A6 (IC_{50} = 210 ± 9.5 μg/mL), CYP2B6 (IC_{50} = 98.5 ± 4.5 μg/mL) and CYP2C8 (IC_{50} = 125 ± 4.7 μg/mL). No inhibitory activity was observed on CYP2D6 and CYP2E1.

These results are important in the light of the many substrates of the CYP2A6, CYP2B6, CYP2C8, CYP2D6 and CYP2E1 isoenzymes and will be clinically relevant for the management of patients who concurrently use conventional medicines and *Lessertia* or *Hypoxis.*

6.6. Conclusion

The effect of the crude extracts of *Lessertia frutescens* and *Hypoxis hemerocallidea* on CYP2A6, CYP2B6, CYP2C8, CYP2D6 and CYP2E1 was investigated. Both *Lessertia* and *Hypoxis* potently inhibited the metabolic activity of CYP2A6, CYP2B6 and CYP2C8. If sufficient *in vivo* concentrations are achieved, *Lessertia* and *Hypoxis* possess the potential for herb drug interactions with the substrates of CYP2A6, CYP2B6 and CYP2C8.

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CHAPTER SEVEN

ASSESSMENT OF THE INFLUENCE OF CRUDE EXTRACTS OF *LESSERTIA FRUTESCENS* AND *HYPOXIS HEMEROCALLIDEA* ON THE *IN VITRO* METABOLISM OF MIDAZOLAM IN HUMAN HEPATOCYTES

7.0. Summary

Graded concentrations of crude extracts of *Lessertia frutescence* (LT) and *Hypoxis hemerocallidea* (HP) were separately co-incubated with 1µM midazolam (MDZ), a CYP3A4/5-specific substrate, in human hepatocytes for up to 6 h. The incubate profiles of MDZ were analyzed by UPLC and known metabolites were identified by LC-MS/MS. The *in vitro* intrinsic clearance values of MDZ (in the presence and absence of LT and HT) were calculated and scaled to hepatic metabolic clearance values *in vivo*.

LT inhibited the production of MDZ metabolites with about 40% reduction in upscaled intrinsic *in vivo* clearance of MDZ (24.98 compared to 40.98 (L/h)/kg body mass). However, neither LT nor HP reduced hepatocyte viability as measured after 6 hours of incubation.

7.1. Introduction and objectives

The main objective of this study was to assess the influence of LT and HP on the metabolism of CYP3A4-mediated metabolism of MDZ in human hepatocytes.

7.2. Material and methods

7.2.1. Materials

The sourcing and preparation of *Lessertia frutescence* and *Hypoxis hemerocallidea* are as described in Chapter Four.

All chemical compounds used were sourced as described in Chapter Five.

Pooled, mixed gender cryopreserved hepatocytes prepared from 20 donors were obtained from Celsis *In vitro* technologies (Pty) Ltd (Baltimore, MD, USA), stored in a liquid nitrogen tank (until use), and thawed according to supplier instructions before use.

7.2.1.1. Test solutions used for the incubations

A 10 mM MDZ solution was prepared and stored at -20°C. A 20µL volume was diluted with 180µL of DMSO to produce 1mM working solution and stored at -20°C, such that the addition of 1 µL of the working solution to 1mL cell incubation mixture would yield a final concentration of 1µM MDZ.

Stock solutions (20mg/mL each) of LT (methanol) and HP (water) were separately prepared, such that an addition of 5 µL of each per 1mL incubation mixture will yield a final incubation mixture of 100µg/mL concentration.

7.2.1.2. Hepatocytes

The characteristics of cryopreserved hepatocytes used in this study are described in Table 26. The hepatocyte mixture was stored in a liquid nitrogen tank. On the day of the incubations, it was thawed according to the instructions provided by the supplier.

Table 26: Characteristics of cryopreserved hepatocytes used in the study (Appendix H)

Species	Human
Strain	n/a
Gender	Pooled (male and female)
Supplier	Celsis/*In vitro* Technologies
Catalog/product number	X008000
Lot number	SSB
Number of individuals pooled	20

7.2.2. Methods

7.2.2.1. Hepatocyte incubations and viability measurements

The cryopreserved hepatocytes were thawed and the cells were suspended in HepatoZYME buffer.

The viability of the suspended hepatocytes was determined by a Guava EasyCyte Mini system using the ViaCount assay according to the instructions of the supplier (Guava Technologies). After cell counting, the cell density was adjusted to approximately 1.3 x 10^6 viable cells/mL with HepatoZYME media. The incubation mixture contained 5 µL of 20 mg/mL LT and HP (separately) in 1mL cell incubation medium. The metabolic activity of the hepatocytes in the incubation mixture was initiated by the addition of 1 µL of 1 mM MDZ into the incubation mixture. The initial concentration of MDZ was 1 µM. The final concentration of organic solvent (methanol and/or DMSO) was 0.5% (v/v). The final concentration of the test inhibitors (LT and HP) was 100 µg/mL. For the cell and MDZ stability test incubation, 12-well Nunclon Surface plates were used. For the incubation to determine the effects of LT and HP on MDZ metabolism, 25 mL tissue culture flasks (Becton Dickinson Franklin Lakes) were used and aliquots of 300 µL were taken at the different time points. Both types of incubations were performed at 37°C under a humidified atmosphere of 95% air and 5% CO2 in a Heraeus incubator/Cytoperm. During the incubations, the plates or flasks were shaken (70 rpm). The viability of the hepatocytes was determined after 0 and 6 h for the metabolites profiles. The metabolic reactions were stopped by the addition of 2 volumes of acetonitrile. Samples were kept at -20°C until analysis.

To monitor the chemical stability of MDZ under the incubation conditions, the compound was incubated in HepatoZYME in the absence of hepatocytes. A second series of control incubations was performed with hepatocytes (without MDZ) in the presence of the test inhibitor compounds to investigate the effect of the latter on the viability of the cells, and

possible cytotoxicity of the compounds. Further details of the incubations are given in Tables 27, 28 and 29. Post-incubation analysis was performed under the conditions highlighted in Tables 30 and 31.

Table 27: Hepatocyte incubation conditions: Incubations for monitoring the effects of test compounds on MDZ metabolism

Hepatocytes	Human, cryopreserved
Initial substrate concentration (µM)	1.0
Volume of substrate (MDZ) solution added (µL/mL final incubate)	1.0
Volume of test inhibitor solution added (µL/mL final incubate)	5.0
Initial density of viable cells (10^6/mL)	1.3
Viability at 0 h (%)	78.3
Incubation times (h)	6
Incubation volume (mL)	3

Table 28: Conditions for control incubation

Initial substrate concentration (µM)	1.0
Volume of test inhibitor solution added (µL/mL final incubate)	5.0
Initial density of viable cells (10^6/mL)	1.3
Viability at 0 h (%)	78.3
Incubation times (h)	6
Incubation volume (mL)	1

Table 29: Summary of the hepatocyte incubation conditions and methodology

Test compound and conditions	1 mM MDZ, 20 mg/mL LT and HP used for hepatocyte incubations.
Sampling times	Kinetic measurements to determine the intrinsic metabolic clearances and metabolite profiles of MDZ were performed at 0, 1, 2, 4 and 6 h incubation.
Incubation methods for intrinsic clearance calculations and metabolite profiles	For the kinetic evaluations, 1 µM MDZ was mixed in a serum-free culture medium containing approximately 1.3 x 10^6 hepatocytes/mL. Sample from each time point was analyzed by HPLC. From these kinetic data, intrinsic clearance was calculated.
Incubation methods for metabolite profiles	Determination of MDZ metabolites was accomplished by LC-MS/MS analysis (including accurate mass measurements) using incubation supernatants from hepatocytes.
Stop solution containing internal standard (d4-1'hydroxymidazolam)	50µL of a solution of d4-hydroxymidazolam was prepared in methanol and stored at -20°C. 15µL of the solution was diluted to 15mL with methanol to give a 0.1µg/mL (0.3µM). This was used as stop solution for enzymatic reactions (stored at 4°C)
Post-incubation sample preparation for analysis	At the end of the incubation period, a 300 µL aliquot was mixed with two volumes of ice-cold acetonitrile (containing 0.3M d4-hydroxymidazolam as internal standard) to stop the enzymatic reactions. The mixture was vortex-mixed and then stored at -20°C (for at least 4 hr) until analysis to complete the protein precipitation. Before use, the samples were thawed and centrifuged (centrifuge 3K30, Sigma) for 20 min at 15,800 *g* and the supernatant was decanted. The supernatants were evaporated under N_2 flow. The dried samples were reconstituted with 25 µL of acetonitrile and 475 µL water. The solution was shaken and sonicated for 10 minutes.

Table 30: HPLC methods for MDZ analysis and metabolite profiling/identification

LC instrumentation	Acquity UPLC system (Waters Corporation, Manchester, U.K.), equipped with a binary solvent manager, a sample manager, a column manager and a photodiode array detector (PDA). UV spectra were monitored in the range of 210-400 nm. The operating software for the UPLC system was MassLynx V4.1.	
Guard column	Acquity UPLC HSS T3 2.1 x 5 mm, 1.8 µm (Waters)	
Analytical column	Acquity HSS T3 1.8 µm, 2.1 x 150 mm (Waters)	
Column conditions	Thermostated at 50°C	
Injection volume	Up to 125 µL	
Mobile phase	A: Water/Acetonitrile: (90/10 v/v) + 0.1% formic acid B: Acetonitrile + 0.1% formic acid	
Flow rate	0.5 mL/min	
Gradient	0 min:	0 % B
	1 min:	0 % B
	17 min:	50 % B
	20.1 min:	95% B
	25 min:	95% B
	25.1 min:	0% B
	30 min:	0 % B
Flow splitting and Radioactivity detection - offline	The LC flow post column was directed to the UV detector, after which it was split (approximately 1:5), as follows. *LC-MS analysis:* The minor split was directed to the LC-MS ESI interface. The major split was diverted to waste	

Table 31: MS conditions used for metabolite analysis

Instrument	Time-of-flight mass spectrometer model Synapt G2 HDMS operated under MassLynx, Version 4.1. (Waters Corporation, Manchester, U.K.)		
Ionization mode	Electrospray in positive ion mode		
Ion source	Spray capillary:		3.0 kV
	Cone voltage:		30 V
	Nebulizer gas:		Nitrogen (7 bar)
	Cone gas:		Nitrogen (25 L/h)
	Desolvation gas:		Nitrogen (800 L/h)
	Source block temperature:		120°C
	Desolvation temperature :		450°C
Collision energy for LC-MS and LC-MS^E or LC-MS/MS fragmentation	Trap cell	MS	4 eV
		MS^E or MS/MS	ramping 10-45 V
	Transfer cell	MS	0 eV
		MS^E or MS/MS	30 eV
Time-of-flight mass analyzer	Mass resolution ~10'000 (full-width at half-maximum definition), Sensitivity-mode		
Accurate mass measurement	The reference channel of the LockSpray interface was operated with a solution of Leucine-Enkephaline (0.2 µg/mL) in acetonitrile at a flow rate of 5 µL/min. The $[M+H]^+$ ion of Leucine-enkephaline at *m/z* 556.2771 was used as lock mass for recalibration of the spectra to obtain exact mass data. The following Lock Spray configuration was set up.		
	Cone voltage:		50 V
	Capillary voltage:		2.4 kV
	Scan frequency:		20 sec.
	Trap collision energy:		4 eV

7.2.2.2. Clearance calculations

Initial half-life ($T^1/_2$) and elimination rate constants (lambda=ln2/$T^1/_2$) of MDZ in hepatocyte incubates were calculated by log-linear regression of MDZ concentrations vs time using data from the sampling points of the 1 µM MDZ incubates showing a linear decrease on the semi-logarithmic plot.

The intrinsic clearance *in vitro* (CLint *in vitro*) was calculated from lambda and the cell density in the respective incubation (number of viable hepatocytes per mL at time zero) and scaled up to the intrinsic clearance *in vivo* (CLint *in vivo*) using the liver mass and the hepatocellularity (number of hepatocytes per gram of liver) of the respective species:

CLint *in vitro* = Lambda / cell density

CLint *in vivo* = CLint *in vitro* x liver mass x heptocellularity

From CLint *in vivo* and the hepatic blood flow (Q), the hepatic metabolic blood clearance (CLh,b) was predicted using the well-stirred model:

CLh,b = (CLint *in vivo* x Q) / (CLint *in vivo* + Q)

No corrections were made for the free fractions *in vitro* or *in vivo* i.e. they were assumed to be identical.

The value for liver mass, hepatocellularity and hepatic blood flow used in these calculations are given in Table 32.

Table 32: Values for liver mass, hepatocellularity and hepatic blood flow (See Davies and Morris, 1993)

Species	Human
Liver mass (g/kg body mass)	25.7
Hepatocellularity (millions cells/g liver)	99
Hepatic blood flow ((mL/min)/kg body mass)	20.7

7.3. Results and discussion

7.3.1. Intrinsic metabolic clearance of MDZ in hepatocytes in the presence of LT and HP

The intrinsic metabolic clearance of MDZ in the presence and absence of LT and HP was measured using human hepatocytes (Table 26). Extrapolation to hepatic blood clearance was performed using the well-stirred liver model (Davies and Morris, 1993). LT reduced the clearance of MDZ by 40% while the presence of HP did not alter the metabolism/clearance of MDZ (Figure 85, Table 33). The viability of the cells was not affected by the presence of the test compounds. Structural characterization of the metabolites was carried out by LC-MS^2 and the results are discussed below.

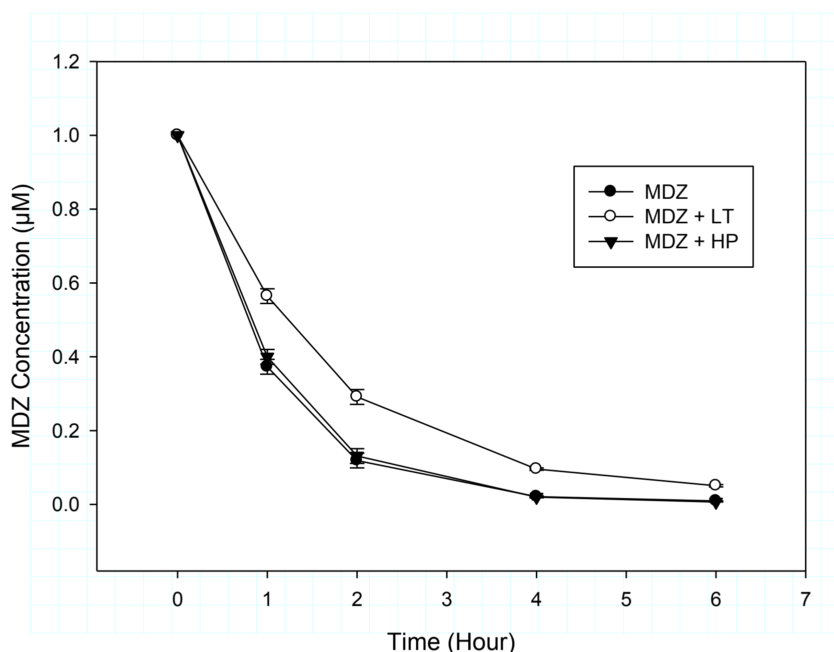

Figure 85: The influence of LT and HP on the *in vitro* clearance of MDZ in hepatocytes.

Table 33: The intrinsic clearance of MDZ in hepatocytes in the presence and absence of LT and HP

Hepatocytes	MDZ	MDZ + ST	MDZ + HP
Half-life (h)[1)]	0.72	1.18	0.70
CLint (µL/min/million cells)[2)]	16	10	16
CLint, scaled (mL/min/kg)[3)]	40.98	24.98	41.85
CLh,b (hepatic blood clearance) (mL/min/kg body weight)[4,5)]	14	11	14

1) Half-lives were evaluated by linear extrapolation
2) (ln2/T1/2) / mio viable cells x 1000
3) (CLint / 1000) * (cells / g liver) * (g liver / kg body weight)
4) CLh = (Qh * CLint, scaled) / (Qh + CLint, 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)

7.3.1.1. Interpretation of mass fragmentation of MDZ

Following the analysis of aliquots of human hepatocyte incubates with MDZ (t = 6h) by LC-MS^2, four metabolites were detected and were assigned as M1, M2, M3 and M4 (Figure 86) Using a combination of accurate mass measurement, elemental composition and MS/MS analysis, the 4 metabolites were identified as a combination of oxygenation and glucuronidation (M1), direct glucuronidation (M2) and a single oxygenation of MDZ (M3 and M4). These metabolites are consistent with previously published data on the metabolism of MDZ (Kronbach et al., 1989; Zhu et al., 2008; Seo et al., 2010).

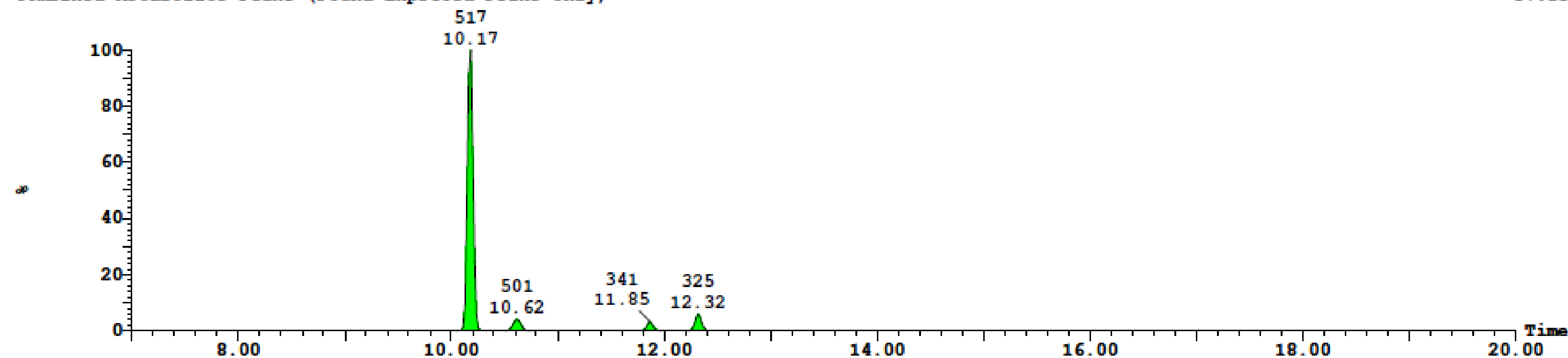

Figure 86: Extracted ion chromatograms showing formation of metabolites M1 (RT = 10.17 min), M2 (RT = 10.62 min), M3 (RT = 11.85 min) and M4 (RT = 12.32 min) of MDZ following incubation with hepatocytes

7.3.1.2. Influence of LT and HP on metabolite production

LT was specifically found to slow the formation of metabolites M1, M3 and M4 of MDZ while HP had no effects compared to controls (Figure 87 and 88). LC-MS peak areas for each compound were measured at 5 time points. The time-course (0-6 hr) influence of LT and HP on the formation of MDZ metabolites is shown in Figures 89 to 95.

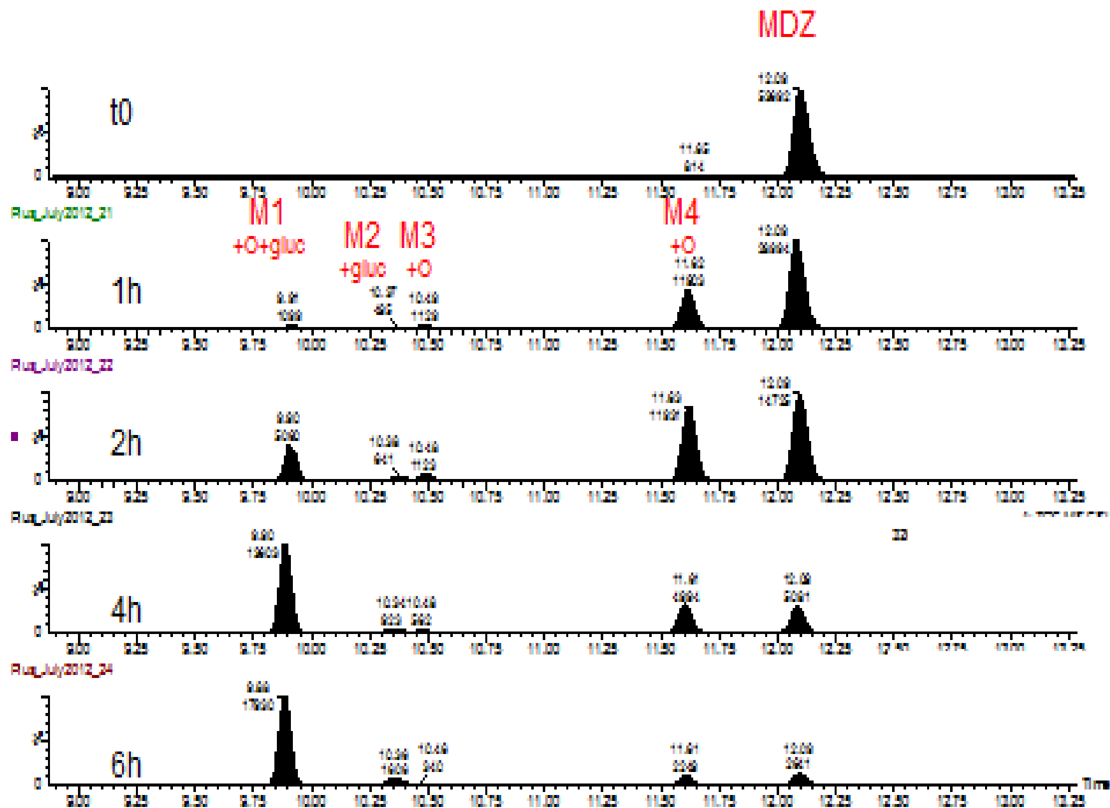

Figure 87: Extracted ion chromatograms showing formation of metabolites M1, M2, M3 and M4 of MDZ in human hepatocytes in the presence of LT

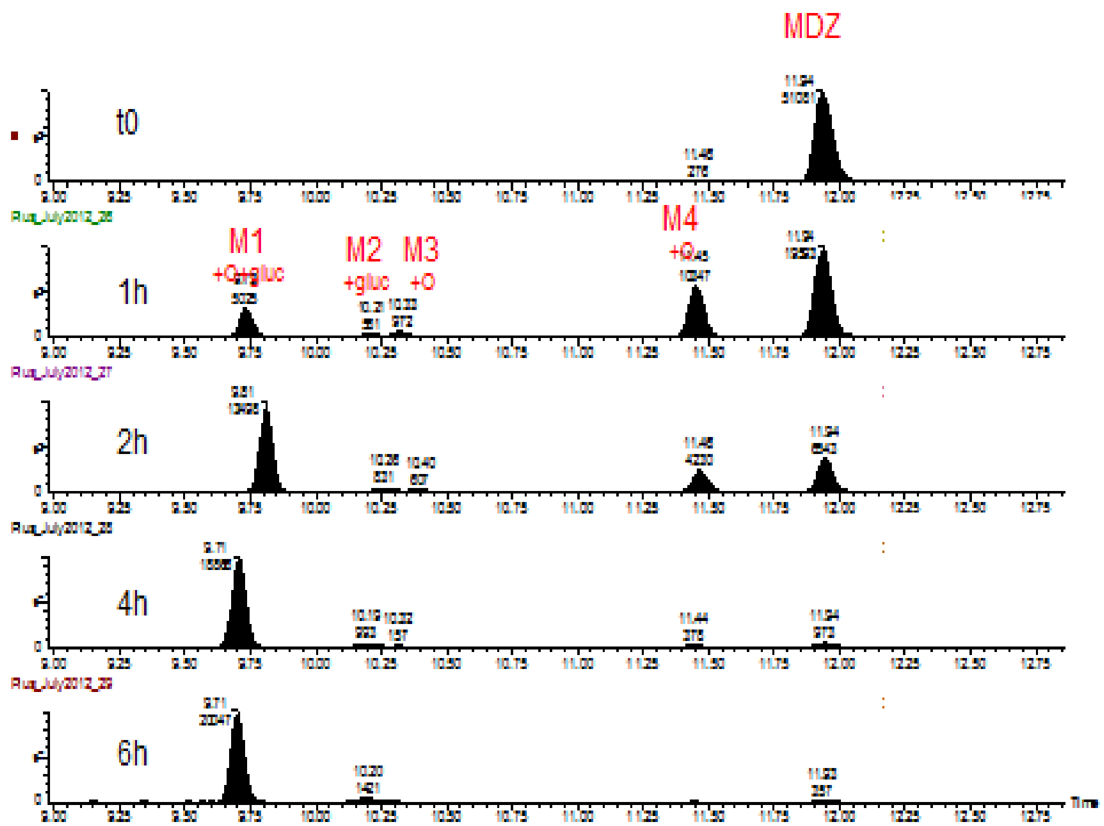

Figure 88: Extracted ion chromatograms showing formation of metabolites M1, M2, M3 and M4 of MDZ in human hepatocytes in the presence of HP

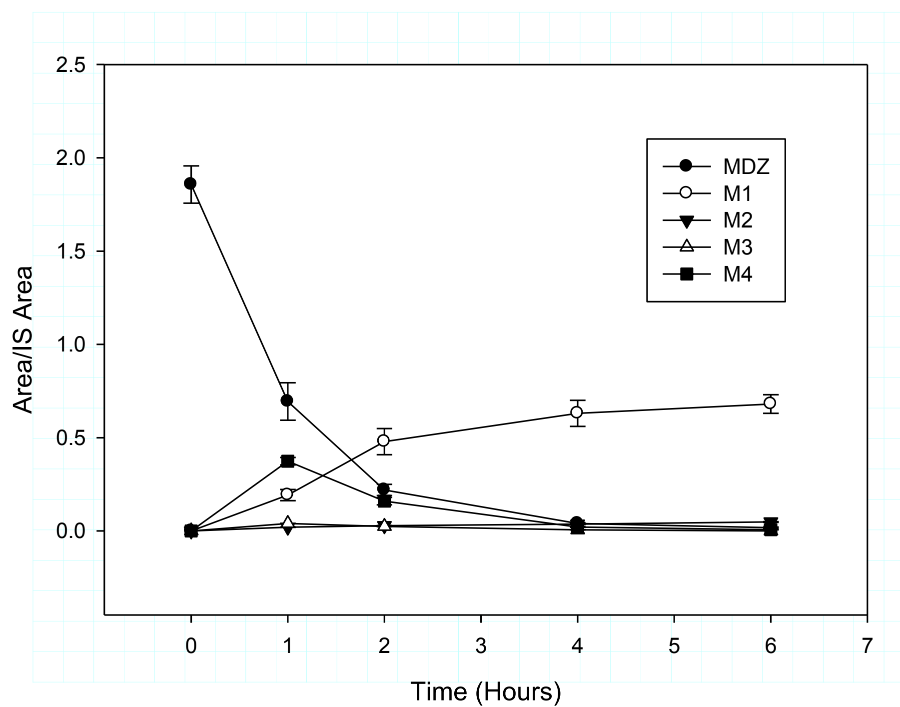

Figure 89: Time course over 6 hours showing the disappearance of MDZ and the formation of metabolites M1, M2, M3 and M4 in human hepatocyte incubations

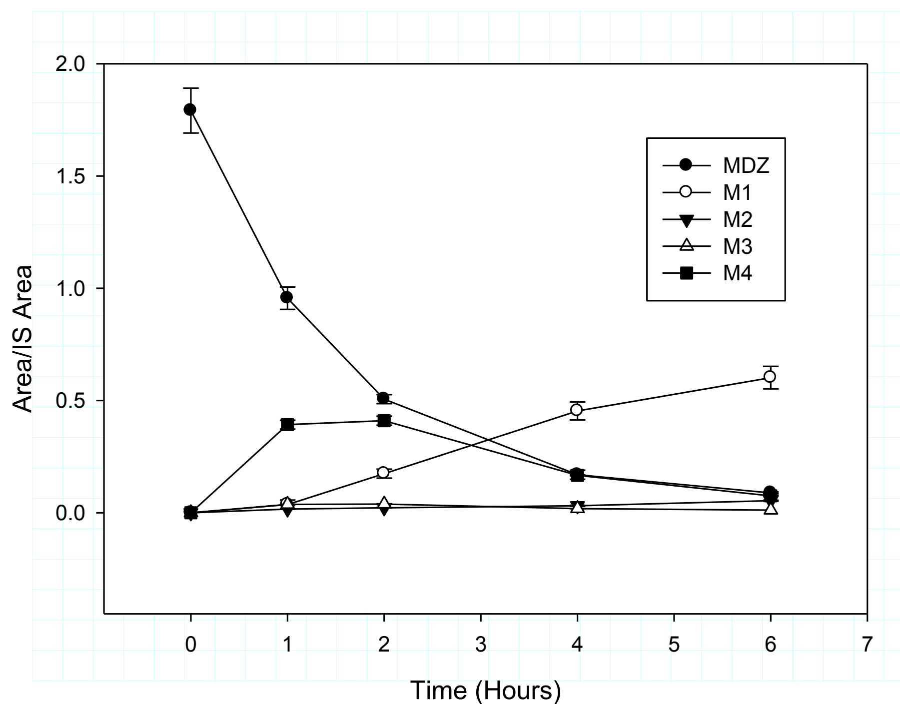

Figure 90: Time course over 6 hours showing the disappearance of MDZ and the formation of metabolites M1, M2, M3 and M4 in human hepatocyte incubations in the presence of ST.

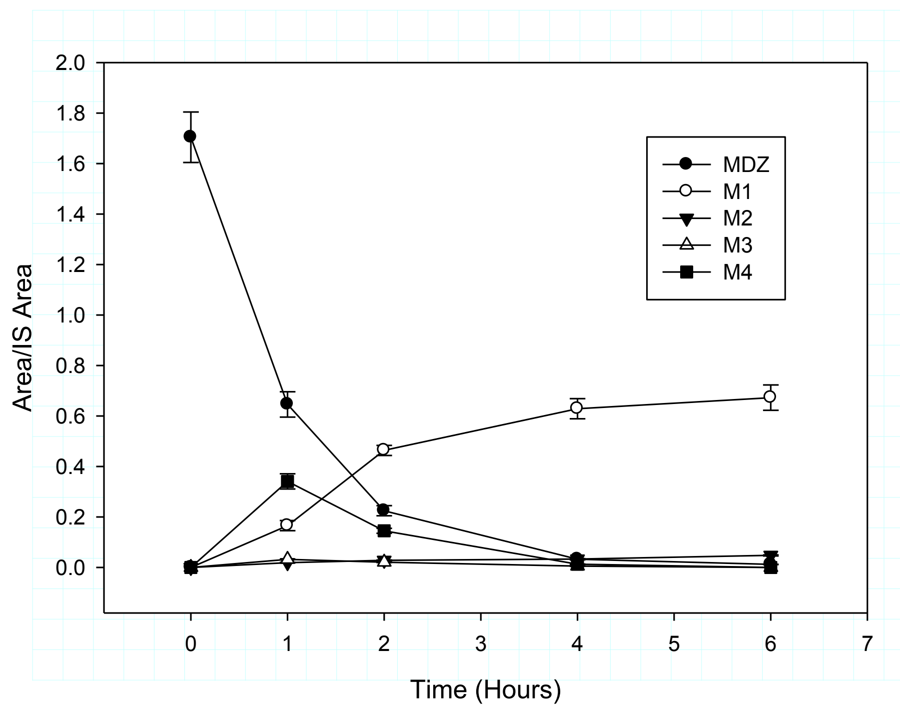

Figure 91: Time course over 6 hours showing the disappearance of MDZ and the formation of metabolites M1, M2, M3 and M4 in human hepatocyte incubations in the presence of HP

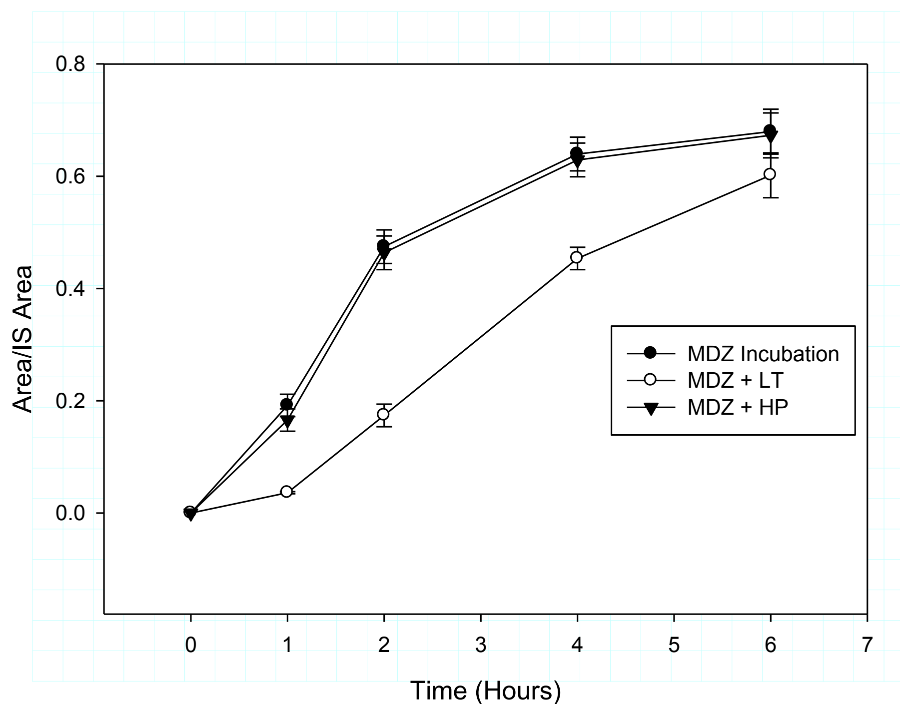

Figure 92: Time course showing the influence of LT and HP on the formation of metabolite M1 in human hepatocyte incubations

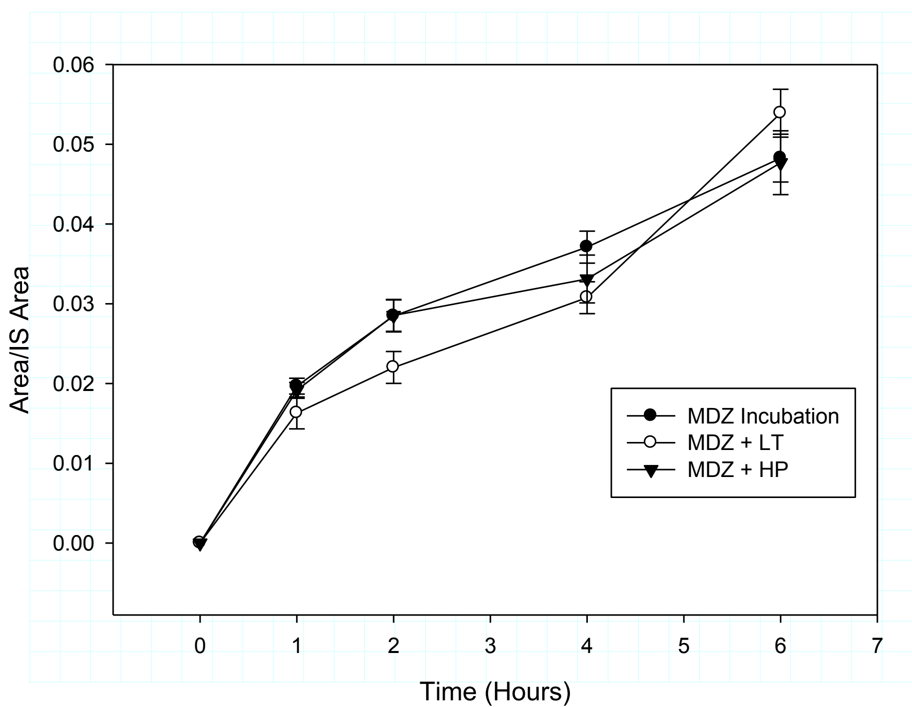

Figure 93: Time course showing the influence of LT and HP on the formation of metabolite M2 in human hepatocyte incubations

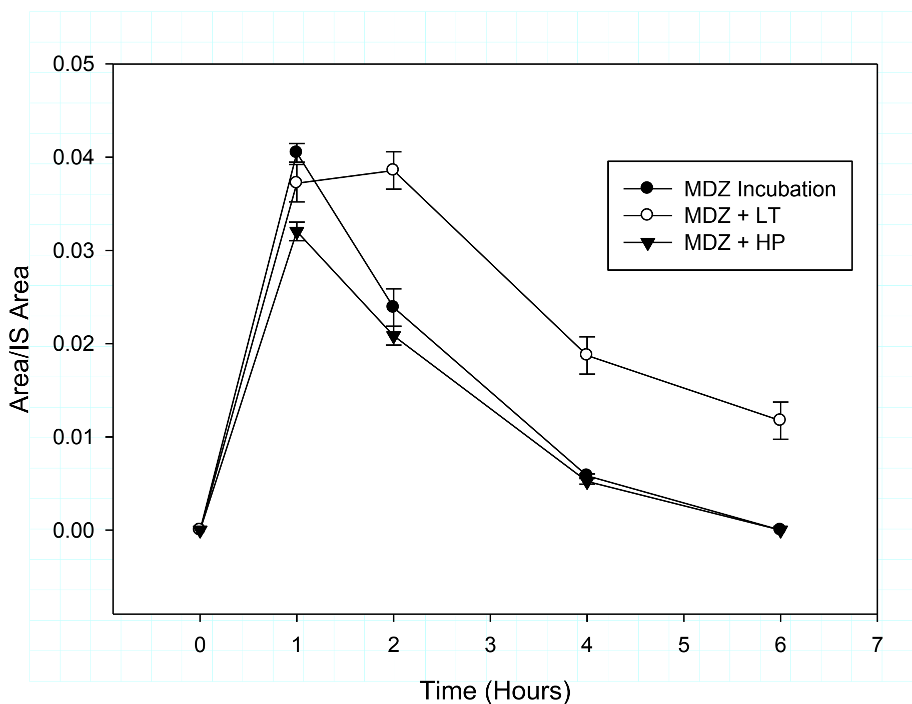

Figure 94: Time course showing the influence of LT and HP on the formation of metabolite M3 in human hepatocyte incubations

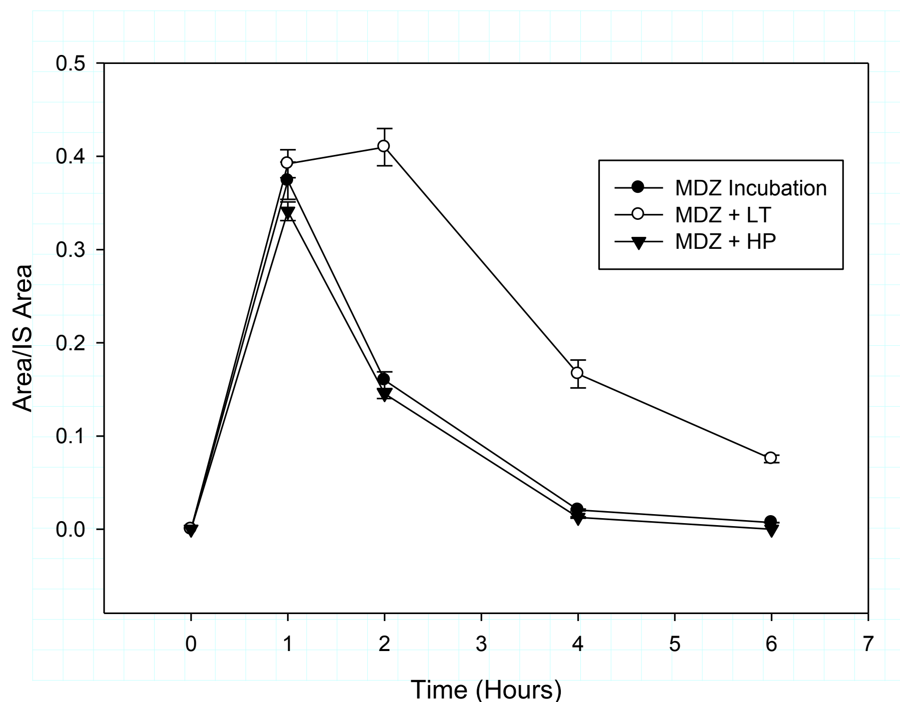

Figure 95: Time course showing the influence of LT and HP on the formation of metabolite M3 in human hepatocyte incubations

7.3.2. Viability data

During the incubation with MDZ, the viability of the hepatocytes (viable cells relative to total cells) decreased from 78.3% at time zero, to 42.7 without test inhibitor, 45.6 with LT and 42.7 with HP. Thus, no major difference was observed in the presence or absence of the compound (Table 34).

Table 34: Viability of hepatocytes at time 0 and 6 h incubation times, determined by the Guava EasyCyte Mini system using the ViaCount assay.

Time point (h)	MDZ	MDZ+ST	MDZ+HP
0	78.3%	-	-
6	42.7%	42.7%	45.6%

7.3.3. Stability of midazolam in the incubation media

As determined at time 0 and 6 h in the negative control, MDZ concentration in the cell media (without hepatocytes) remained the same. Normalization of MDZ LC-MS peak area to the d4-1'hydroxymidazolam internal standard showed that the peak area ratio did not change between 0 and 6 hours of incubation, indicating that MDZ was stable in the incubation media over the time course of the experiments. This implies the absence of other forms of degradation (Figure 96).

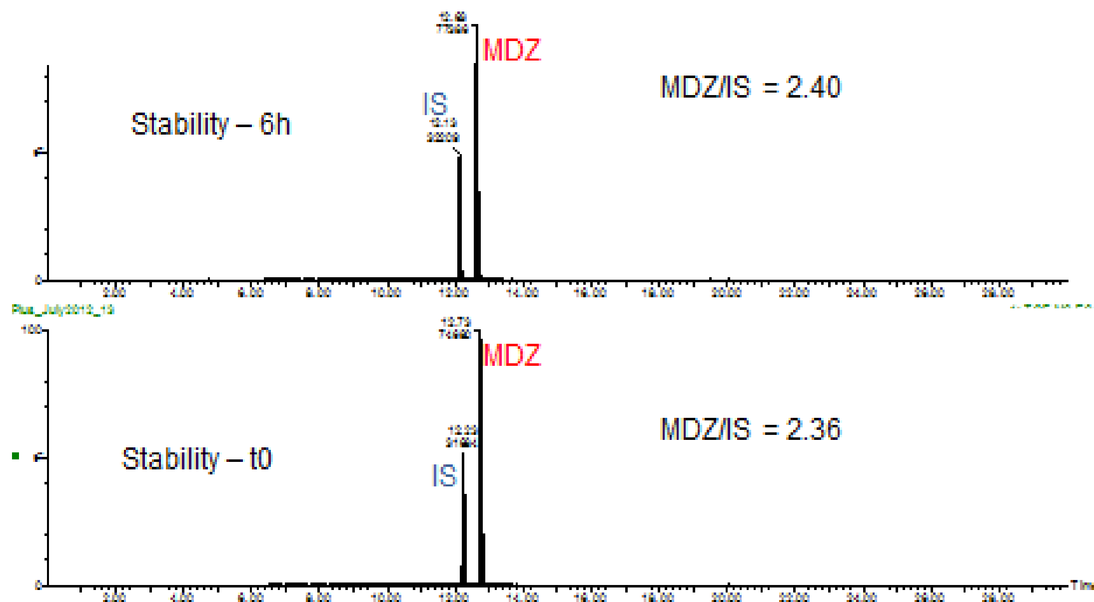

Figure 96: Extracted ion chromatograms showing the stability of MDZ over 6 hours in a no-hepatocyte control incubation

7.4. Discussion

Graded concentrations of the crude extracts of *Lessertia frutescence* and *Hypoxis hemerocallidea* were separately co-incubated with 1µM MDZ in human hepatocytes for up to 6 h. The *in vitro* intrinsic clearance values of MDZ as calculated (in the presence and absence of LT and HP) were scaled to hepatic metabolic clearance values *in vivo*

LT inhibited the production of MDZ metabolites with about 40% reduction in upscaled intrinsic *in vivo* clearance of MDZ (24.98 compared to 40.98 (L/h)/kg body mass). However, neither LT nor HP reduced hepatocyte viability as measured after 6 hours of incubation.

The use of hepatocyte for *in vitro* drug metabolism is considered very close to *in vivo* reality because of the total expression of metabolic enzymes in the whole cells. The results from the use of hepatocytes have been variously employed for *in vivo* extrapolation. Thus, LT is a potential inhibitor of CYP3A4. It should also be noted that, the 40% reduction in MDZ clearance observed in this study is a reflection of a single dosage. In practice where herbal preparations are taken concomitantly with prescription medicines over a long period of time, the inhibitory effects of LT could be cumulative, and higher.

7.5. Conclusion

LT caused 40% inhibition of CYP3A4-dependent MDZ metabolism in hepatocytes over 6 hour period, following a single addition. HP did not exert any inhibitory effects on MDZ metabolism in this study. If sufficient concentrations of the phytochemical constituents of LT are achieved *in vivo*, there is a potential for HDI.

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CHAPTER EIGHT

ASSESSMENT OF THE INHIBITORY EFFECTS OF *LESSERTIA FRUTESCENS* AND *HYPOXIS HEMEROCALLIDEA* ON EFFLUX PROTEINS (ABC TRANSPORTERS): P-GLYCOPROTEIN AND BCRP

8.0. Summary

The influence of *Lessertia frutescens* and *Hypoxis hemerocallidea* on the activity of efflux (ABC) transporters (P-glycoprotein and BCRP) was investigated in monolayers of LLC-PK1 cells expressing human P-gp; and monolayers of MDCKII cells expressing human BCRP monolayers. This was performed by determining the concentration dependent ability of the crude extracts (LT and HP) of the two herbs to inhibit efflux transporters leading to increased cellular uptake of radiolabeled probe substrates ([^{3}H]Digoxin for P-gp and [^{14}C]PhIP for BCRP).

Within the concentrations tested, LT inhibited P-glycoprotein (IC_{50} = 324.31 µg/mL; K_i = 324.31 µg/mL; maximum observed inhibition = 75.85 ± 7.45 %) with no inhibition observed for BCRP. HP did not inhibit the activity of any of the two transporters.

Thus *Lessertia frutescens* may inhibit P-gp if sufficiently high concentrations of its extracts are achieved *in vivo*. It may not inhibit BCRP transporters at therapeutic concentrations (provided such concentrations are within the tested range). *In vivo* inhibition of P-gp and BCRP transporters at therapeutic concentrations of *Hypoxis* corresponding to concentrations tested is not expected.

8.1. Introduction and objectives

The roles of transport proteins have been well established in the pharmacokinetic profiles of drugs. ABC transporters particularly play significant roles in drug interactions including HDI. Two important ABC transporters are the human P-glycoproteins (P-gp) and human breast cancer resistant polypeptide (BCRP) which are expressed in several human tissues including the gastrointestinal tracts and the liver. As discussed in Chapter three, the inhibition of the activity of these transporters alters the absorption, distribution and clearance of administered drugs.

The objective of this *in vitro* study was to determine the potential of *Lessertia frutescence* (*Lessertia*, LT) and *Hypoxis hemerocallidea* (*Hypoxis*, HP) to inhibit human ABC transporters-mediated drug efflux via P-gp) and BCRP.

8.2. Materials and methods

8.2.1. Materials

The procedure for sourcing and preparation of *Lessertia* and *Hypoxis* were detailed in Chapter Four.

The necessary chemical compounds were obtained as indicated: digoxin and cyclosporin A from Sigma-Aldrich (Pty) Ltd (St. Louis, USA); (3S,6S,12aS)-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1',2':1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester (Ko143) from Tocris Bioscience (Pty) Ltd (Ellisville, MI); 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-2 (PhIP) and its radio-labeled from (1.85 MBq/nmol, radiochemical purity >99%) from Toronto Research Chemicals (Pty) Ltd (North York, Canada); radio-labeled [^{3}H]Digoxin (1.103 MBq/nmol, radiochemical purity >97%) and [^{3}H]estradiol-17β-D-glucuronide (1.72 MBq/nmol, radiochemical purity >97% from Perkin Elmer Radiochemicals (Pty) Ltd (Waltham, MA, USA)

All other chemicals and materials were sourced as described in earlier chapters.

8.2.2. Methods

8.2.2.1. Working solutions

Incubation solutions were prepared by serial dilution of *Lessertia* in assay buffer (see Table 35) to obtain the final incubation concentrations of 5, 10, 50, 100, 150, 200, 250, 300, 350,

400, 450, 500, 550, 600, 650 and 700µg/mL for P-gp and 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10, 20, 40, 60, 80, 100, 125, 150, 175 and 200µg/mL for BCRP. *Lessertia* was soluble up to a final concentration of 700µg/mL in P-gp uptake buffer and 200µg/mL in BCRP uptake buffer.

Similar dilutions of *Hypoxis* were made in assay buffer to obtain the final incubation concentrations of 0.5, 1, 2, 5, 7.5, 10, 20, 50, 75, 100, 200, 300 and 500 µg/mL for P-gp and 1, 5, 10, 20, 50, 100, 200, 300, and 500µg/mL for BCRP. *Hypoxis* was soluble up to a final concentration of 500µg/mL in both P-gp and BCRP uptake buffer (Table 35).

Transporter inhibitors were added at concentrations given in Table 36. Methanol/DMSO was always below 1% of the total volume (v/v). All solutions used in cell growth were maintained at pH 7.4. Solutions for P-gp transporter studies were maintained at pH 7.4 whereas solutions for BCRP transporter assays were maintained at pH 7.8.

Table 35: Probe substrate concentrations and incubation conditions

Probe substrate	ABC transporter	Conc (µM)	Literature K_m value (µM)	Incubation time (min)	Buffer system	pH adjustment
Digoxin	P-gp	1	650 [a)]	40	HBSS + 12.5 mM HEPES	7.4
PhIP	BCRP	1	not assessed [b)]	40	OPTI-MEM I + 12.5 mM HEPES	7.8

[a)] (Cavet et al., 1996) [b)] (Van Herwaarden et al., 2003)

8.2.2.2. Cell Culture

MDCKII cells stably transfected with human BCRP were grown and maintained in DMEM supplemented with 10% FBS, 1% L-Glutamine and 1% penicillin/streptomycin at 37°C under an atmosphere of 5% CO_2. Once they were defrosted, cells were not cultured for more than 2 months.

LLC-PK1 cells stably transfected with human P-gp were grown and maintained in Medium 199 supplemented with 10% FBS, 50 ng/µL Gentamycin and 100 ng/µL Hygromycin B at 37°C under an atmosphere of 5% CO2. Once they were defrosted cells were not cultured for more than 2 months.

8.2.2.3. Drug uptake/efflux studies

Cells (~0.6 x 10^5 cells per well for LLC-PK1-P-gp and MDCKII-BCRP transfectants) were seeded into Falcon® clear bottom 96 well plates (Becton Dickinson) with 200 µL of culture medium. The assay was performed after 24 hr of seeding to allow for confluencing. On the day of the study, the culture medium was aspirated and replaced by pre-incubation solution (buffer system of choice containing the herbal extracts or the positive control inhibitor of interest) (Tables 11.1 and 11.2). Plates were subsequently incubated at 37°C for 10 min. To start the uptake experiment, the pre-incubation solution was replaced with the final incubation solution (buffer system of choice containing the probe substrate of choice and herbal extract or the positive control inhibitor of interest) (Tables 35 and 36). The experiment was terminated at designated time-points (Table 35) by removing the incubation solution. Subsequently, the cells were washed twice with ice-cold PBS buffer. Cell viability was assessed optically. The cells were then dissolved by the addition of 0.2 mL of 0.2 N NaOH solution to each well.

After a further incubation for 20 min at 37°C (to dissolve the cells), aliquots (0.195 mL) were transferred into scintillation vials containing a scintillation cocktail solution, followed by the liquid scintillation counting to determine the radioactive substrates in the cells. All uptake studies were performed in triplicate in an incubator without shaking. In control experiments, incubations were performed using the same incubation conditions in the absence of cellular material. Based on this control data, unspecific binding to the plastic support was negligible.

Table 36: Positive control inhibitor compounds

Inhibitor	Conc (µM)	ABC transporter(s)	Literature K_i value (µM)	Substrate
Cyclosporin A	10	P-gp	1.3; 2.2[a)]	yes
Ko143	1	BCRP	0.2 (IC_{50}) [b, c)]	no
LY335979	1	P-gp	0.5 (IC_{50}) [d)]	yes

[a)] (Ekins et al., 2002) [b] (Allen et al., 2002) [c)] (Matsson et al., 2009) [d)] (Weiss et al., 2003), et al 2002)

8.2.2.4. Drug uptake clearance calculations

Probe substrate uptake clearance (nL/min/mg protein) by LLC-PK1 or MDCKII cells stably expressing P-gp or BCRP, respectively, was determined from the specific amount of radiolabeled probe substrate inside the cells divided by the concentration in the incubation medium and normalized to the incubation time and the mean protein concentration measured in test wells.

8.2.2.5. Method suitability and limit of quantitation

To assess the passive cellular uptake (= background permeation) and to demonstrate the functional expression of the transporter system of interest in the recombinant cellular uptake models; the inhibition of uptake activity was assessed against that afforded by positive control transporter inhibitors shown in Table 36.

For labeled compounds, the limit of quantitation (LOQ) was taken as the lowest measurement from the radioactive scale which was statistically seen to be significantly higher than the measured blank value and for which the standard error of the measurement is lower than 20%. Under the conditions of this study, the LOQ of absolute radioactivity was 60 dpm for [^{3}H]-labeled probe substrates and 20 dpm for [^{14}C]-labeled probe substrates.

8.2.2.6. Sample and data analysis

The probe substrate transport in the presence of a competitive and non-competitive inhibitor can be described as follows (Gao et al., 2001):

$$PSapp = PSm \pm \frac{V\max}{S + Km \cdot (1 + I/Ki)}$$

Equation 8.1

Where, PSapp represents the overall membrane permeability (nL/min/mg protein) of a probe substrate of choice (Table 35) at 37°C. S is the concentration (µM) of the probe compound in the medium, V_{max} is the maximal uptake velocity/rate (pmol/min/mg protein), K_m is the Michaelis-Menten constant (µM), PSm is the nonspecific (passive) membrane permeability

(nL/min/mg protein), K_i is the inhibition constant (µM) and I is the concentration of the inhibitor in the medium (µM).

8.2.2.7. IC_{50} and kinetic parameter calculations

All absolute transporter uptake data were converted into relative inhibition values (y) by defining membrane permeability of the probe substrate without addition of inhibitor (baseline) as 0% inhibition and with addition of positive control inhibitor as 100% transporter inhibition and recalculating all the other uptake data relative to this number.

$$y = 100 - (100/((PSapp,p - PSapp,0)(PSapp,p - PSapp,i)))$$ *Equation 8.2*

Where, PSapp,0 represents the initial membrane permeability without addition of inhibitor (=baseline) whereas PSapp,i and PSapp,p represent the membrane permeability in the presence of test inhibitor and positive control inhibitor, respectively.

The IC_{50} values (inhibitor concentration that causes 50% inhibition of the maximal drug effect) were calculated using the equation 11.3 (Rautio et al., 2006):

$$y = y0 + \frac{a \cdot I^n}{IC50^n + I^n}$$ *Equation 8.3*

Where, n is the slope factor (Hill coefficient), y0 is the relative baseline inhibition and a is the maximal transporter inhibition (%).

To obtain estimates of the kinetic parameters, data were fitted to the above mentioned equations by a nonlinear least-square method using SigmaPlot® Version 12.1. All figures were drawn using SigmaPlot® Version 12.1.

8.2.2.8. Statistical analysis

The nonlinear-regression method of least squares was used to calculate the kinetic parameters from the respective uptake versus concentration curves. Testing for significant

differences between means was performed by unpaired Student's t test statistics, with $p < 0.05$ as the minimum level of significance.

8.3. Results

8.3.1. Influence of Lessertia on P-gp

The effect of the varying *Lessertia* concentrations on digoxin [nominal 1 µM] uptake by P-gp-expressing LLC-PK1 cells is shown in Figure 97. Data are given as relative values by defining the transporter activities in the absence of *Lessertia* as 0% inhibition. The data are averages of triplicate determinations and the bars represent the standard deviations. The line represents the "best fit" of the data to the equation 8.3 (IC_{50} = 324.8 ± 27.8 µg/mL, a = 69.5 ± 7.7 %, n = 7.9 ± 4.7, R2 = 0.88). The inhibitory activity of *Lessertia* is compared to standard inhibitor in Figure 98a and 98b.

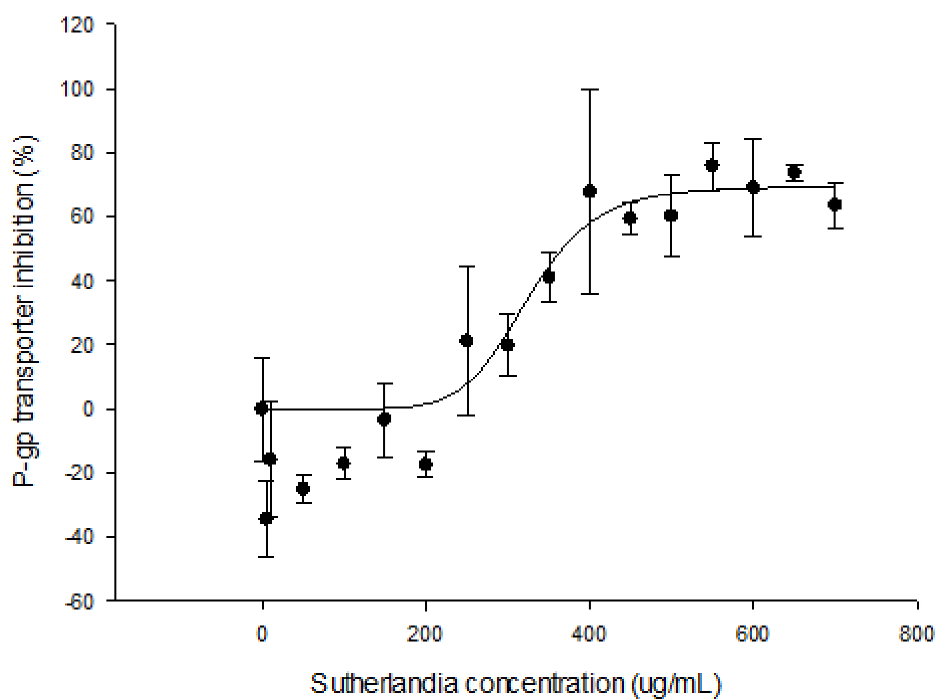


Figure 97: Concentration-dependent effect of *Lessertia* on probe substrate uptake by P-gp transporter-expressing LLC-PK1 cells. The data are averages of triplicate determinations and the bars represent the standard deviations

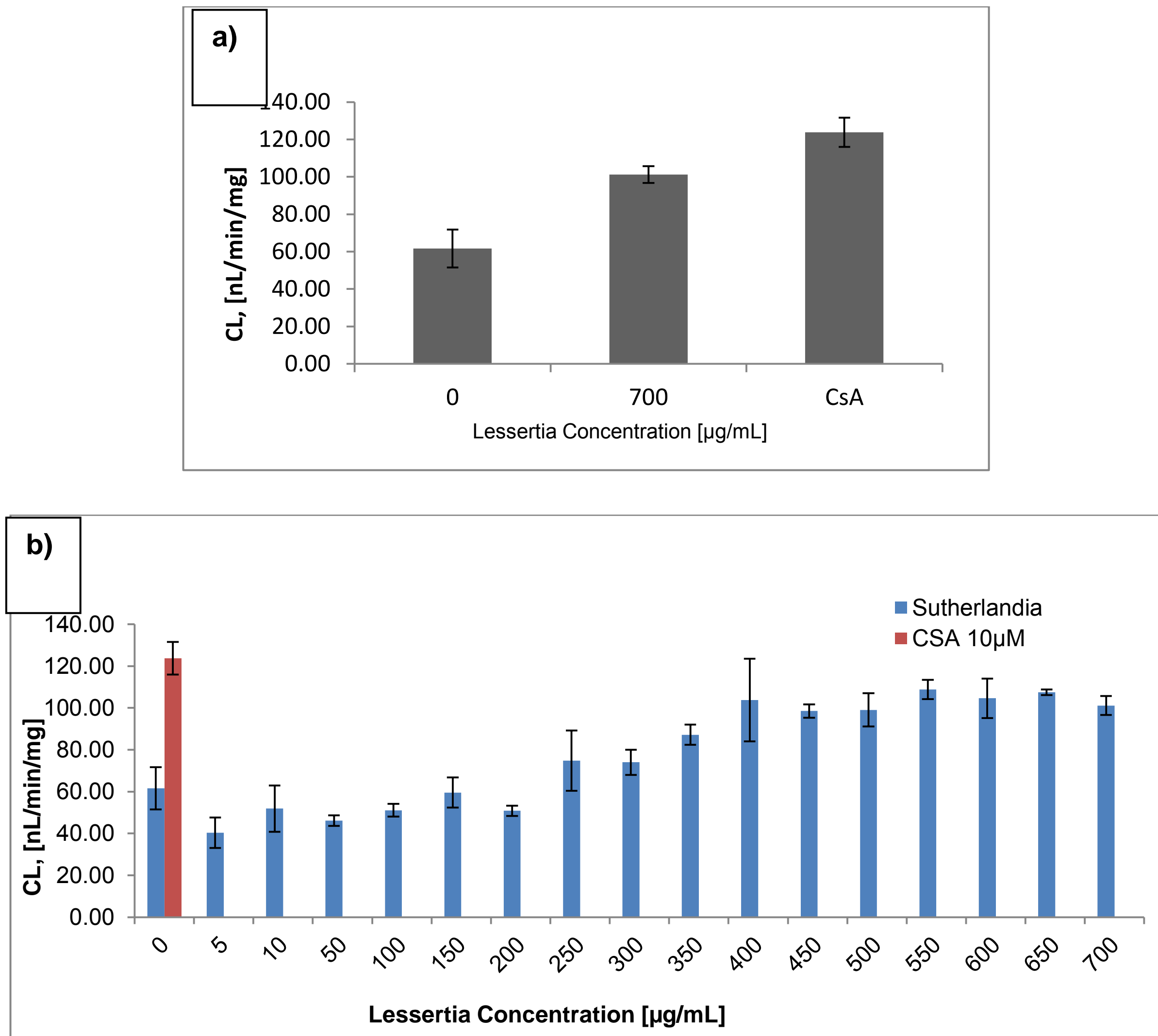
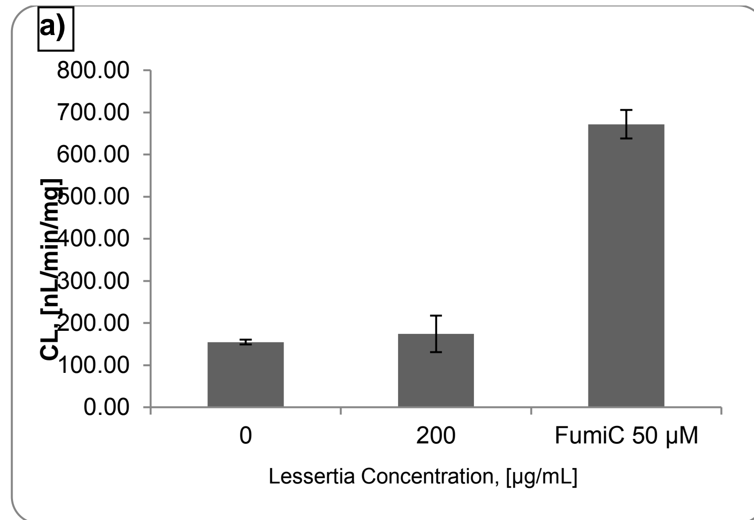


Figure 98: Comparison of the inhibitory activity of LT and cyclosporine A on P-gp mediated drug uptake in LLC-PK1 cells: a) digoxin (1 µM) uptake in the presence of no inhibitor compared to uptake in the presence of 700 µg/mL ST, positive control inhibitor LY335979 [1 µM]/CsA [10 µM]; b) effects of graded LT concentration on digoxin uptake. The data are averages of triplicate determinations and the bars represent the standard deviations

8.3.2. Influence of Lessertia on BCRP

The effect of the extracts of *Lessertia* on PhIP [1 µM] uptake by BCRP expressing MDCKII cells as compared to known BCRP inhibitor is shown in Figure 99a and Figure 99b. Data are given as relative values by defining the transporter activities in the absence of *Lessertia* as 0% inhibition. The data are averages of triplicate determinations and the bars represent the standard deviations.



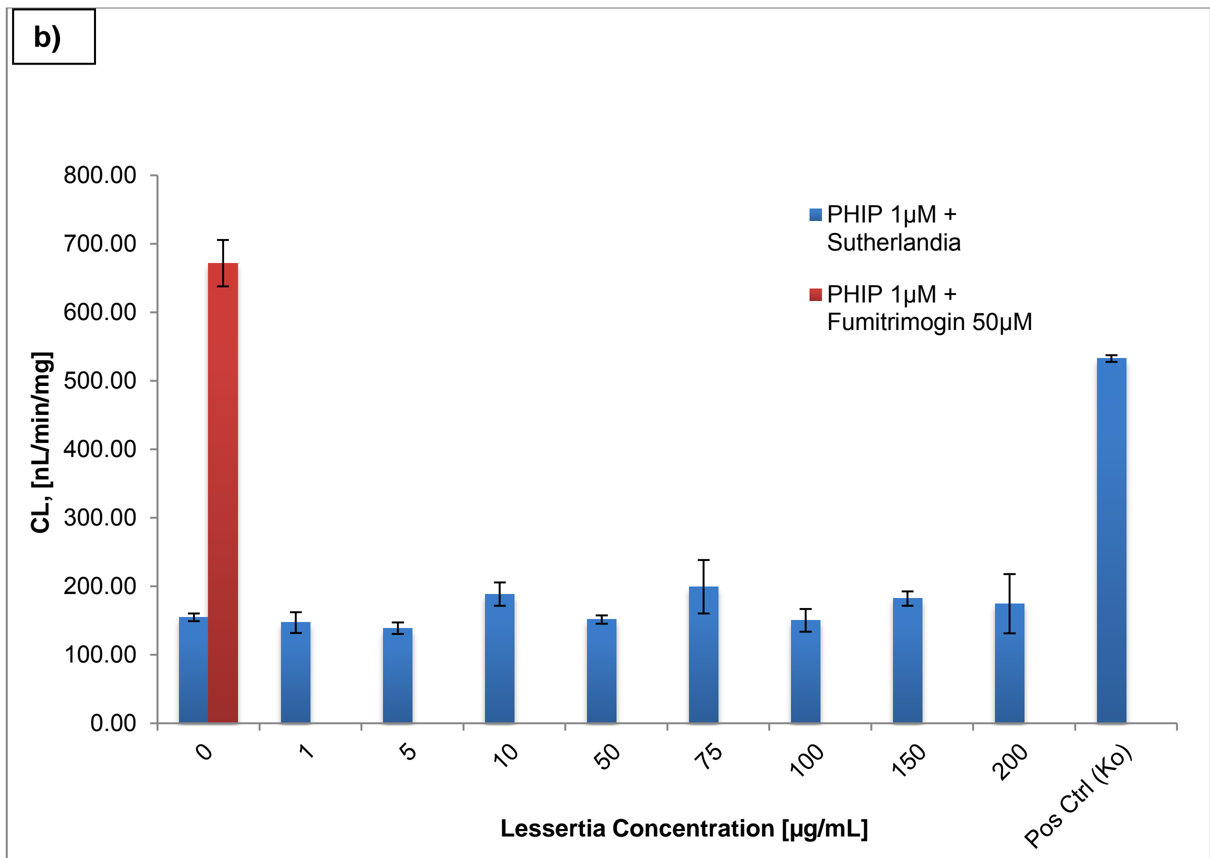


Figure 99: Concentration-dependent effect of LT on probe substrate uptake by BCRP transporter-expressing MDCKII cells: a) comparison of the effects of 200 µg/mL LT and standard control and b) effects of varying LT concentrations on PhIP [1 µM] uptake by BCRP-expressing MDCKII cells. The data are averages of triplicate determinations and the bars represent the standard deviations

8.3.3. Influence of Hypoxis on P-gp

The effect of varying *Hypoxis* concentrations on Digoxin [nominal 1 µM] uptake by P-gp expressing LLC-PK1 cells as compared to standard P-gp inhibitor is shown in Figure 100a and 100b. Data are given as relative values by defining the transporter activities in the absence of *Hypoxis* as 0% inhibition.

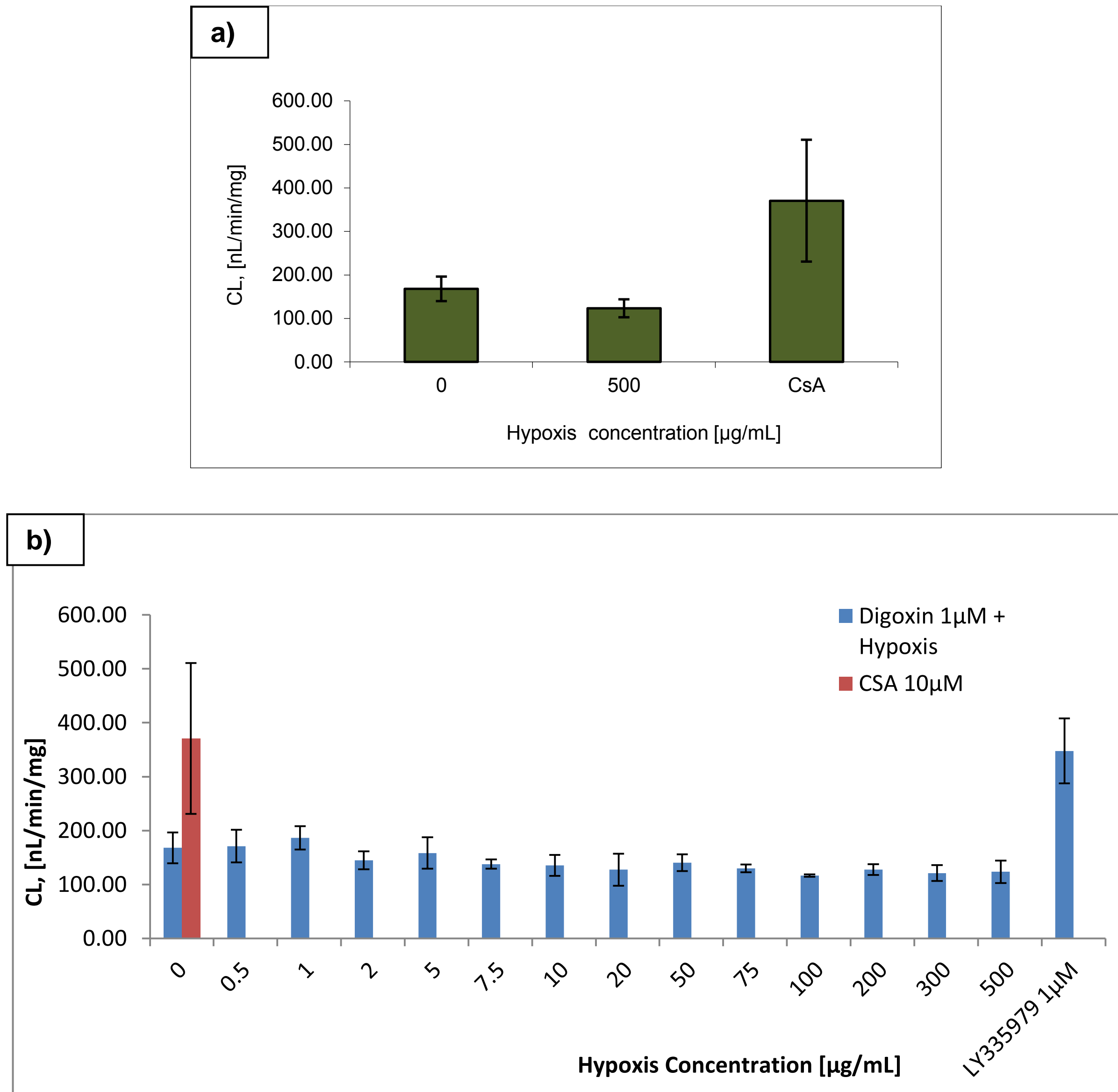


Figure 100: a) Comparison of the inhibitory activity of HP and cyclosporine A on P-gp mediated drug uptake in LLC-PK1 cells: a) digoxin (1 µM) uptake in the presence of no inhibitor compared to uptake in the presence of 500 µg/mL HP, positive control inhibitor LY335979 [1 µM]/CsA [10 µM]; b) effects of graded HP concentration on digoxin uptake. The data are averages of triplicate determinations and the bars represent the standard deviations

8.3.4. Influence of Hypoxis on BCRP

The effect of the extracts on PhIP [1 μM] uptake by BCRP expressing MDCKII cells as compared to known BCRP inhibitor is shown in Figure 101a and 101b. Data are given as relative values by defining the transporter activities in the absence of *Lessertia* as 0% inhibition.

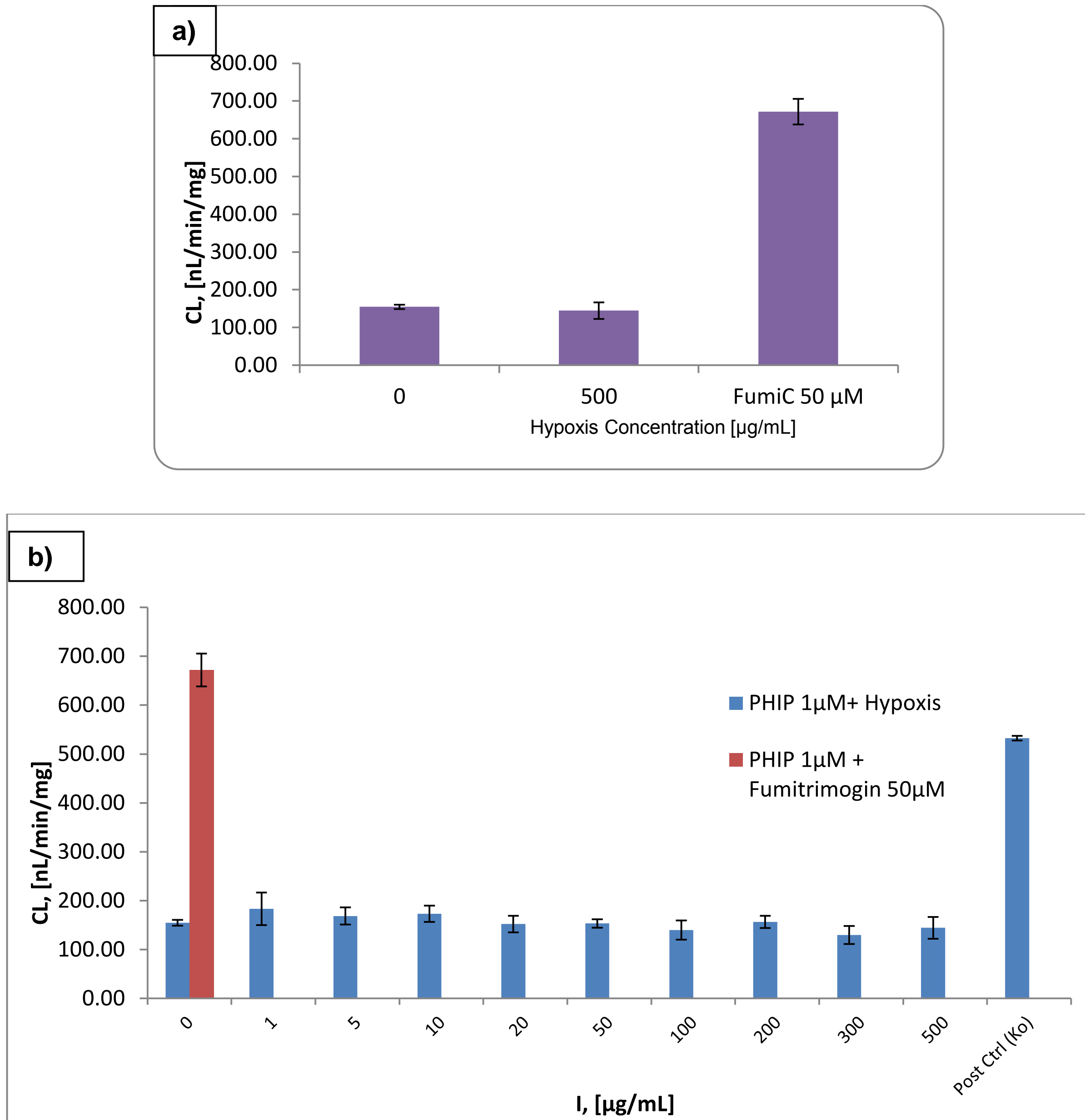


Figure 101: Concentration-dependent effect of HP on probe substrate uptake by BCRP transporter-expressing MDCKII cells: a) comparison of the effects of 500 µg/mL HP and standard control and b) effects of varying HP concentrations on PhIP [1 µM] uptake by BCRP-expressing MDCKII cells. The data are averages of triplicate determinations and the bars represent the standard deviations.

8.4. Discussion

P-gp- or BCRP-transporter expressing cells were incubated with probe substrates in the absence and presence of increasing concentrations of the crude herbal extracts: *Lessertia* - up to 700 µg/mL for P-gp and up to 200 µg/mL for BCRP, respectively; and *Hypoxis* - up to 500 µg/mL for both transpoters. The transporter activities without addition of test substances represent the control values (100% activity, 0% inhibition, baseline). Maximum inhibition of transporter activity was accomplished by the addition of known inhibitors of P-gp (LY335979, 1 µM/CsA, 10 µM) and of BCRP (Ko143, 1 µM). The effect of the increasing LT and HP concentrations on the uptake of probe substrate by the ABC transporters is summarized in Table 37.

Table 37: Summary of the influence of *Lessertia* and *Hypoxis* on the uptake activity of ABC transporters

	ABC transporter	Probe substrate	IC_{50} value [a, b] (µg/mL)	K_i [c] (µg/mL)	Max. inhibition [b] (%)
ST	P-gp	Digoxin	324.8	324.31	75.85 ± 7.45[d]
	BCRP	PhIP	not observed	not applicable [f]	not observed [e]
HYP	P-gp	Digoxin	not observed	not applicable [f]	not observed [e]
	BCRP	PhIP	not observed	not applicable [f]	not observed [e]

[a)] Extract concentration estimated to inhibit transporter activity by 50%
[b)] Data used for curve fitting are mean ± SD of N = 3
[c)] Calculated with $K_i = IC_{50}/(1+S/K_m)$, where S is the substrate concentration of the probe substrate and K_m is the transporter affinity of the probe substrate according to Michaelis-Menten (see Table 35)
[d)] Maximal observed inhibition with respect to positive control (CsA/LY335979)
[e)] Maximal observed inhibition with respect to positive control (Ko143)
[f)] K_i determination not applicable since K_m of PhIP is unknown

Based on these *in vitro* inhibition results, *Lessertia* was found to be an inhibitor of P-gp with an IC_{50} of 324.8 µg/mL with a maximal inhibition of 75.85 %. None of *Lessertia* and *Hypoxis* (up to the highest concentration 200 and 500 µg/mL respectively) was found to be an inhibitor of BCRP. *Hypoxis* did not inhibit the activity of P-gp.

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8.5. Conclusion

Lessertia may inhibit P-gp if sufficiently high concentrations are achieved *in vivo*. Based on the present data, it will not inhibit BCRP transporters at *in vivo* concentrations similar to those investigated. *In vivo* inhibition of P-gp and BCRP transporters by *Hypoxis*, within the concentrantions tested is not expected.

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CHAPTER NINE

ASSESSMENT OF THE INHIBITORY EFFECTS OF *LESSERTIA FRUTESCENCE* AND *HYPOXIS HEMEROCALLIDEA* ON DRUG UPTAKE TRANSPORTERS (OATP1B1, OATP1B3)

9.0. Summary

The influence of *Lessertia frutescence* and *Hypoxis hemerocallidea* on the human solute carrier (SLC) transporter-mediated uptake activity via the human organic anion transporting polypeptide family (OATPs) was investigated in monolayers of HEK293 cells expressing human OATP1B1 or human OATP1B3. This was performed by determining the concentration-dependent ability of the crude extracts (LT and HP) of the two herbs to inhibit cellular uptake of radiolabeled probe substrates ([^{3}H]Estradiol-17β-D-glucuronide) of the OATP-transporters

Within the concentrations tested, LT inhibited OATP1B1 (IC_{50} = 10.4 ± 0.6 µg/mL; K_i = 8.19 ± 0.47 µg/mL; maximum observed inhibition = 100.08 ± 0.16 %) and OATP1B3 (IC_{50} = 6.6 ± 0.9 µg/mL; K_i = 5.57 ± 0.76 µg/mL; maximum observed inhibition = 88.2 ± 2.27 %). Similarly, HP inhibited OATP1B1 (IC_{50} = 118.7 ± 33.3 µg/mL; K_i = 93.44 ± 26.21 µg/mL; maximum observed inhibition = 71.291 ± 5.22 %) and OATP1B3 (IC_{50} = 290.1 ± 59.7 µg/mL; K_i = 244.79 ± 50.38 µg/mL; maximum observed inhibition = 93.95 ± 0.68 %).

Thus, both *Lessertia frutescens* and *Hypoxis hemerocallidea* may inhibit OATP1B1 and OATP1B3 if sufficiently high concentrations of their extracts are achieved *in vivo*. They may not inhibit BCRP transporters at therapeutic concentrations (provided such concentrations are within the tested range). *In vivo* inhibition of P-gp and BCRP transporters at therapeutic concentrations of *Hypoxis* corresponding to concentrations tested is not expected.

9.1. Introduction and objectives

The solute carrier (SLC) group of membrane transport proteins are drug uptake transporters responsible for the movement of drug molecules across the cell membranes into the cell. They are present in the liver (and not in the gastrointestinal tract) (Tamai et al., 2001). They play roles in drug distribution and clearance, and are also responsible for carrier-mediated DDI and HDI.

The objective of this *in vitro* study was to determine the potential of the crude extracts of *Lessertia* and *Hypoxis* to inhibit the human SLC transporter-mediated uptake via the human organic anion transporting polypeptide family (OATPs).

9.2. Materials and Methods

9.2.1. Material

The procedure for sourcing and preparation of *Lessertia* and *Hypoxis* were detailed in Chapter Four.

The chemical compounds were obtained as indicated: atorvastatin calcium (MW 1155.3 g/mol; purity > 98%) from AK Scientific (Pty) Ltd (CA, USA), estradiol-17β-D-glucuronide (sodium salt; MW 470.5 g/mol; purity = 98%) and rifamycin SV (sodium salt; MW 719.8 g/mol; purity = 98.8%) from Sigma-Aldrich (Pty) Ltd (St. Louis, USA), [^{3}H]estradiol-17β-D-glucuronide (1.72 MBq/nmol; MW 448 g/mol; radiochemical purity > 97%) from Perkin Elmer Radiochemicals (Pty) Ltd (Waltham, MA, USA). All other chemicals used were of analytical grade.

9.2.2. Methods

9.2.2.1. Working solutions

Incubation solutions were prepared by serial dilution of the crude extracts of *Lessertia* in assay buffer (Table 38) to obtain the final incubation concentrations of 0.5; 1; 2; 5; 7.5; 10; 20; 50; 75; 100; 200; 300 and 500 µg/mL for OATP1B1 and 0.5; 1; 2; 5; 7.5; 10; 20; 50; 75; 100; 200; 300; 500 µg/mL for OATP1B3. Similar dilutions for *Hypoxis* was carried out in the assay buffer to yield final incubation concentrations of 0.5, 1, 2,5, 7.5, 10, 20, 50, 75, 100, 200, 300, 500 µg/mL for OATP1B1 and 1, 2, 5, 10, 20, 50, 100, 200, 300, 400, 500, 600 and

800µg/mL for OATP1B3. The solubility of the crude extracts uptake buffer, up to the highest concentrations employed was ascertained by optical inspection.

Transporter inhibitors were added at concentrations given in Table 39. Methanol was always below 1% of the total volume (v/v) and all solutions used in cell growth and transporter studies were maintained at pH 7.4.

Table 38: Probe substrate concentrations and incubation conditions

Probe substrate	OATP transporter	Conc (µM)	Literature K_m value (µM)	Incubation time (min)	Buffer system	pH adjustment
Estradiol-17β-D-glucuronide	OATP1B1	1	3.7 [a)]	5	HBSS with 12.5 mM HEPES	7.4
Estradiol-17β-D-glucuronide	OATP1B3	1	5.4 [b)]	5	HBSS with 12.5 mM HEPES	7.4

[a)] (Tamai et al., 2001) [b)] (Koenig et al., 2000)

Table 39: Inhibitors of the uptake transporters

Inhibitor	Conc (µM)	OATP transporter(s)	Literature K_i value (µM)	Substrate
Rifamycin SV (RSV)	20	OATP family	2-3 [a]	no
Atorvastatin (ATO)	10	OATP1B1	2.6 [b] (IC_{50})	yes

[a] (Vavricka et al., 2002) [b] (Chen et al., 2005)

9.2.2.2. Cell Culture

HEK293 cells stably transfected with human OATP1B1 were grown and maintained in DMEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 50 ng/µL hygromycin B at 37°C under an atmosphere of 5% CO_2. HEK293 cells stably transfected with human OATP1B3 were grown and maintained in DMEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin and 0.8 mg/mL G418 at 37°C under an atmosphere of 5% CO_2. Once they were defrosted, cells were not cultured for more than 2 months.

9.2.2.3. Drug uptake studies

Cells (~0.2 x 10^6 cells per well for OATP1B1 and OATP1B3 overexpressing cells) were seeded into pre-coated (poly-L-lysine, poly-L-ornitin, 0.1 mg/mL) clear bottom 96 well plates (Corning Product No 734-1795) with 200 µL of culture medium. Uptake studies were performed 72 h after seeding.

On the day of the study, the culture medium was aspirated and replaced with the final incubation solution (buffer system of choice containing the probe substrate of choice and *Lessertia* or the positive control inhibitor of interest) (Table 38 and 39). The experiment was terminated at designated time-points (Table 38) by removing the incubation solution. Subsequently, the wells were washed twice with ice-cold PBS buffer. Cell viability was assessed optically. The cells were then dissolved by the addition of 0.2 mL of 0.2 N NaOH solution to each well. After incubation for 20 min at 37°C, aliquots (0.195 mL) were transferred into scintillation vials containing scintillation cocktail. All uptake studies were

performed in triplicate in an incubator without shaking. In control experiments incubations were performed using the same incubation conditions in the absence of cellular material. Based on this control data, unspecific binding to the plastic support was negligible.

9.2.2.4. Drug uptake Clearance calculations

Drug uptake (nL/min/mg protein) by the stably transfected OATP1B1, OATP1B3 cells was determined from the specific amount of radiolabeled probe substrate inside the cells divided by the concentration in the incubation medium and normalized to the incubation time and the mean protein concentration measured in test wells.

9.2.2.5. Method suitability and limit of quantitation

To assess the passive cellular uptake (= background permeation) and to demonstrate the functional expression of the transporter system of interest in the recombinant cellular uptake models known transporter inhibitors along with the above mentioned probe substrates for active carrier mechanism were included (Table 39).

For labeled compounds, the limit of quantitation (LOQ) was taken as the lowest measurement from the radioactive scale which was statistically seen to be significantly higher than the measured blank value and for which the standard error of the measurement is lower than 20%. Under the conditions of this study, the LOQ of absolute radioactivity was 60 dpm for [^{3}H] estradiol-17β-D-glucuronide.

9.2.2.6. Analysis of inhibition kinetics

The inhibition kinetics were analyzed employing the procedure and formula expressed in section 8.2.2.6.

9.2.2.7. IC_{50} and Kinetic parameter calculations

All absolute transporter uptake data were converted into relative inhibition values (y) by defining membrane permeability of the probe substrate without addition of inhibitor (baseline) as 0% inhibition and with addition of positive control inhibitor as 100% transporter inhibition

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and recalculating all the other uptake data relative to this number. Further determinations were done as presented in section 8.2.2.7.

9.2.2.8. Statistical analysis

The nonlinear-regression method of least squares was used as described earlier in section 8.2.2.8.

9.3. Results

9.3.1. The influence of Lessertia on OATP1B1 and OATP1B3

The transporter activities without the addition of test substances represent the control values (100% activity, 0% inhibition, baseline). The inhibitory effect of increasing *Lessertia* extract concentrations on the OATP-transporter dependent uptake is presented in Figures 102 to 105. The extracts inhibited the uptake activity of both OATP1B1 and of OATP1B3.

The profile, in comparison with known inhibitors is provided in Figure 103 and 105. The calculated IC_{50} values were 10.4 µg/mL for OATP1B1 and 6.6 µg/mLM for OATP1B3, respectively.

The effect of varying *Lessertia* concentrations on estradiol-17β-D-glucuronide [nominal 1 µM] uptake by OATP1B1 expressing HEK293 cells is shown in Figure 102. Data are given as relative values by defining the transporter activities in the absence of *Lessertia* as 0% inhibition. The line represents the “best fit” of the data to the equation 9.3 (IC_{50} = 10.4 ± 0.6 µg/mL, a = 101.4 ± 1.7 %, n = 1.2 ± 0.08, R^2 = 0.99).

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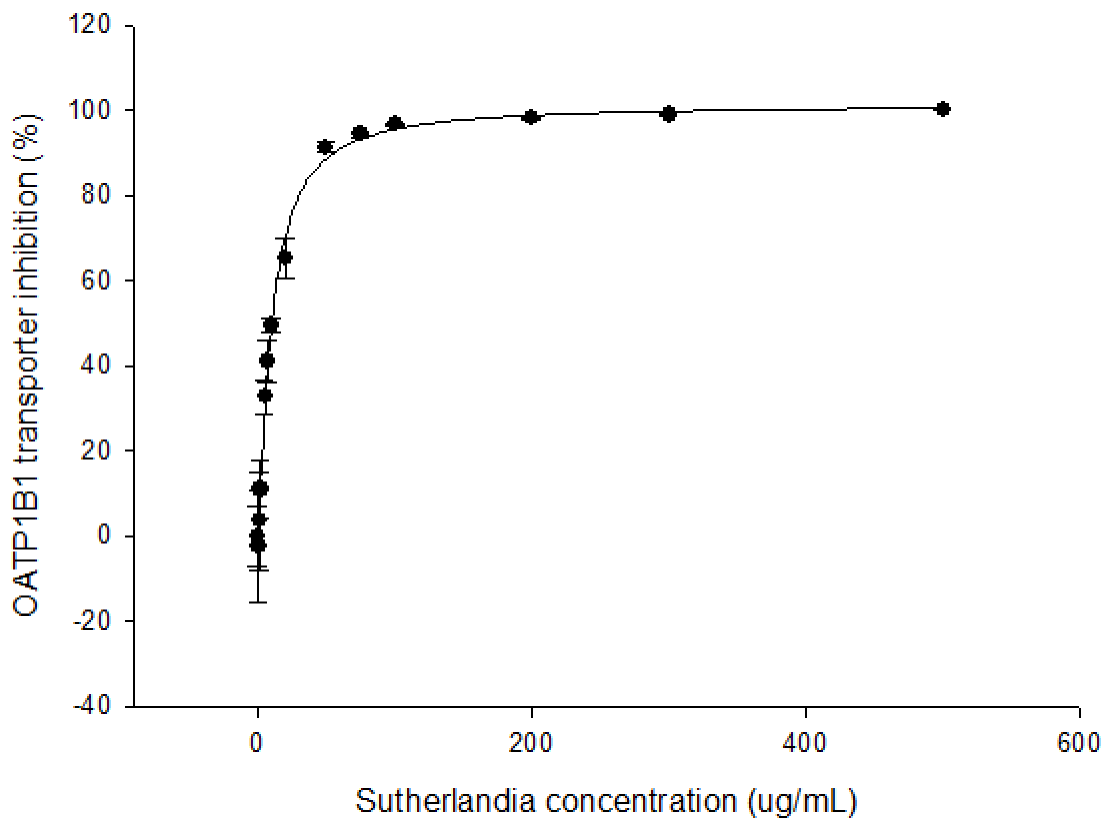
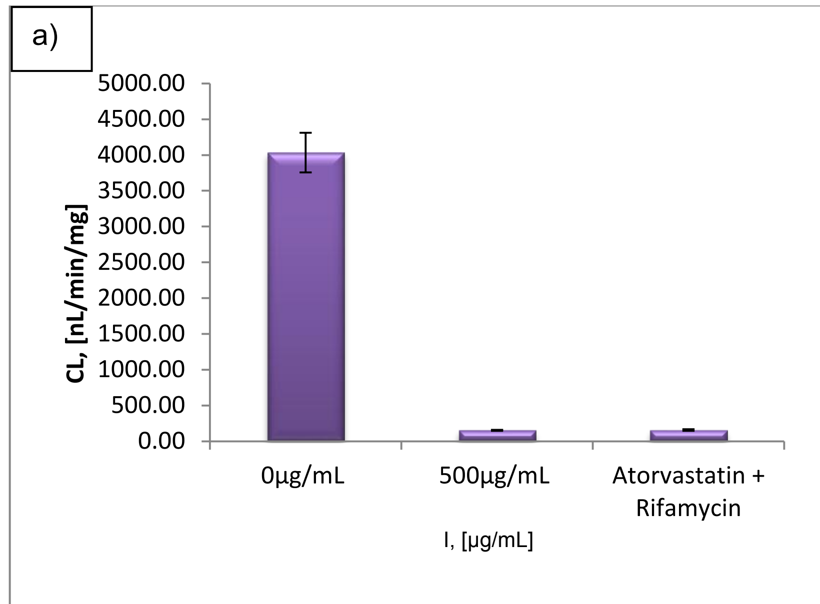


Figure 102: A concentration dependent effect of LT on probe substrate uptake by OATP1B1 transporter-expressing HEK293 cells. The data are averages of triplicate determinations and the bars represent the standard deviations.



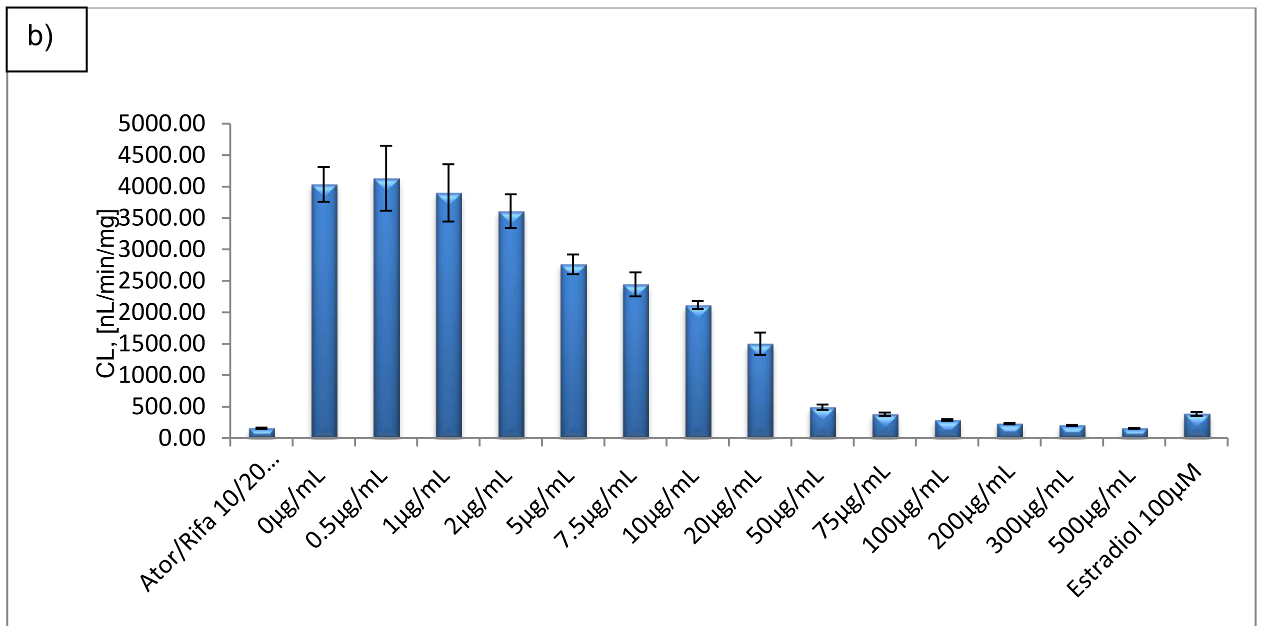


Figure 103: OATP1B1-mediated estradiol-17β-D-glucuronide [nominal: 1 µM] uptake a) in the presence of 500 µg/mL LT extract compared to positive controls; and b) in the presence of increasing concentration of LT extract positive control inhibitor 20 µM rifamycin SV and 10 µM atorvastatin (RSV/ATO). The data are averages of triplicate determinations and the bars represent the standard deviations

The inhibitory effects (%) of *Lessertia* on estradiol-17β-D-glucuronide [nominal 1 μM] uptake by OATP1B3 expressing HEK293 cells as profiled against extract concentration is shown in Figure 104. Data are given as relative values by defining the transporter activities in the absence of *Lessertia* as 0% inhibition. The line represents the “best fit” of the data to the equation 9.3 (IC_{50} = 6.6 ± 0.9 μg/mL, a = 85.3 ± 5.3 %, n = 1.16 ± 0.2, R^2 = 0.98). The data are averages of triplicate determinations and the bars represent the standard deviations.

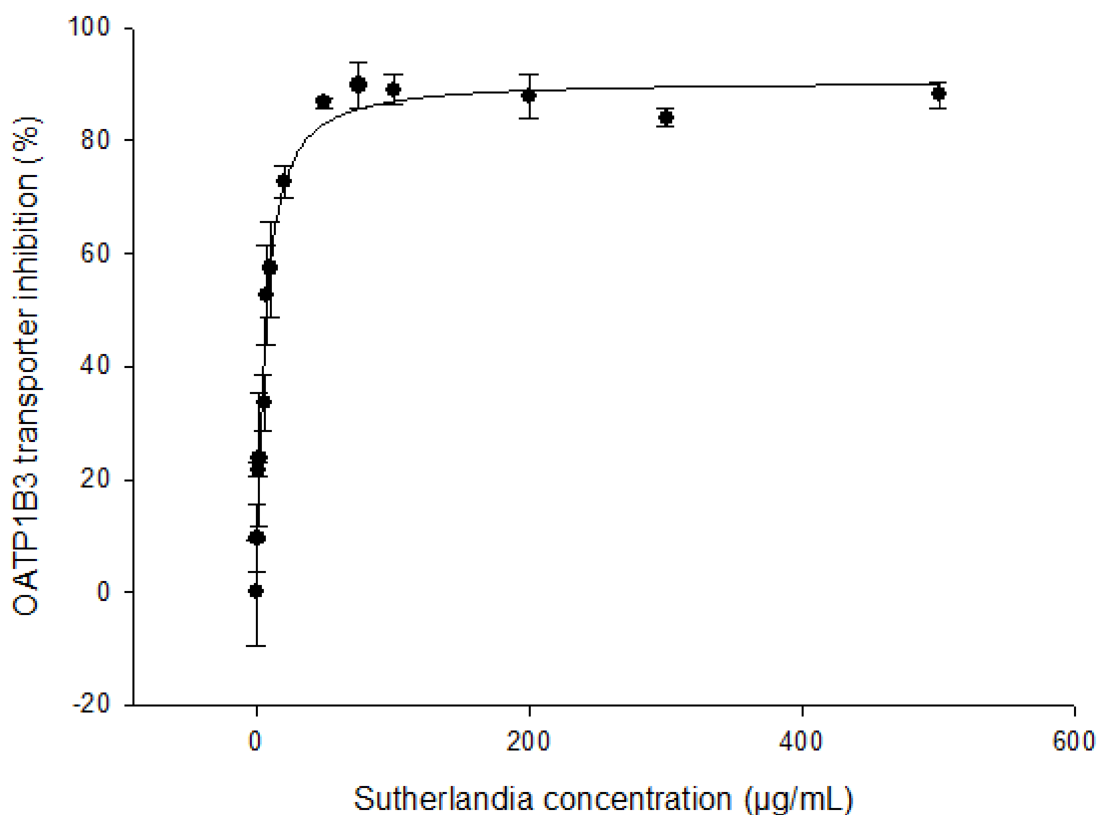
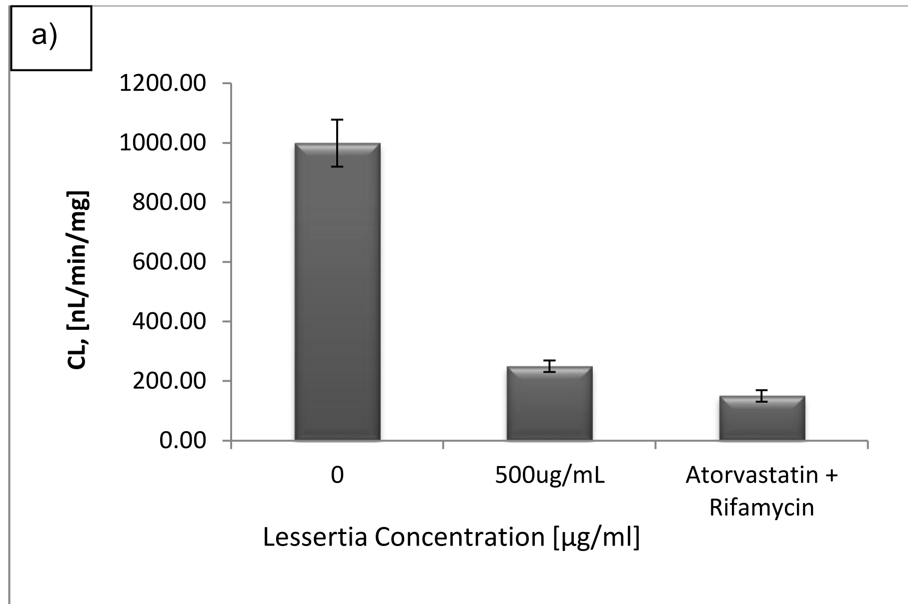


Figure 104: A concentration dependent effect of LT on probe substrate uptake by OATP1B3 transporter-expressing HEK293 cells. The data are averages of triplicate determinations and the bars represent the standard deviations.



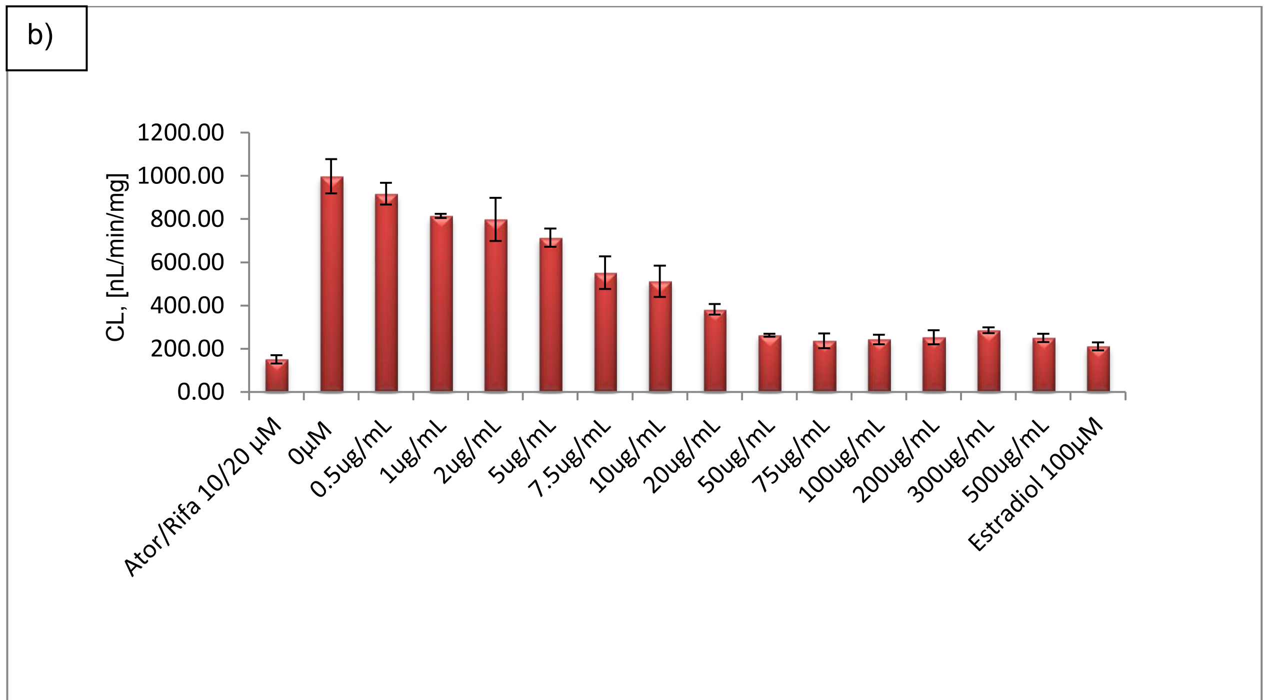


Figure 105: OATP1B3-mediated estradiol-17β-D-glucuronide [nominal: 1 µM] uptake **a**) in the presence of 500 µg/mL LT extract compared to positive controls; and **b**) in the presence of increasing concentration of LT extract positive control inhibitor 20 µM rifamycin SV and 10 µM atorvastatin (RSV/ATO). The data are averages of triplicate determinations and the bars represent the standard deviations

9.3.2. The influence of Hypoxis on OATP1B1 and OATP1B3

Extracts of *Hypoxis* inhibited OATP1B1 and of OATP1B3 (Figures 106 - 109). The calculated IC_{50} values were 118.7 µg/mL for OATP1B1 and 290.1 µg/mL for OATP1B3, respectively.

The effect of varying *Hypoxis* concentrations on Estradiol-17β-D-glucuronide [nominal 1 µM] uptake by OATP1B1-expressing HEK293 cells is shown in Figure 106. Data are given as relative values by defining the transporter activities in the absence of *Hypoxis* as 0% inhibition. The line represents the "best fit" of the data to the equation 9.5 (IC_{50} = 118.7 ± 33.3 µg/mL, a = 62.9 ± 7.5 %, n = 67.8 ± 20.8.1, R^2 = 0.86).

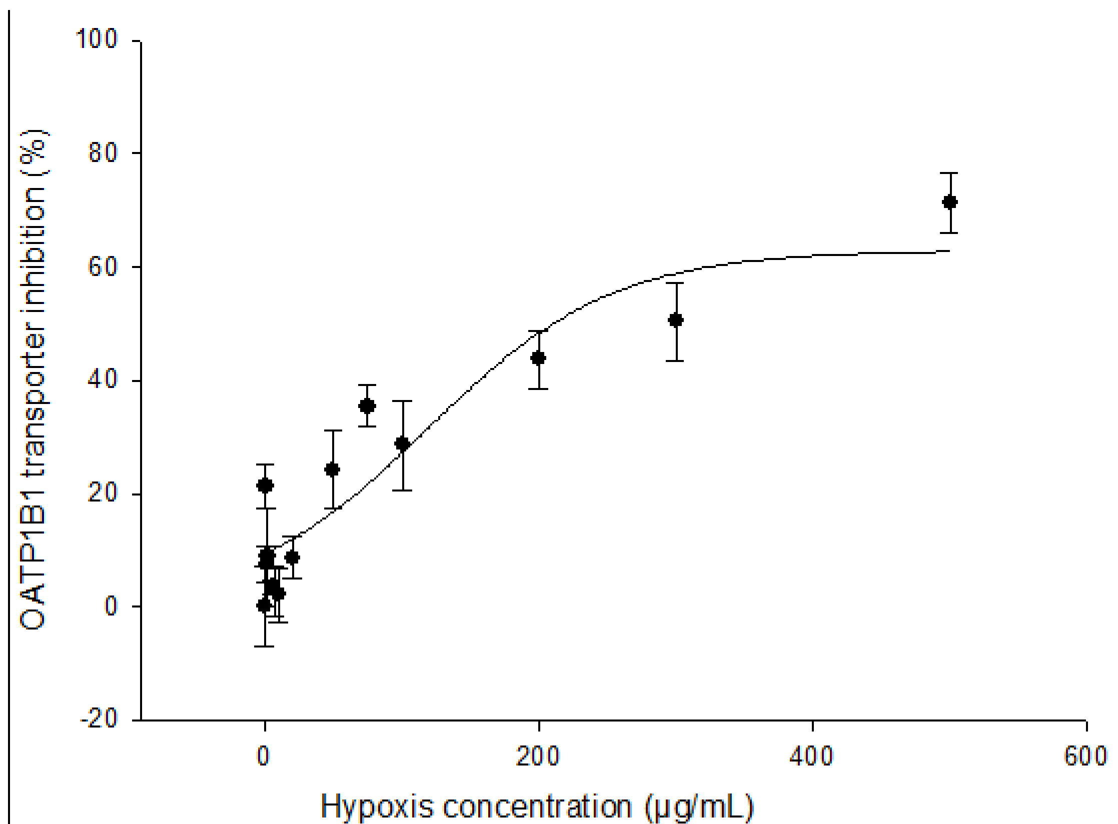
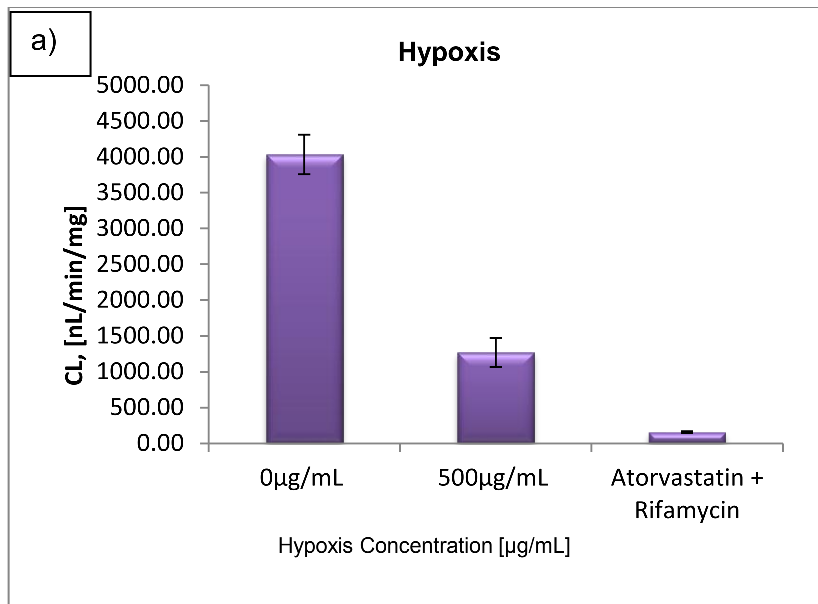


Figure 106: A concentration dependent effect of *Hypoxis* extracts on probe substrate uptake by OATP1B1 transporter-expressing HEK293 cells. The data are averages of triplicate determinations and the bars represent the standard deviations.



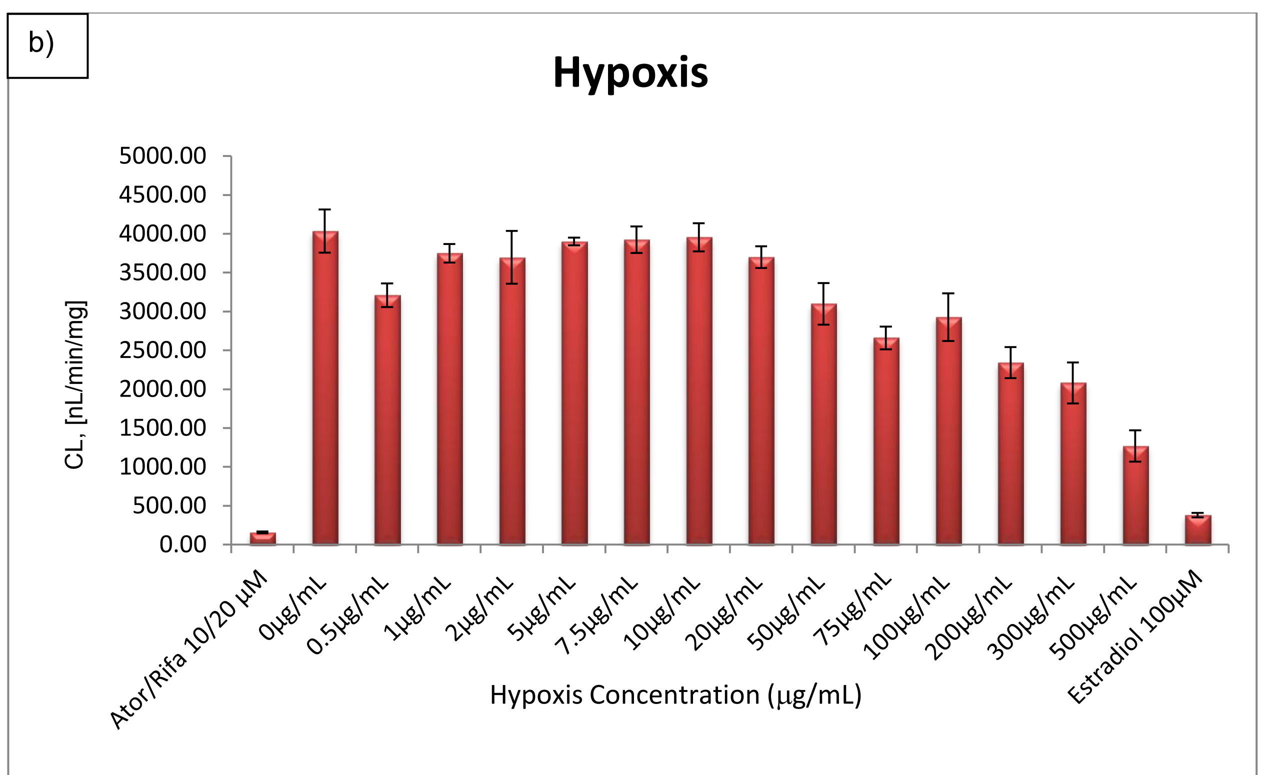


Figure 107: OATP1B1-mediated estradiol-17β-D-glucuronide [nominal: 1 µM] uptake **a**) in the presence of 500 µg/mL *Hypoxis* extract compared to positive controls; and **b**) in the presence of increasing concentration of LT extract positive control inhibitor 20 µM rifamycin SV and 10 µM atorvastatin (RSV/ATO). The data are averages of triplicate determinations and the bars represent the standard deviations

The inhibitory effects (%) of *Hypoxis* on estradiol-17β-D-glucuronide [nominal 1 µM] uptake by OATP1B3 expressing HEK293 cells as profiled against extract concentration is shown in

Figure 108. Data are given as relative values by defining the transporter activities in the absence of *Lessertia* as 0% inhibition. The line represents the "best fit" of the data to the equation 9.5 (IC_{50} = 290.1 ± 59.7 µg/mL, a = 99.2 ± 10.3 %, n = 195.5 ± 33.7, R^2 = 0.95).

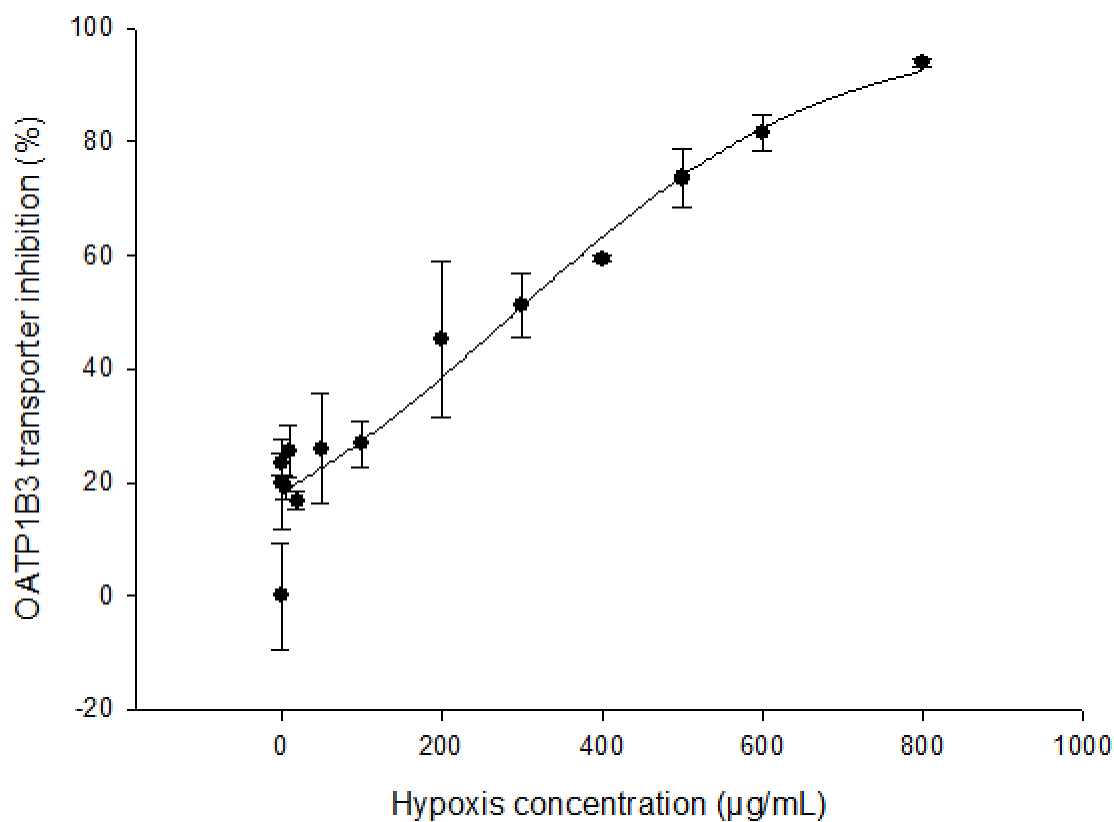
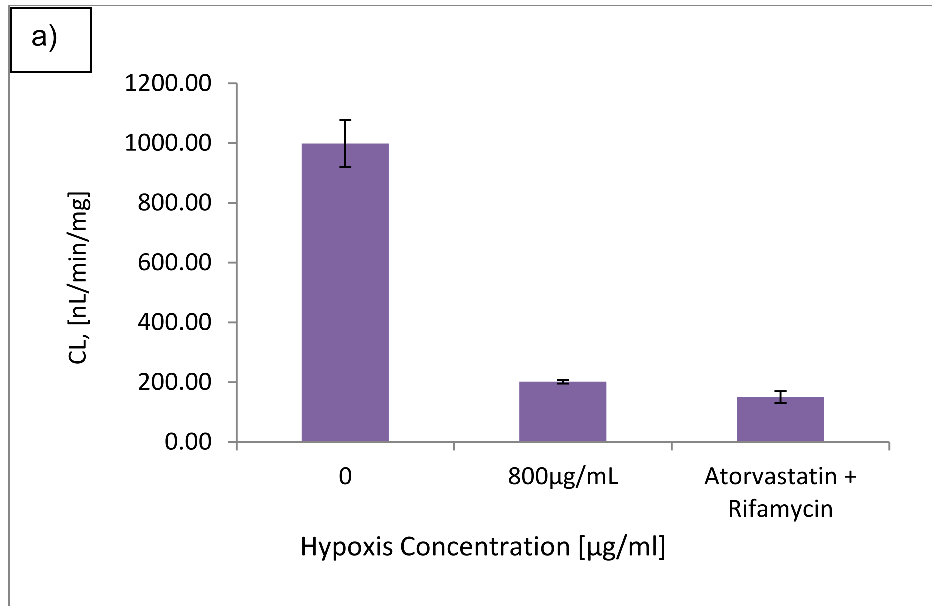


Figure 108: A concentration dependent effect of *Hypoxis* extracts on probe substrate uptake by OATP1B3 transporter-expressing HEK293 cells. The data are averages of triplicate determinations and the bars represent the standard deviations



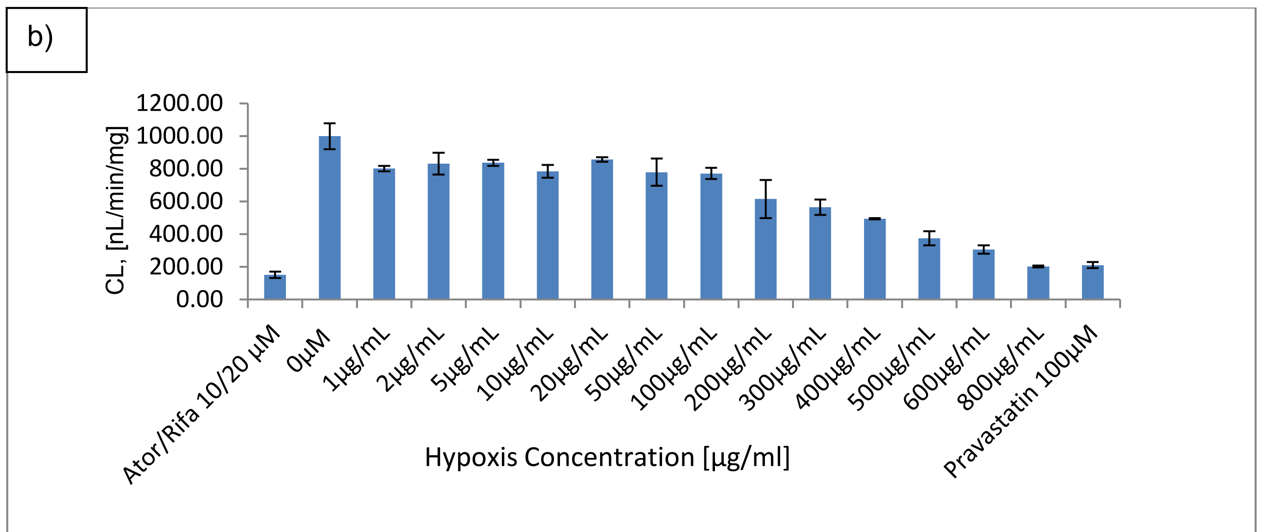


Figure 109: OATP1B3-mediated estradiol-17β-D-glucuronide [nominal: 1 µM] uptake **a**) in the presence of 500 µg/mL *Hypoxis* extract compared to positive controls; and **b**) in the presence of increasing concentration of LT extract positive control inhibitor 20 µM rifamycin SV and 10 µM atorvastatin (RSV/ATO). The data are averages of triplicate determinations and the bars represent the standard deviations.

9.4. Discussion

OATP1B1 and OATP1B3 are important drug transporters. Both *Lessertia* and *Hypoxis* inhibited uptake activity of these uptake drug transporters (Table 40).

Table 40: Summary of the influence of *Lessertia* and *Hypoxis* on the uptake activity of ABC transporters

	OATP transporter	Probe substrate	IC_{50} value [a, b] (µg/mL)	K_i [c] (µg/mL)	Max. inhibition [b] (%)
ST	OATP1B1	Estradiol-17β-D-glucuronide	10.4 ± 0.6	8.19	100 [d]
	OATP1B3	Estradiol-17β-D-glucuronide	6.6 ± 0.9	5.57	88.2 [d]
HYP	OATP1B1	Estradiol-17β-D-glucuronide	118.7 ± 33.3	93.44	71.29 [d]
	OATP1B3	Estradiol-17β-D-glucuronide	290.1 ± 59.7	244.79	93.95 [d]

[a] *Lessertia* concentration estimated to inhibit probe substrate uptake by 50%
[b] Mean ± SD of N = 3
[c] Calculated with Ki= $IC_{50}/(1+S/K_m)$, where S is the substrate concentration of the probe substrate and K_m is the transporter affinity of the probe substrate according to Michaelis-Menten (see Table 38)
[d] Maximal observed inhibition with respect to positive control (RSV/ATO)

The OATP transporter families are not expressed in the gastrointestinal tract. Their rich expression in the liver however, makes their effects on the pharmacokinetics of drugs very important (Emami-Riedmaier et al., 2012). Thus, any *Lessertia*- and/or *Hypoxis*-induced interaction with OATP transporter family will depend on the extent of the absorption of the herbs. Information on the absorption and bioavailability of *Lessertia* and *Hypoxis* phytoconstituents is currently not available. However, the long history of the use of *Lessertia* and *Hypoxis* for systemic disorders can be an indication of substantial systemic bioavailability (See Chapter 4). Caution should therefore be taken in combining *Lessertia* and/or *Hypoxis* with conventional medicines, especially OATP substrates. The systemic inhibition of OATP can prevent cellular uptake of important drugs with the potential of therapy failure, especially drugs intended for intracellular activity. Such inhibition can also lead to drug accumulation, and alteration in the intra- and extra-cellular concentration of drugs.

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9.5. Conclusion

Lessertia and *Hypoxis* may inhibit OATP1B1 and OATP1B3 if sufficiently high concentrations are achieved *in vivo*. This indicates a potential for herb-drug interactions.

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CHAPTER TEN

DISCUSSION, STUDY LIMITATION, CONCLUSION AND RECOMMENDATIONS

10.1. General Discussion

This study has demonstrated the potential of commonly consumed medicinal herbs in South Africa to inhibit metabolic enzymes and drug transporters. A summary of the results demonstrates the high likelihood of *in vivo* inhibitory effects, as shown in Table 41.

In traditional practice, all selected herbs are taken as aqueous extracts, as gathered from the THPs. Thus, an aqueous extraction was used in this study to reflect what most consumers are exposed to during medical use of the plants. *Lessertia* and a growing number of medicinal herbs are available commercially in capsule formulations. A difference in the nature and quantity of phytochemicals exposed to in the body following the consumption of aqueous extract compared to whole-leaf capsules can be anticipated. This is because, phytoconstituents that are not water-soluble may be extracted from whole-leaf (or root, stem, bulb, rhizome) preparations by the gastrointestinal fluids. 50% methanol was thus used in a separate experiment to extract *Lessertia*, to facilitate the extraction of constituents that are not soluble in water.

As shown in Table 41, both aqueous and methanolic extracts of *Lessertia* inhibited CYP1A2, 2C9, 2C19 and 3A4. Table 42 further shows the inhibitory effects of methanolic extracts of *Lessertia* on all the CYP isozymes and transport proteins except BCRP.

The extent of inhibition of the enzymes has been presented in relative terms in Table 41 and Table 42. Using arbitrary range of potency, inhibition with IC_{50} value of less than 100 μg/mL is considered potent (+++), IC_{50} values between 100 and 1000 μg/mL are considered moderate (++) and values greater than 1000 μg/mL mild/negligible (+). This is because, such high concentrations are not likely achievable *in vivo*.

In Table 41 and Table 42, traditional doses of the medicinal herbs are provided based mainly on the information gathered from the THPs. According to the THPs, the traditional doses are estimated and not subjected to conventional weighing in practice. While single-dose (Tables 41) decoctions are common as dosage regimes, higher amounts of herbal products (about 10 -50 g) are often decocted and taken several days in the course of treatment. Thus, it is difficult to determine uniform doses of traditional medications. The estimates provided however, are determined based on the information gathered from the THPs and the determined weight of sample doses.

Based on the approximated intestinal fluid volume of 250 mL and the laboratory extraction yields, putative achievable concentrations of the herbal extracts in the GIT were determined as shown in Table 41. The extent and efficiency of extraction of the herbs in the intestine may vary from laboratory extraction due to several factors: intestinal fluid composition, GIT transit time, disease state and dosage form (whole leaf/root, ground herbs or refined products as in capsules, tablets etc). The putative values of achievable intestinal concentration of the extractds are higher than the determined IC_{50} of the herbal extracts. This is important because of the rich expression of CYP and ABC transporters in the intestines (Ufer et al., 2008). Information on the absorption and bioavailability of the herbs is not available in literature. However, with the high putative GIT concentrations, high systemic blood levels are likely with the potential for post-absorption HDI.

Lessertia and *Hypoxis* are popular among people living with HIV/AIDS. The combination of *Lessertia* and/or *Hypoxis* with antiretroviral drugs presents the potential for HDI (Mills et al., 2005b). This is because, most antiretroviral drugs are substrates of the enzymes and transporters inhibited by the two herbs.

All herbs investigated in this study inhibited at least one metabolic enzyme. Considering the traditional use of such herbs, and conventional medications, important HDI can be inferred. For example, *Ranunculus multifidus* is traditionally used for the treatment of STIs, genital sores, warts and haemorrhoids (See Table 4.1 in Chapter 4). Patients who consume this herb for these indications may combine them with antibiotics. Results from this study (Table 41) showed that extract of *Ranunculus multifidus* is a potent inhibitor of CYP3A4. Antibiotics like erythromycin are substrates of CYP3A4.

Within the context of the literature, similar but limited investigations have been conducted most of which agree with the findings of the current study. A study by Mills and co-workers (2005b) had suggested that extracts of *Hypoxis hemerocallidea* and *Lessertia frutescens* may inhibit CYP3A4 and P-gp. The study investigated the potential of *Hypoxis hemerocallidea* and *Lessertia frutescens* to inhibit CYP3A4-mediated metabolism and P-gp-dependent drug transport as a way of assessing potential HDI with ARVs. While a number of ARVs are metabolized by CYP3A4 and/or transported by P-gp, others are not. There are 6 major classes of drugs approved by the US-FDA for the treatment of HIV/AIDS which include nucleotide reverse transcriptase inhibitors (NRTI), non-nucleotide reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), fusion inhibitors, integrase inhibitors, and chemochine receptor CCR5 antagonists. Entry inhibitor, maraviroc, is a CYP3A4 substrate making it a potential candidate for CYP3A4-dependent drug interactions. While protease inhibitors (PIs) are known substrates of both CYP3A4 and p-glycoprotein, other enzymes are involved in their metabolism. These include CYP2C19, 2D6, 2C9 and 2E1. Etravirine inhibits

CYP2C9 and CYP2C19. Efavirenz is a substrate of CYP2B6. In addition to these, most HIV/AIDS patients have co-morbidities managed with other drugs. These other drugs are substrates of a wide range of enzymes/transporters which were not covered in the study by Mills and others.

The findings of the current study however, agree with those of Mills and co-workers (2005b) with both herbs inhibiting the activity of CYP3A4. Only *Lessertia frutescens* inhibited the transport activity of P-gp. The difference in methodology (sourcing of herbal specimen, extraction and incubation method, concentration range etc) may account for why Mills and co-workers observed inhibition of P-gp by *Hypoxis hemerocallidea* while similar inhibition was not observed in this study. For example, HLM and standard recommended probe reactions of the various CYP isozymes were used in this study. This presents a major difference when compared to the inhibition of the conversion of dibenzylfluorescein to fluorescein with a measurement of fluorescence at 538 used by Mills and co-workers to probe CYP3A4-dependent reactions. Such reaction has not been sufficiently proven to be exclusively CYP3A4-catalysed in a microsome mixture containing all CYP isoforms (Bjornsson et al., 2003). The assessment of the influence of the herbal extracts on most CYP enzymes, BCRP, OATP1B1 and OATP1B3 makes this study more extensive than that of Mills and co-workers.

In a study to investigate the effect of the aqueous extracts of *Hypoxis hemerocallidea* and *Lessertia frutescens* on the transport of nevirapine across human intestinal epithelial cells using caco-2 cell monolayers model, Brown and co-workers (2008) reported that the extracts of *Hypoxis hemerocallidea* inhibited nevirapine transport. The effect of *Lessertia frutescens* on nevirapine transport was not significantly different from the control. Compared to the current study, extracts of *Lessertia frutescens* inhibited the acitivity of P-gp although in a different *in vitro* model.

Nair and co-workers (2007) investigated the influence of the phytochemical constituents (hypoxoside, rooperol, β-sitosterol, stigmasterol and stigmastanol) of *Hypoxis hemerocallidea* on the metabolic activity of CYP 3A4/5, CYP2C19 using *in vitro* fluorometric microtitre plate assay with isolated CYP isozymes. The potential interaction with P-gp was investigated by measuring the efflux of the fluorescent dye rhodamine 123 (Rh 123) in the caco-2 cell line model. The study reported that stigmasterol and rooperol inhibited the activity of CYP3A4/5 and CYP2C19 while hypoxoside induced P-gp. The use of phytochemical constituents for HDI studies is common but often non-reflective of the method of herbal use. Thus, apart from the extensive coverage of the CYP and transporters, the use of crude extracts in the current study makes a major difference from the work of Nair and colleagues (2007). Both studies

however, demonstrated the inhibitory activity of *Hypoxis hemerocallidea* on CYP3A4/5 and CYP2C19.

Gwaza and co-workers (2009) investigated the inhibitory activity of the extracts of a different specie of *Hypoxis* (*Hypoxis obtusa*) on CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 using HLM. Inhibitory effect of the extracts on P-gp using caco-2 cell monolayers model was also assessed. The authors reported that the extracts inhibited the CYP isozymes tested with no significant activity on P-gp. Compared to the current study, the inhibitory behaviour is similar except for CYP2C9 where no inhibition was observed in the current study.

In another study, Mogatle and co-workers (2008) evaluated the effect of *Hypoxis hemerocallidea* on the pharmacokinetics of efavirenz in a single-dose, two-phase sequential study conducted over 31 days in 10 healthy volunteers. Subjects were administered with a 600 mg single-dose efavirenz on Day 1 followed by blood sampling for analysis. From Day 16 to 30, subjects consumed preparations of *Hypoxis hemerocallidea* with 600 mg efavirenz added on Day 29. No significant pharmacokinetic HDI between *Hypoxis hemerocallidea* and efavirenz was observed. The finding from this study is important but may be difficult to generalize. This is because efaviranz is metabolized by both CYP2B6 and CYP3A4. Other substrates of each of the enzymes may behave differently.

Two more studies conducted on *Lessertia frutescens* are those by Minocha and colleagues (2011) and Müller and co-workers (2012). Müller and co-workers investigated the effects of the extracts and phytochemical components on the *in vitro* absorption and metabolism of atazanavir, a protease inhibitor, in HLM and caco-2 cell monolayers. The authors reported that aqueous extract and D-pinitol (a phytoconstituent of *Lessertia frutescens*) significantly reduced atazanavir accumulation by caco-2 cells (which implied a decrease in atazanavir absorption). Both the aqueous and methanolic extracts inhibited atazanavir metabolism in HLM.

Minocha and colleagues (2011) investigated the effects of short term and chronic exposure of *Lessertia frutescence* on the oral bioavailability and pharmacokinetics of nevirapine in Sprague Dawley rats. No significant difference in the pharmacokinetic parameters of nevirapine was found upon short-term co-administration. However, there was a 50% decrease in the AUC and C(max) values of nevirapine after 5 days of chronic exposure with *Lessertia frutescens*. The quantitative reverse transcription polymerase chain reaction (RT-PCR) studies demonstrated a 2-3-fold increase in the hepatic and intestinal mRNA expression of CYP3A2, relative to control. Employing LS-180 cells as an *in vitro* induction model for human CYP3A4, and using rifampicin as a positive control, extracts of *Lessertia frutescens* elevated m-RNA expression levels and functional activity of CYP3A4 (human

homologue of rodent CYP3A2). The results suggested a potential for HDI between *Lessertia frutescens* and nevirapine on chronic administration.

The findings of the current study agree with those of Minocha, Müller and co-workers. By inhibiting the activity of CYP3A4, other enzymes and transporters involved in the metabolism of ARVs, *Lessertia frutescens* has been shown to be a potential candidate for HDI in HIV/AIDS drug management. The popularity of *Hypoxis Hemerocallidea and Lessertia Frutescens* in Africa, and the various interests involved in their use and promotion make them deserving herbal products for multiple scientific studies.

Apart from *Hypoxis hemerocallidea* and *Lessertia frutescens*, information on the potential of other herbal products used in this study for HDI potential is sparse. Extracts of *Spirostachys africana* has previously shown inhibitory activity against CYP2C9, CYP2C19 and CYP3A4 in a study conducted using CYP inhibition screening that employed pregnane X (PXR) receptor in Chinese hamster ovary cell lines expressing human PXR (van den Bout-van den Beukel et al., 2008). The study reported induced CYP3A4 mRNA levels in the presence of the extracts. The current study reports a potent inhibitory effect of the aqueous extracts of *Spirostachys africana* on CYP3A4.

Diosmetin, a phytochemical constituent of a different specie of *Acacia* (*Acacia farnesiana*) has been shown to be a potent inhibitor of CYP1A1 and CYP1B1 enzyme activity by Androutsopoulos and co-workers (2009). Its crude extracts have been shown to inhibit xanthine oxidase (Chang and Yu-tang, 2012).

In general, the findings of the current study agree largely with previous studies (where available). This study thus provides a more elaborate assessment of HDI potential in commonly used South African medicinal herbs.

From the foregoing discussion, it is important to put the findings of this study in the following perspective:

1. Medicinal herbs are widely used and their indications vary from place to place.
2. Methods of use also vary. All the herbs investigated in this study are taken traditionally as aqueous decoction/infusions.
3. Conventional medications that may be combined with herbal products vary widely.
4. The *in vitro* inhibition of metabolic enzymes can reliably predict similar effects *in vivo*, with variations in the extent of inhibition, as earlier studies have shown.
5. The long history of the folkloric use of the medicinal herbs used in this study, for systemic disorders is an indication of their potential systemic availability. Systemic

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availability of phytoconstituents increases the potential for enzyme/transporter inhibition and HDI.

6. In the absence of systemic availability of phytoconstituents of the selected herbs, the effects of high GIT concentrations on drug absorption can be clinically potent especially with drugs with high presystemic intestinal metabolism, and narrow therapeutic window.

7. Compared to microsomes, results from *in vitro* use of cryopreserved hepatocytes are closer to *in vivo* condition due to the complete expression of the cell matrix and the presence of other enzymes. Thus, the predicted reduction of midazolam clearance by 40% by *Lessertia*, as shown with the *in vitro*-*in vivo* correlation may be clinically relevant.

Table 41: Summary of the findings of this study, *in vivo* predictions and possible clinical significance: extent of enzyme inhibition is provided as strong inhibition with IC_{50} of 0-100 μg/mL (**+++**); moderate inhibition with IC_{50} of 100-1000 μg/mL (**++**); mild/negligible inhibition with IC_{50} greater than 1000 μg/mL (**+**); and no inhibition (**-**).

Scientific name	CYP1A2	CYP2C9	CYP2C19	CYP3A4	Yield[a] (%$^w/_w$)	Usual (single) dose (mg)[b]	Estimated extract per dose (mg)[c]	Putative GIT conc (μg/mL)[d]
Acacia karroo	++	++	+++	+++	15.3	1000	153	612
Alepidea amatymbica	++	+++	+	-	14.5	1000	145	580
Bowiea volubilis	+++	-	+	+++	15.6	1000	156	624
Capparis sepiaria	++	+++	+++	+++	17.3	1000	173	692
Chenopodium album	+++	-	+++	++	17.7	1000	177	708
Emex australis	-	+++	+	+++	19.0	1000	190	760
Hypoxis hemerocallidea	+++	-	++	+++	12.6	1000	126	504
Kedrostis africana	+++	+++	++	+++	20.6	1000	206	824
Pachycarpus concolor	-	+++	+++	+++	16.5	1000	165	660
Pentanisia prunelloides	-	+++	+	+++	19.2	1000	192	768
Ranunculus multifidus	++	++	+++	+++	14.1	1000	141	564
Spirostachys africana	+++	-	-	+++	18.1	1000	181	724

Scientific name	CYP1A2	CYP2C9	CYP2C19	CYP3A4	Yield[a] (%$^w/_w$)	Usual (single) dose (mg)[b]	Estimated extract per dose (mg)[c]	Putative GIT conc (μg/mL)[d]
Lessertia frutescens (Methanolic)	+++	+++	+++	+++	28.5	300[e]	85.5	340
Lessertia frutescens (aqueous)	+++	+++	+++	+++	20	1000	200	800
Tulbaghia violacea	++	++	+	+	16.4	1000	164	656
Zantedeschia aethiopica	+	++	+++	+++	15.2	1000	152	608

[a] This is laboratory extraction yield using water in all cases except with *Lessertia* where both water and methanol were used as extraction solvents
[b] Usual traditional doses vary widely. On the average, up to 10g of the herbal materials are extracted and the decoctions are taken over time (3-6 times). Thus each consumption represent an extract of >1g
[c] This is based on the assumption that similar yield is obtained in the traditional process of decoction or extraction
[d] The estimation of putative GIT concentration is based on the average volume of GIT fluid (250mL)
[e] The 300mg traditional dose is based on the commercially available 300mg capsules of *Lessertia* made of its dried leaves. Aqueous extraction will not be a close reflection of a whole-leave consumption, as the patients are exposed to non-aqueous-soluble contents, thus the choice of 50% methanol for extraction
Note: Extent of CYP inhibition is given in relative terms: no inhibition (**-**); strong inhibition with IC_{50} of 0-100 μg/mL (**+++**); moderate inhibition with IC_{50} of 100-1000 μg/mL (**++**); and mild/negligible inhibition with IC_{50} greater than 1000 μg/mL (**+**)

Table 42: The relative inhibitory effect of *Lessertia* and *Hypoxis* on CYP isozymes and transport proteins: extent of inhibition is provided as follows: strong inhibition with IC_{50} of 0-100 μg/mL (**+++**); moderate inhibition with IC_{50} of 100-1000 μg/mL (**++**); mild/negligible inhibition with IC_{50} greater than 1000 μg/mL (**+**); and no inhibition (**-**).

Enzyme/transporter	Relative inhibitory effect of	
	Lessertia	***Hypoxis***
CYP2A6	++	++
CYP2B6	+++	+++
CYP2C8	+++	++
CYP2D6	++	-
CYP2E1	+	-
P-gp	++	-
BCRP	-	-
OATP1B1	+++	++
OATP1B3	+++	++

10.2. Study Limitation

For this study, all herbal samples were obtained from THPs, just as consumers/clients do. It is important to note that many other consumers of herbal products obtain them commercially (for instance, in the open markets). The phytochemical composition of herbs is known to vary depending on the place and time of harvesting. Thus, as representative as the collection procedure in this study may be (within the South African context), same herbs obtained from other provinces and localities may exhibit variations in phytochemical constituents. Modern commercialization of medicinal herbs involves the deliberate cultivation (including nursing, application of fertilizers/organic manure, irrigation and shedding) of the herbs of interest. Since THPs rely principally on wild sources, the extent of phytochemical composition from the different sources may vary.

Another important limitation to the generalization of the findings in this study is the known variation of the phytochemical composition of medicinal plants due to time of harvest. A study conducted by Ozkan and co-workers (2010) found that the chemical compositions, free radical scavenging activities and reducing/antioxidant capacities of the extracts and essential oils of the studied plant (Turkish oregano) changed significantly depending on the vegetative periods of growing season. Thus, such quantitative and qualitative differences in the phytochemical expression of medicinal plants may lead to variations in the inhibitory activity of the herbal extracts on metabolic enzymes.

In traditional use, estimates of dosages are often consumed. There can be variation on the extraction/decoction process (temperature, time). This may lead to variation in the concentration of extracts consumed by the individual patients. The yield from laboratory extraction may vary from the actual yield from the consumers' decoctions. Thus, values and correlations made herein are estimates only. The non-standardized dosage system of the THPs makes it practically difficult to predict what GIT concentration may have important interaction with metabolic enzymes and transporters.

It is important to note that, there may be variations in what THPs indicate for various medicinal herbs. What is consumed for a particular ailment in a province may be indicated for a different or additional disease in another locality. This will influence the conventional drugs that are candidates for concomitant administration.

There was no analysis of the individual phytochemical composition of the extracts. The findings in this study are based on crude extracts only. The determination of the exact compounds exerting inhibitory activity on the drug metabolism and/or transport process was beyond the scope of this study.

The extracts that may be systemically available in significant quantities are not known. With no information on the extent of oral absorption of the crude extracts of the individual herbs, the extent of systemic inhibition of enzymes/transporters, especially in the liver could not be estimated. These herb-specific factors have been recognised as major limitations of *in vitro* assessment of herb-drug interactions (Markowitz and Zhu, 2012).

Certain limitations are associated with the *in vitro* models employed in this study. Ideally, clinical pharmacology studies should comprehensively investigate drug biotransformation and interaction in humans. This will ensure the most reliable clinical data. Some of the major setbacks of such studies are the risk to the human subjects and the stringent ethical and regulatory requirements. The use of *in vitro* technologies has thus evolved and advanced as suitable alternative. In the current study, human liver microsomes, cryopreserved hepatocytes and transfected mammalian cell lines were used.

In all models, there is a continuous contact between the test inhibitors, enzyme/transporters and their substrates. This is not representative of the *in vivo* system where blood circulation provides mobility to the drugs and the inhibitors. Non-specific plasma protein binding, organ perfusion, concentration of drugs in body areas with no or less metabolic activity, solubility in the surrounding lipid layers, the contribution of other enzyme systems and non-metabolic clearance are not reflected in *in vitro* systems (Poulin, 2013).

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HLM has poor quantitative extrapolative values. Microsomes consist of the vesicles of the hepatocyte endoplasmic reticulum prepared by differential centrifugation (Brandon et al., 2003). As a result, they contain almost only CYP and UGT enzymes. The absence of other enzymes in the microsomes makes *in vitro* metabolism faster compared to *in vivo* scenario. The artificiality of exogenous supply of co-factors to the microsomal incubation is a major variation from *in vivo* reality (Ong et al., 2013). The relevance of the drug concentration employed in microsomal incubations is often questionable as only microsomal binding is available compared to additional and often more pronounced plasma protein binding *in vivo* (Poulin, 2013). The choice of incubation conditions may influence the outcomes of *in vitro* studies. In the *in vivo* scenario, for example, the ionic strength and pH are kept constant through homeostatic processes. Maintaining these same conditions *in vitro* is difficult. While the initial pH can be regulated, the addition of water, methanol, extract solution and co-factors can greatly alter the physicochemical conditions (Ong et al., 2013).

Organic solvent, although used at the minimum permissible level, may still influence the activity of the enzyme system. This is because of its continuous and persistent presence in the incubation mixture unlike in the *in vivo* setting. Although the use of pooled HLM and hepatocytes is supposed to neutralize the donor-to-donor variations, the efficiency of pooling depends to a large extent on the balanced blend of donors from various racial, gender and genetic groups. This is difficult to achieve. Further limitations in extrapolating *in vitro* data to predict *in vivo* relevance is the implicit assumption that the drug candidate to be affected is solely metabolized in the liver. In reality, multiple enzymes are responsible for the metabolism of a large number of drugs (Camenisch and Umehara, 2013).

Although cryopreserved hepatocytes often retain their metabolic enzyme activity, the effect of cryoprotectants on the primary hepatocytes is not completely understood. There has been no characterization of cryopreserved hepatocytes, including the quantitation of transcription factors, co-activators and co-repressors, the effectiveness of the inherent co-factors and the maintenance of cellular integrity, all of which are important regulators of drug metabolizing enzymes (Hariparsad et al., 2006). The cycle of freezing and thawing of the cryopreserved hepatocytes is known to reduce their viability (Terry et al., 2010). *In vitro* use of hepatocytes does not require an exogenous supply of co-factors. Unlike the *in vivo* situation where energy supply for metabolic activity is consistent, the viability of hepatocytes decreases with time of activity during incubation. This may influence outcomes of rate and extent of metabolism in *in vitro* settings, and make *in vivo* extrapolations inaccurate. The absence of other liver-based cells such as Kupffer cells in this model makes the metabolic environment different and non-representative of the liver.

In vitro drug transport assays employ cell lines transfected with the desired transporter protein. This study used Madin-Darby canine kidney (MDCK-II and LLC-PK1), transfected with human MDR1 (ABCB1) as a recombinant models for efflux transport. Human Embryonic Kidney (HEK293) cells transfected with human OATP1B1 and OATP1B3 were used for the drug uptake transport assays. Endogenous transporters in these cells may contribute to the activities of the recombinant transporters. The expression of such endogenous transporters may vary from cell to cell, and therefore difficult to account for in the result interpretation. According to Kuteykin-Teplyakov and co-workers (2010), this difference in the expression of endogenous efflux transporters provide a potential bias for *in vitro* studies on drug transport.

In the liver, hepatocytes express both metabolic enzymes and transport proteins. The absence of metabolic enzymes in transfected cell lines thus, is at variance with *in vivo* scenario. The isolation and concentration of the transport proteins in the transfected cells provides a high drug transport rate which is not the case *in vivo* (Masimirembwa et al., 2012; Alqahtani et al., 2013).

Due to these highlighted limitations, accurate measurement of kinetic parameters to correspond with values *in vivo* or through extrapolation is difficult. The results obtained from this study are largely qualitative, and only provide indications for the potential for herb-drug interactions.

While *in vitro* HDI studies provide the indication for *in vivo* relevance, *in vivo* human studies are the ultimate proof of clinically significant HDI.

10.3. Conclusion

All the selected medicinal herbs inhibited one or more of the CYP isozymes and drug transport proteins. The confirmation of inhibitory effects of *Lessertia* using human hepatocytes is a stronger indication of *in vivo* reflection of the findings of this study. The study clearly shows potential for clinically relevant HDI between the studied herbs and conventional medicines. It is important for health practitioners, patients and policy makers to be aware of HDI potential in concurrent herb-drug use.

10.4. Recommendations

In vitro liver-based drug metabolism is still the standard for drug interaction studies especially for new chemical entities and poorly understood compounds like phytochemicals. However, due to the increasing knowledge in phytopharmacology, *in vivo* human studies can be recommended to expand the knowledge of HDI with respect to the medicinal herbs selected in

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this study. Such study will provide definite clinical significance of the observed inhibition of metabolic enzymes and transport proteins.

There is also a need to research into the identification of the active phytochemical entities in the herbal products. A further understanding of the systemic bioavailability of such phytochemicals will be beneficial in elucidating clinical benefits, and open opportunities in drug discovery. Future findings in this regard will also be important in understanding phytotoxicity and drug safety.

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APPENDIX A: ABSTRACTS OF PAPERS PUBLISHED/SUBMITTED FROM THIS THESIS

APPENDIX A1

Current Drug Metabolism, 2012, *13*, 215-224 215

Liver-Based *In Vitro* Technologies for Drug Biotransformation Studies - A Review

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Abstract: Early understanding of the metabolic pathway and potential interaction of new drug candidates with other drugs is one of the goals of preclinical studies in the drug discovery process. Although other body organs are involved in drug biotransformation, the liver is the predominant organ of metabolism for a wide range of endogenous compounds and xenobiotics. The set of enzymes contained in the cytochrome P450 superfamily present predominantly in the liver have been identified as the single most important agent of drug metabolism and have formed the bedrock of most matured technologies for *in vitro* drug biotransformation studies. With the development of a number of liver-based technologies, *in vitro* metabolism has gained significant popularity in the past three decades. This has come in response to several demanding factors including the questionable relevance of data from animal studies; the high cost and stringent regulatory and ethical requirement, as well as safety issues involved with studies using human subjects; and the need for high throughput due to the wide range of chemical entities for routine investigations. These technologies which vary from whole liver to subcellular fractions have found ready application in generating the desired information on the substrate and inhibitor specificity of most metabolic enzymes. This paper reviews such technologies as isolated fresh liver; liver slices; primary, cultured and cryopreserved hepatocytes; microsomes; cytosolic fractions; and purified or heterologously expressed drug-metabolizing enzymes. It highlights the general principles of *in vitro* enzyme kinetics and the factors that determine the choice of each *in vitro* technology for biotransformation studies.

Keywords: biotransformation, cytochrome P450, *in vitro* metabolism, liver cytosol, liver slices, microsomes, S9 fractions, subcellular fractions, UDT-glucuronosyltransferase.

INTRODUCTION

Drug biotransformation study is an integral part of preclinical screening for new drug candidates [1]. This assessment usually involves both *in vitro* and *in vivo* models in animal species where the main pharmacokinetic, pharmacodynamic and toxicological

The use of human subjects for comprehensive clinical pharmacology studies seems to be ideal. However, the risks involved, and the stringent regulatory demands make this practically challenging with attendant high cost and longtime duration. Results from animal studies also pose correlation challenges and clinical significance.

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APPENDIX A2

frontiers in
PHARMACOLOGY

REVIEW ARTICLE
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An overview of the evidence and mechanisms of herb–drug interactions

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Despite the lack of sufficient information on the safety of herbal products, their use as alternative and/or complementary medicine is globally popular. There is also an increasing interest in medicinal herbs as precursor for pharmacological actives. Of serious concern is the concurrent consumption of herbal products and conventional drugs. Herb–drug interaction (HDI) is the single most important clinical consequence of this practice. Using a structured assessment procedure, the evidence of HDI presents with varying degree of clinical significance. While the potential for HDI for a number of herbal products is inferred from non-human studies, certain HDIs are well established through human studies and documented case reports. Various mechanisms of pharmacokinetic HDI have been identified and include the alteration in the gastrointestinal functions with consequent effects on drug absorption; induction and inhibition of metabolic enzymes and transport proteins; and alteration of renal excretion of drugs and their metabolites. Due to the intrinsic pharmacologic properties of phytochemicals, pharmacodynamic HDIs are also known to occur. The effects could be synergistic, additive, and/or antagonistic. Poor reporting on the part of patients and the inability to promptly identify HDI by health providers are identified as major factors limiting the extensive compilation of clinically relevant HDIs. A general overview and the significance of pharmacokinetic and pharmacodynamic HDI are provided, detailing basic mechanism, and nature of evidence available. An increased level of awareness of HDI is necessary among health professionals and drug discovery scientists. With the increasing number of plant-sourced pharmacological actives, the potential for HDI should always be assessed in the non-clinical safety assessment phase of drug development process. More clinically relevant research is also required in this area as current information on HDI is insufficient for clinical applications.

Keywords: Herb–drug interaction, traditional medicine, phytochemicals, transport proteins, cytochrome P450

INTRODUCTION

There is increasing consumptions of medicinal herbs and herbal

Canada (Calixto, 2000), Australia (Bensoussan et al., 2004), as well as Europe where the highest sales of herbal products have been

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APPENDIX A3

Drug Metab Dispos. 2012 Dec 4. [Epub ahead of print]

The Potential of Sutherlandia Frutescens for Herb-drug Interaction.

Fasinu PS, Gutmann H, Schiller H, James AD, Bouic PJ, Rosenkranz B.

1 Division of Pharmacology, Stellenbosch University;

Abstract

Sutherlandia frutescens (ST) is a popular medicinal herb widely consumed in Africa by people living with HIV/AIDS. Concomitant use with antiretroviral drugs has generated concerns of herb-drug interaction. This study investigated the inhibitory effects of the crude extracts of ST on the major cytochrome P450 isozymes employing pooled human liver microsomes. Its effect on the metabolic clearance of midazolam using cryopreserved hepatocytes was also monitored. The potential of ST to inhibit human ATP-binding cassette (ABC) transporters (P-gp and BCRP) and the human organic anion transporting polypeptide (OATP1B1 and OATP1B3) activity was assessed using cell lines overexpressing the transporter proteins. ST showed inhibitory potency for CYP1A2 (IC(50) = 41.0 µg/mL), CYP2A6 (IC(50) = 160 µg/mL), CYP2B6 (IC(50) = 20.0 µg/mL), CYP2C8 (IC(50) = 22.4 µg/mL), CYP2C9 (IC(50) = 23.0 µg/mL), CYP2C19 (IC(50) = 35.9 µg/mL) and CYP3A4/5 (IC(50,) = 17.5 µg/mL [with midazolam1'-hydroxylation]; IC(50) = 28.3 µg/mL [with testosterone 6β-hydroxylation]). Time-dependent (irreversible) inhibition by ST was observed for CYP3A4/5 (KI = 296 µg/mL, kinact = 0.063 min(-1)) under the conditions of this study. ST also delays the production of midazolam metabolites in the hepatocytes, decreasing its clearance by 40%. Further, ST inhibited P-gp (IC(50) = 324.8 µg/mL); OATP1B1 (IC(50) = 10.4 µg/mL, and of OATP1B3 (IC(50) = 6.6 µg/mL). The result indicates the potential for HDI between ST and the substrates of the affected enzymes, if sufficient in vivo concentration of ST is attained.

PMID: 23209194 [PubMed - as supplied by publisher]

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APPENDIX B: ABSTRACTS OF CONFERENCE PROCEEDINGS FROM THIS THESIS

APPENDIX B1

Herb-drug interaction potential of popular South African medicinal herbs: an in vitro assessment

Fasinu P[1*], Gutmann H[2], Schiller H[2], Birlinger B[2], Bijasson L[2], Faller S[2], Seifart H[1], Bouic P[3,4], and Rosenkranz B[1]

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Background and Purpose
The incidence of concurrent use of herbal preparation and orthodox medicine has been shown to be high (about 70%) in South Africa. Pharmacokinetic herb-drug interaction (HDI) is known to result from such practices with clinically significant alterations in drug efficacy or toxicity. While studies in developed countries have largely documented common HDI, information on the potential of African medicinal herbs to interact with concomitantly administered drugs is largely unavailable. Cytochrome P450 (CYP) and transport proteins are responsible for most pharmacokinetic drug interactions. The aim of this study was to investigate the influence of popular South African medicinal herbs on these enzymes and transporters.

Methods
With the approval of the Health Research Ethics Committee of the University of Stellenbosch, medicinal herbs were sourced from traditional medical practitioners and identified. Extracts were freeze-dried (aqueous) or dried using vacuum rotary evaporator (methanolic). Graded concentrations were incubated with human liver microsomes to monitor phenacetin O-deethylation, coumarin 7-hydroxylation, bupropion hydroxylation, paclitaxel 6α-hydroxylation, diclofenac 4'-hydroxylation, S-mephenytoin 4'-hydroxylation, bufuralol 1'-hydroxylation, chlorzoxazone 6-hydroxylation, midazolam 1'-hydroxylation and testosterone 6β-hydroxylation as markers for the metabolic activities of CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4/5 respectively. Substrate consumption and generation of metabolites were monitored and quantified with the aid of LC/MS/MS. Each substrate was incubated at concentrations below pre-determined *Km* value. All assays were performed in duplicates. The influence on transport proteins – efflux (p-glycoprotein and BCRP) and uptake (OATP1B1 and OATP1B3) were also investigated using radiolabelled substrates.

Results and Discussion
Of the 15 plants investigated, *Sutherlandia frutescens, Tulbaghia violacea, Hypoxis hemerocallidea Spirostachys Africana, Ranunculus multifidus, Chenopodium album and Emex australis* demonstrated very potent inhibitory activity against different CYP enzymes. There was wide difference in the inhibitory activity of aqueous and methanolic extract of *Hypoxis hemerocallidea*. All extracts inhibited at least 3 of the enzymes tested in a concentration-dependent manner with IC_{50} values varying from 10.6 to 240.3 µg/mL. Near-total enzyme inhibition (98%) was observed in a number of extracts. Time-dependent (irreversible) inhibition by *Sutherlandia frutescens* was observed for CYP3A4/5 (K_I = 295.653 µg/mL, K_{inact} = 0.0632 min^{-1}). *Sutherlandia frutescens* and *Hypoxis hemerocallidea* inhibited the activity of p-glycoprotein, OATP1B1 and OATP1A3 to varying degrees.

Conclusions:
The result showed significant potential for relevant HDI between popular SA medicinal herbs and orthodox medicines which should be considered during concurrent herb-drug administration.

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APPENDIX B2

ABSTRACT NUMBER / ABSTRAKNOMMER 24

THE POTENTIAL OF H. HEMEROCALLIDEA AND S. FRUTESCENS FOR HERB-DRUG INTERACTION WITH ANTIRETROVIRAL DRUGS

MR PS FASINU (*Pharmacology*)

Background/Purpose:
Two of the most popular herbs widely consumed for medicinal purposes among HIV/AIDS patients who are on ART are Hypoxis hemerocallidea and Sutherlandia frutescens. Cytochrome P3A4 is responsible for the metabolism of majority of drugs including the protease inhibitors (PI) and non-nucleotide reverse transcriptase inhibitors (NNRI). The international standard for in vitro assessment of CYP3A4 activity and potential interaction consists of monitoring testosterone metabolism in a liver-based medium. Thus, the current study was aimed at investigating the potential for HDI between the extracts of the two herbs and CYP3A4 substrates employing human liver microsomes (HLM)

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Methods:
Aqueous extracts of fresh herbal samples, sourced from two traditional health practitioners, were freeze-dried. In vitro metabolism was optimized by the incubation of graded concentrations of testosterone in NADPH-regenerating system and 0.25mg/mL HLM suspension where kinetic parameters (Km and Vmax) were determined. Metabolic reaction was halted by the addition of ice-cold acetonitrile and the resulting mixture centrifuged (5000rpm, 10 mins) to precipitate microsomal proteins. Supernatants were analyzed by LC/MS. Further incubations were performed in the presence of graded concentrations (µg/mL) of the herbal extracts. Control incubations with ketoconazole, a CYP3A4-specific inhibitor, were performed concurrently. The influence of the aqueous herbal extracts on CYP3A4 activity was determined and compared. All determinations were performed in duplicates (intra-day and inter-day).

Results and Discussion:
Both H. hemerocallidea and S. frutescens demonstrated concentration-dependent inhibition of CYP3A4 activity with IC50 values of 25.7 and 24.3µg/mL respectively. At 100µg/mL, a concentration estimated to fall within the putative in vivo situation, CYP3A4 activity was inhibited by 91% and 85% respectively.

Conclusions:
The result showed significant potential for relevant HDI between CYP3A4 substrates and H. hemerocallidea and S. frutescens. Concomittant herb and ART consumption may lead to HDI precipitated by CYP3A4 inhibition.

Acknowledgement:
SURMEPI and Hope Cape Town, for financial support.

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APPENDIX B3

11.1.6.3 Fasinu, PS

In vitro Investigation of the Effects of Commonly Used South African Medicinal Herbs on CYP1A2 activity Employing Human Liver Microsomes

Pius Fasinu[1]*, Heiner Seifart[1], Patrick Bouic[2], and Bernd Rosenkranz[1]
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Purpose:
Herb-drug interaction (HDI) is a major clinical concern especially with concomitant consumption of medicinal herbs and prescription medicines. *In vitro* liver-based technologies present scientifically relevant medium for investigating the potential for HDI. CYP1A2 is one of the major metabolic enzymes accounting for 11% of all CYP-mediated drug metabolism. The purpose of the current study therefore, was to investigate the effects of 15 commonly used herbal medications in South Africa on the metabolic activity of CYP1A2 employing human liver microsomes (HLM) and phenacetin as a standard probe substrate.

Methods:
Graded concentrations of phenacetin (0.25 – 50µM) were incubated with 0.25mg/mL HLM in Eppendorf tubes for 15 min in a shaking water bath (100rpm; 37°C). An NADPH-regenerating system prepared with glucose-6-phosphate dehydrogenase and reduced NADP+ in a cofactor solution was used to initiate the metabolic reaction. The reaction was halted by the addition of ice-cold acetonitrile (-20°C) containing 2µg/mL thiacetazone as an internal standard. The resulting mixture was centrifuged (5000g, 10 min) to precipitate the microsomal proteins. Supernatants were analysed by HPLC/MS. The methodology was optimized by profiling the rate of metabolism against the substrate concentrations to determine the enzyme kinetic parameters (K_m and V_{max}). Further incubations were performed at the determined K_m in the presence of the herbal extracts, prepared in percentage dilutions, according to the recipe of the traditional healers. Control incubations with α-naphthoflavone, a CYP1A2-specific inhibitor, were performed concurrently. The influence of the aqueous herbal extracts on CYP1A2 activity was determined and compared. All determinations were performed in triplicates.

Results and Discussion:
The results showed that 7 out of the 15 herbal extracts investigated demonstrated significant inhibition of phenacetin metabolism to varying degrees (30-86%). These included *Hypoxis hemerocallidea, Spirostachys Africana, Bowiea volubilis, Chenopodium album, Kedrostis Africana, Lauridia tetragonia* and *Pachycarpus concolor*.

Conclusions:
Given the role of CYP1A2 in the metabolism of a wide range of therapeutically important drugs, the results showed a potential for herb-drug interaction especially if such drugs are administered concomitantly with the herbal medications investigated.

Ethics:
This research was approved by the University of Stellenbosch Human Research Ethics Committee with Ethics Reference number N10/09/307

References:
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Acknowledgement:
Hope Cape Town, for financial support.

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APPENDIX B4

ABSTRACT NUMBER / ABSTRAKNOMMER 7

IN VITRO INVESTIGATION OF HERB-DRUG INTERACTION POTENTIAL: THE INFLUENCE OF 15 COMMONLY USED SOUTH AFRICAN MEDICINAL HERBS ON CYP1A2 ACTIVITY

MR PS FASINU, DR HI SEIFART, PROF PJD BOUIC, PROF B ROSENKRANZ (*University of Stellenbosch*)

Purpose: CYP1A2 is one of the major metabolic enzymes accounting for 11% of all CYP-mediated drug metabolism. The purpose of the current study therefore, was to investigate the effects of 15 commonly used herbal medications in South Africa on the metabolic activity of CYP1A2 employing human liver microsomes (HLM) and phenacetin as a standard probe substrate.
Methods: Graded concentrations of phenacetin (0.25 – 50µM) were incubated with 0.25mg/mL HLM in eppendorf tubes for 15 min in a shaking water bath (100rpm; 37°C). An NADPH-regenerating system prepared with glucose-6-phosphate dehydrogenase and reduced NADP+ in a cofactor solution was used to initiate the metabolic reaction. The reaction was halted by the addition of ice-cold acetonitrile (-20°C) containing 2µg/mL thiacetazone as an internal standard. The resulting mixture was centrifuged (5000g, 10 min) to precipitate the microsomal proteins. Supernatants were analysed by HPLC/MS. The methodology was optimized by profiling the rate of metabolism against the substrate concentrations to determine the enzyme kinetic parameters (Km and Vmax). Further incubations were performed at the determined Km in the presence of the herbal extracts, prepared in percentage dilutions, according to the recipe of the traditional healers. Control incubations with α-naphthoflavone, a CYP1A2-specific inhibitor, were performed concurrently. The influence of the herbal extracts on CYP1A2 activity was determined and compared. All determinations were performed in triplicates.
Results and Discussion: The results showed that 7 out of the 15 herbal extracts investigated demonstrated significant inhibition of phenacetin metabolism to varying degrees (30-86%). These included Hypoxis hemerocallidea, Spirostachys Africana, Bowiea volubilis, Chenopodium album, Kedrostis Africana, Lauridia tetragonia and Pachycarpus concolor.
Conclusions: Given the role of CYP1A2 in the metabolism of a wide range of therapeutically important drugs, the results showed a potential for herb-drug interaction especially if such drugs are administered concomitantly with the herbal medications investigated.

ABSTRACT NUMBER / ABSTRAKNOMMER 8

THE PLATELET ACTIVATION MARKER, CD62P CORRELATES WITH LOWER CD4 COUNTS IN ASYMPTOMATIC HIV INFECTION.A PILOT STUDY

MR BB NKAMBULE (*Haematology*), DR RH GLASHOFF (*Virology*), DR H IPP (*Haematology and Virology*)

Stellenbosch University http://scholar.sun.ac.za

APPENDIX C: ABSTRACTS OF COLLABORATIVE PUBLICATIONS AND CONFERENCE PROCEEDINGS

APPENDIX C1

RESEARCH ARTICLE

Flavonoids and Polymer Derivatives as CYP3A4 Inhibitors for Improved Oral Drug Bioavailability

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ABSTRACT: Molecular modeling computations were utilized to generate pharmaceutical grade CYP3A4-enzyme inhibitors. *In vitro* metabolism of felodipine in human intestinal and liver microsomes (HLM and HIM) was optimized yielding a Michaelis–Menten plot from where the K_m and V_{max} values were estimated by nonlinear regression. The flavonoids, naringin, naringenin, and quercetin, were subsequently incubated with felodipine at the determined K_m value in HLM. Comparing results obtained from a known CYP3A4 inhibitor, verapamil, the flavonoids inhibited felodipine metabolism. In-depth computational analysis of these flavonoids in terms of CYP3A4 binding, sequencing, and affinity, computational biomimetism was employed to validate the potential CYP3A4 inhibitors. The modeled compounds were comparatively evaluated by incubation with felodipine in both HLM and HIM. Results showed that the polymers 8-arm-PEG, o-(2-aminoethyl)-o-methyl-PEG, 4-arm-PEG (molecular weight = 10,000 g/mol and 20,000 g/mol, respectively), and poly(L-lysine) were able to inhibit the felodipine metabolism with the half maximal inhibitory concentration (IC_{50}) values ranging from 7.22 to 30.0 μM (HLM) and 6.78 to 41.03 μM (HIM). Molecular docking confirmed drug–enzyme interactions by computing the free energies of binding (ΔE) and inhibition constants (K_i) of the docked compounds utilizing a Lamarckian Genetic Algorithm. Comparative correlations between the computed and experimental K_i values were obtained. Computational modeling of CYP3A4 inhibitors provided a suitable strategy to screen pharmaceutical grade compounds that may potentially inhibit presystemic CYP3A4-dependent drug metabolism with the prospect of improving oral drug bioavailability. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: oral bioavailability; drug delivery; polymers; CYP3A4 inhibitors; computational molecular modeling; cytochrome P450; computer aided drug design; docking studies; drug metabolism; kinetics.

INTRODUCTION

Research in drug metabolism has attracted more at-

P450 (CYP) enzyme system present in the liver and intestine is responsible for the metabolism of a wide

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APPENDIX C2

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Review

Drug-drug interactions in ageing HIV-infected individuals

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Accepted 24 September, 2012

The effectiveness of antiretroviral therapy has significantly improved life expectancy of HIV-infected individuals. Global campaigns and awareness programmes have led to substantial drop in the rate of new infections. Consequently, the proportion of ageing HIV-infected individuals continues to increase. HIV-associated and age-related comorbidity necessitates polypharmacy in ageing individuals living with HIV/AIDS. The risk of drug-drug interaction increases with the number of administered drugs. Age-related changes in the body physiology are known to influence pharmacokinetic and pharmacodynamic profile of administered drugs. These changes include reduction in blood flow to major organs, decline in metabolic activities, body mass shrinking and changes in body water and fat proportion. These factors contribute to the perceived and reported higher incidence of drug-drug interaction in this population. The current paper reviews the reported incidence of drug-drug interactions in ageing HIV-infected individuals, providing relative mechanisms and possible factors responsible in comparison to younger population. Health professionals should be aware of the drug interaction risks involved in the management of HIV/AIDS in the ageing population; be able to anticipate them based on concomitant medications and manage them as necessary.

Key words: Drug-drug interaction, drug metabolism, drugs, ageing, HIV/AIDS, cytochrome P450.

INTRODUCTION

Since the advent of highly active antiretroviral therapy (HAART) in 1990s, the management of HIV has resulted in improved quality of life, survival and life expectancy of infected individuals (Antiretroviral Therapy Cohort Collaboration, 2008; van Sighem et al., 2010; Mills et al., 2011; Nakagawa et al., 2012). Although without a cure, HIV infection with early diagnosis and treatment adherence has become a chronic disease albeit life-gaining prominence as the number of affected people continues to increase (Grabar et al., 2006). However, there are no specific guidelines targeted at drug management of HIV in older patients. Information on the safety and efficacy of current antiretroviral (ARV) regimen in older patients are not sufficiently available. Clinical trials have often excluded older individuals until recently (Limb, 2011). Meanwhile, little research has been done to

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APPENDIX C3

Comparative evaluation of warfarin utilization in two primary health care clinics in the Cape Town area

Xolani W. Njovane, MBChB, BSc (Hons), Pius S. Fasinu, MSc MED, Bernd Rosenkranz, MD, PhD, FFPM

Division of Pharmacology, Faculty of Medicine and Health Sciences, University of Stellenbosch, Tygerberg, Cape Town, South Africa

Abstract

Background: Warfarin remains the anticoagulant drug of choice in a wide range of patients. Its narrow therapeutic window makes patients susceptible to high risk of bleeding complications or failure to prevent clotting. This has necessitated therapeutic monitoring in warfarinized patients. Factors that could be responsible for the fluctuating responses to warfarin vary from pharmacogenetic to concomitant morbidity, diet and medication. In order to assess the quality of the management of warfarin treatment in a local primary care setting, the current study evaluated warfarin utilization and monitoring records in two hospitals with different patient groups.

Methods: A retrospective study was undertaken in the special warfarin clinics at Wesfleur and Gugulethu hospitals (Western Cape, South Africa) covering all warfarin related therapy records in a 12-month period. Data extracted from the patients' folders included age, sex, race, weight, address, concurrent chronic illnesses, treatment and medication, indication for warfarin and INR history.

Results: A total of 119 patient's folders were analysed. Attendance at the clinics reflects the demographic and racial distribution of the host location of the hospitals. While all the patients were maintained above the minimum INR value of 2, about 50% had at least one record of INR above the cut-off value of 3.5. None of the patient records showed a INR less than 2; however, over a third of the patients (32.2%) had at least one record of INR greater than 3.5 in Gugulethu hospital compared to over half (58.3%) in Wesfleur hospital.

In total, atrial fibrillation was the most common indication for warfarinization while hypertension was the most common concurrent chronic condition in warfarinized patients. All patients who received quinolone antibiotics had INR values above the cut-off point of 3.5 within the same month of the initiation of the antibiotic therapy suggesting drug-induced warfarin potentiation. Other co-medications including beta-lactam antibiotics, NSAIDs and anti-ulcers appeared to alter warfarin responses as measured by recorded INR values.

Conclusion: The study found inter-individual variability in the response to warfarin therapy which cut across the racial classifications. It also confirms the possible influence of concomitant morbidity on patient response to anticoagulant therapy.

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APPENDIX C4

BIOPHARMACEUTICS & DRUG DISPOSITION
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Review

Diverse approaches for the enhancement of oral drug bioavailability

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ABSTRACT: In conscious and co-operating patients, oral drug delivery remains the preferable route of drug administration. However, not all drugs possess the desirable physicochemical and pharmacokinetic properties which favor oral administration mainly due to poor bioavailability. This has in some cases led to the choice of other routes of administration, which may compromise the convenience and increase the risk of non-compliance. Poor bioavailability has necessitated the administration of higher than normally required oral doses which often leads to economic wastages, risk of toxicity, erratic and unpredictable responses. The challenge over the years has been to design techniques that will allow oral administration of most drugs, irrespective of their properties, to achieve a therapeutic systemic availability. This will be a worthy achievement since over 90% of therapeutic compounds are known to possess oral bioavailability limitations. In this review, an attempt has been made to explore various approaches that have been used in recent years to improve oral drug bioavailability, including physical and chemical means. This review strives to provide a comprehensive overview of advances made over the past 10 years (2000–2010) in the improvement of the oral bioavailability of drugs. Briefly, the design of prodrugs to bypass metabolism or to enhance solubility as well as modification of formulation techniques such as the use of additives, permeation enhancers, solubilizers, emulsifiers and non-aqueous vehicles have been discussed. Arising approaches, such as formulation modification techniques; novel drug delivery systems, which exploit the gastrointestinal regionality of drugs, and include the pharmaceutical application of nanotechnology as an emerging area in drug delivery; inhibition of efflux pumps; and inhibition of presystemic metabolism have been more extensively addressed. This critical review sought to assess each method aimed at enhancing the oral bioavailability of drugs in terms of the purpose, scientific basis, limitations, commercial application, as well as the areas in which current research efforts are being focused and should be focused in the future. Copyright © 2011 John Wiley & Sons, Ltd.

Key words: absorption; bioavailability; cytochrome P450; efflux transporters; enzymes; inhibitors; permeation enhancers; P-glycoproteins; poorly water-soluble drugs; pre-systemic metabolism

Introduction

The ability of an administered drug to elicit the desired pharmacological response and reverse a disease condition is the ultimate goal of drug therapy [1]. Such pharmacological response depends on the availability of the drug at the receptor site, which in turn is influenced by plasma drug concentrations [2]. The goal of an effective drug delivery system is therefore to achieve and sustain therapeutic blood levels of the drug except in some pathological conditions where drugs are intended for local action, e.g. antacids. Drug delivery has traditionally taken the form of ingestion, inhalation, injection, infusion

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E-mail: viness.pillay@wits.ac.za

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Revised 23 November 2010
Accepted 28 January 2011

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APPENDIX C5

YOUNG SCIENTIST

YS 09 Pharmacokinetic Drug Interactions as a Route for Improving Oral Drug Bioavailability

PS Fasinu
Department of Medicine, Division of Pharmacology, Stellenbosch University, Tygerberg Campus, South Africa
16669967@sun.ac.za

The poor bioavailability of drugs has been identified as the single most important challenge in oral drug delivery. Prominent among the factors responsible for this are the metabolic activity of the intestinal and hepatic cytochrome P450 (CYP) enzyme family. In the current study, novel CYP inhibitors were generated through the application of computational modeling of known CYP3A4-substrate and CYP3A4-inhibitor interactions. *In vitro* metabolism of felodipine by CYP3A4-expressed human liver microsomes (HLM) was optimized yielding a typical Michaelis-Menten plot through the application of Enzyme Kinetic Module software from where the enzyme kinetic parameters were determined. Phytochemical components of grapefruit juice, a well known CYP3A4 inhibitor, were separately incubated in HLM together with felodipine at concentration equivalent to the determined Michaelis-Menten Constant (*Km*) value. All of quercetin, naringin and naringenin inhibited felodipine metabolism with IC_{50} values of 208.65, 177.81 and 121.97µM respectively. Following a detailed study of the quantitative structure-activity relationship of these flavonoids, their binding properties with CYP3A4, the amino acid sequence and binding affinity of CYP3A4, computational modeling software on a non-silicon graphic system was employed to generate pharmaceutical grade and commercially available polymers based on activity prediction aided by computational biomimetism and simulations. The modeled compounds including 8-arm-poly(ethylene glycol), o-(2-aminoethyl)-o-methyl poly(ethylene glycol), 4-arm-poly(ethylene glycol) (M_w=10000g/mol and 20000g/mol) and poly (L-lysine) were investigated for inhibitory activity against felodipine metabolism by HLM and human intestinal microsomes (HIM) where 8-arm-poly(ethylene glycol) demonstrated the highest inhibitory potency with an IC_{50} value of 7.22µM. *In vivo* studies of the effects of 8-arm-poly(ethylene glycol) on the oral bioavailability of felodipine were performed on the Large White pig model. Compared to controls, a >100% increase in plasma felodipine levels was observed. The outcome of this research presents 8-arm-poly(ethelyne glycol) as a promising oral bioavailability enhancer.

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APPENDIX D: ETHICS APPROVAL

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04 November 2010

MAILED

Mr P Fasinu
Department of Pharmacology
7th Floor Clinical Building
Room 7070

Dear Mr Fasinu

"In Vitro Assessment of Some Traditional Medications Used in South Africa for Pharmacokinetic Drug Interaction Potential."

ETHICS REFERENCE NO: N10/09/307

RE : APPROVAL

At a meeting of the Health Research Ethics Committee that was held on 20 October 2010, the above project was approved on condition that further information is submitted.

This information was supplied and the project was finally approved on 3 November 2010 for a period of one year from this date. This project is therefore now registered and you can proceed with the work.

Please quote the above-mentioned project number in ALL future correspondence.

Please note that a progress report (obtainable on the website of our Division: www.sun.ac.za/rds 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 and subjected to an external audit.
Translations of the consent document in the languages 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).

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 Hélène 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.

Approval Date: 3 November 2010 Expiry Date: 3 November 2011

04 November 2010 13:01 Page 1 of 2

Verbind tot Optimale Gesondheid · Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun · Division of Research Development and Support
Posbus/PO Box 19063 · Tygerberg 7505 · Suid-Afrika/South Africa
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Yours faithfully

MS CARLI SAGER
RESEARCH DEVELOPMENT AND SUPPORT
Tel: +27 21 938 9140 / E-mail: carlis@sun.ac.za
Fax: +27 21 931 3352

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15 March 2012 **MAILED**

Mr P Fasinu
Department of Pharmacology
7th Floor Clinical Building
Room 7070

Dear Mr Fasinu

"In Vitro Assessment of Some Traditional Medications Used in South Africa for Pharmacokinetic Drug Interaction Potential."

ETHICS REFERENCE NO: N10/09/307

RE : PROGRESS REPORT

At a meeting of the Health Research Ethics Committee that was held on 14 March 2012, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 14 March 2012 Expiry Date: 14 March 2013

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352

15 March 2012 15:35 Page 1 of 1

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19 March 2012 **MAILED**

Mr P Fasinu
Department of Pharmacology
7th Floor Clinical Building
Room 7070

Dear Mr Fasinu

"In Vitro Assessment of Some Traditional Medications Used in South Africa for Pharmacokinetic Drug Interaction Potential."

ETHICS REFERENCE NO: N10/09/307

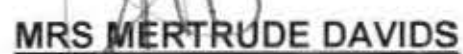
RE : AMENDMENT

Your letter dated 24 February 2012 refers.

The Chairperson of the Health Research Ethics Committee approved the amended documentation in accordance with the authority given to him by the Committee.

The following amendments were approved:
1. The amendments as stipulated in your letter dated 12 March 2012.
Please note STIPULATION: Explain in lay terms the concept 'human metabolic enzymes' in the informed consent form.

Yours faithfully



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APPENDIX E: PLANT MATERIALS TRANSFER AGREEMENT

Agreement for Transfer of Plant Material

Stellenbosch University through its Division of Pharmacology, , Tygerberg Campus

and

____

_____

PROVIDER:

Organization:

Address:

RECIPIENT:

Organization: Stellenbosch Unversity

Address: Administration B, B3223, Victoria Street, Stellenbosch, 7602

In response to the RECIPIENT's request for the PLANT MATERIAL identified as
plant and herbal products

---

the PROVIDER and the RECIPIENT agree to the following before the RECIPIENT receives the PLANT MATERIAL:

1. The PLANT MATERIAL is the property of PROVIDER's institution and is made available only as a service to the research community. The RECIPIENT shall not represent to any person that it is the owner of the PLANT MATERIAL.

2. The PLANT MATERIAL will be used for non-commercial research purposes only. The RECIPIENT will not use the PLANT MATERIAL except pursuant to this Agreement.

3. The RECIPIENT agrees to maintain in confidence PROVIDER’S PLANT MATERIAL with the same degree of care it holds its own confidential and proprietary information, but not less than a reasonable degree of care.

4. The PLANT MATERIAL will not be further distributed to others without the PROVIDER's written consent. The RECIPIENT will either return or destroy PROVIDER’S PLANT MATERIAL upon termination of this Agreement.

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5. The RECIPIENT agrees to acknowledge the source of the PLANT MATERIAL in any publications reporting use of it. Notwithstanding anything else in this agreement, the RECIPIENT may publish the results of the research individually, or in joint collaboration with the PROVIDER.

6. The PROVIDER retains ownership of the PLANT MATERIAL, including any PLANT MATERIAL contained or incorporated in substances created by RECIPIENT (hereinafter, “MODIFICATIONS”). The RECIPIENT retains ownership of: (a) MODIFICATIONS (except that, the PROVIDER retains ownership rights to the PLANT MATERIAL included therein), and (b) those substances created through the use of the PLANT MATERIAL or MODIFICATIONS, but which are not unmodified descendant(s) from the PLANT MATERIAL (hereinafter, “PROGENY”), or substances created by RECIPIENT that constitute an unmodified functional sub-unit or an expression product of the PLANT MATERIAL (hereinafter, “UNMODIFIED DERIVATIVES”). If either 6 (a) or 6 (b) result from the collaborative efforts of the PROVIDER and the RECIPIENT, joint ownership may be negotiated.

7. Any PLANT MATERIAL delivered pursuant to this Agreement is understood to be experimental in nature and may have hazardous properties. The PROVIDER MAKES NO REPRESENTATIONS AND EXTENDS NO WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED. THERE ARE NO EXPRESS OR IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, OR THAT THE USE OF THE PLANT MATERIAL WILL NOT INFRINGE ANY PATENT, COPYRIGHT, TRADEMARK, OR OTHER PROPRIETARY RIGHTS. The RECIPIENT agrees to be responsible for all claims and damages that directly result from the negligent acts or omissions of the RECIPIENT, its employees, or agents, to the extent permitted by law. The PROVIDER will not be liable to the RECIPIENT for any loss, claim or demand made by the RECIPIENT, or made against the RECIPIENT by any other party, due to or arising from the use of the PLANT MATERIAL by the RECIPIENT, except to the extent permitted by law when caused by the gross negligence or willful misconduct of the PROVIDER.

8. The RECIPIENT acknowledges that the PLANT MATERIAL is or may be the subject of a patent application. Except as provided in this Agreement, no express or implied licenses or other rights are provided to the RECIPIENT under any patents, patent applications, trade secrets or other proprietary rights of the PROVIDER, including any altered forms of the PLANT MATERIAL made by the PROVIDER. In particular, no express or implied licenses or other rights are provided to use the PLANT MATERIAL, MODIFICATIONS, or any related patents of the PROVIDER for commercial purposes. If RECIPIENT desires to use the PLANT MATERIAL or MODIFICATIONS for a commercial purpose, RECIPIENT agrees, in advance of such use, to negotiate in good faith with PROVIDER to establish the terms of a commercial license.

9. The RECIPIENT agrees to use the PLANT MATERIAL in compliance with all applicable statutes and regulations, including, for example, those relating to research involving the use of human and animal subjects or recombinant DNA.

10. This Agreement shall expire three (3) years from the last signature date indicated below, unless terminated by either party upon sixty (60) days prior written notice, or unless extended by mutual written agreement.

If the terms and conditions set forth above are acceptable, please sign and return two copies to:

Upon execution of both copies of this document by all parties, the BIOLOGICAL MATERIAL will be forwarded to the RECIPIENT SCIENTIST.

PROVIDER:

Organization:

Address:

Name:

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Title:

Signature: ____

Date: ____

WITNESS OF PROVIDER:

Organization:

Address:

Name:

Title:

Signature: ____

Date: ____

RECIPIENT:

Organization:

Address:

Name:

Title:

Signature: ____

Date: ____

WITNESS OF RECIPIENT:

Organization:

Address:

Name:

Title:

Signature: ____

Date: ____

RECIPIENT SCIENTIST:

Organization:

Address:

Name:

Title:

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Signature: ____

Date: ____

WITNESS OF RECIPIENT:

Organization:

Address:

Name:

Title:

Signature: ____

Date: ____

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APPENDIX F: PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: In vitro Assessment of Some Traditional Medications Used in South Africa for Pharmacokinetic Drug Interaction Potential

REFERENCE NUMBER: N10/09/307

PRINCIPAL INVESTIGATOR: Mr Pius Fasinu

ADDRESS: Division of Pharmacology, 7th Floor, Clinical Building, Faculty of Health Sciences, University of Stellenbosch, Tygerberg 19063

CONTACT NUMBER: 021 938 9336

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

This study is designed to assess the effects of traditional herbal medicine on human metabolic enzymes. This type of laboratory-based study is generally used to investigate interaction between traditional and orthodox medicine. It is applicable to infer on the safety or otherwise, of the concurrent use of traditional and orthodox medicines. It involves laboratory tests between the traditional and orthodox medicines to find out if the metabolism of one is affected by the presence of the other.

The study will be conducted in the laboratories in the Division of Pharmacology, University of Stellenbosch. The researchers will like to source for certain medicinal herbs and document the traditional use of these samples, to be used in the study. This is why people with knowledge in traditional health practice will be needed to voluntarily help to provide information on the use of these medicinal herbs.

At least two traditional health practitioners will be needed to participate in this study. The study does not involve the administration of any medication or enquiries on the medication/health records of the participants.

Why have you been invited to participate?

You have been invited to participate in this study because you are identified as a traditional health practitioner with knowledge on the use of medicinal herbs.

What will your responsibilities be?

If you volunteer to participate in this study, we would ask you to do the following things:

We will ask you to assist us with providing any or all of the following herbal plants (a – o) and ask you

1. What medicinal use do they serve in your practice?
2. How are they used?
3. In case of publications or public presentations arising from this study, will you like to be acknowledged in person?

a. *Hypoxis hemerocallidea* (African Potato (Eng) Afrika patat (Afrk), Inkomfe, ilabatheka, sterblom, lotsane, molikharatsa)
b. *Spirostachys africana Sond.* (Mthombothi)
c. *Bowiea volubilis Harv. Ex Hook. F* (Umagaqana)
d. *Zantedeschia aethiopica L. Spreng.* (Inyibiba)
e. *Chenopodium album L.* (Imbikicane embomvu)
f. *Kedrostis Africana L. Cogn.* (uTuvana)
g. *Alepidea amatymbica Eckl & Zeyh.* (Iqwili)
h. *Lessertia frutescens* (Cancer bush, Umnwele)
i. *Acacia karroo* (Sweet thorn, UmNga)
j. *Emex australis* (Inkunzane)
k. *Pachycarpus concolor E. May.* (Itshongwe)
l. *Ranunculus multifidus Forssk* (Igangashane)
m. *Capparis sepiaria L. var citrifolia (Lam) Toelken* (Isihlo esimbovu)
n. *Pentanisia prunelloides* (Isicimamlilo, Itshamlilo)
o. *Tulbaghia violacea (Harv)* (Wild garlic, wildenoflok, wilde knoffel (Afr)

Will you benefit from taking part in this research?

There will be no direct and immediate benefit of this study to the participants. However, in the long term, this study will benefit the participants and the general society. The findings from this study will contribute to the available knowledge on the safety of

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concomitant use of traditional and orthodox medicine. It will help the practice of the traditional health practitioner as in the future, such information will translate to applicable advice to patients if the administration of traditional medicines should be spaced from orthodox medicine for risk of interaction.

The presentations of the results of this study either in conferences or as published academic articles will acknowledge the participants' contribution. The identities of the participants will be disclosed only if they expressly permit so.

Are there any risks involved in your taking part in this research?

There is no direct risk or harm in participating in this study. However, participation may look like divulging professional and indigenous information to non-practitioner of traditional healing. Information should be voluntary and if you feel uncomfortable in disclosing the indication of any of the herbs, you are free not to do so. Any information provided will be used for research purposes only. There will be no commercial application of the information so provided whatsoever.

If you do not agree to take part, what alternatives do you have?

Your participation in this study is absolutely voluntary. You therefore have the right to decline participating in this study with no consequence whatsoever.

Who will have access to your medical records?

The participants will not be asked any personal questions relating to their medical records. The researchers will not have any access to the medical records of the participants.

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission or as required by law. Confidentiality will be maintained by means of describing participants with letter codes 'a', 'b', 'c' etc while the true identities of the participants will be known to the investigator only. The data generated from individual participants will be kept in the Division of Pharmacology, safeguarded from public.

Published results will acknowledge participants as a whole. The identities of participants will be disclosed in acknowledgment sections of such publications only if they expressly permit.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

No possible injury is anticipated in this study. There will not be personal and invasive questions to be asked the participants.

Will you be paid to take part in this study and are there any costs involved?

The participants will be compensated for participating in this study. The investigator understands that traditional health practitioners charge their clients who consult them. R10 will be paid for each of the samples provided and a compensation for the consultation and information will be paid for at a flat rate of R100.

Is there any thing else that you should know or do?

You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study staff.

You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……………………………….………… agree to take part in a research study entitled ‘In vitro Assessment of Some Traditional Medications Used in South Africa for Pharmacokinetic Drug Interaction Potential’.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at......................…........…………….. on …………...……….. 20….

....	....
Signature of participant	**Signature of witness**

Declaration by investigator

I …………………………………………….……… declare that:

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- I explained the information in this document to ………………………..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at......................….......…………….. on …………...……….. 20...

....	....
Signature of investigator	**Signature of witness**

Declaration by interpreter

I ……………………………………………..……… declare that:

- I assisted the investigator (*name*) ………………………………………. to

  explain the information in this document to (*name of participant*)

  ……………..…………………………….. using the language medium of

  Afrikaans/Xhosa.

- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at......................….......…………….. on …………...…………… 20…..

....	....
Signature of interpreter	**Signature of witness**

APPENDIX G: SOME OF THE MEDICINAL HERBS USED IN THIS STUDY

Hypoxis hemerocallidea in its natural habitat

Lessertia frutescens germinating in a nursery

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Tulbaghia violacea in a natural habitat

A wild tree of *Acacia karoo*

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Botanical parts of a) *Hypoxis hemerocallidea* (corm) and b) *Tulbaghia violacea* (bulb) used in traditional medical practice

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APPENDIX H: HEPATOCYTES CHARACTERISTICS

SEP-05-2007 02:43 P.001/001

Celsis | IN VITRO **Certificate of Analysis**

Product Number: FX008001
Product: LiverPool™ Cryopreserved Hepatocytes (10 donor) Human, Female pool
Quantity: 5 million
Lot Number: TXB
storage condition: below -150° C

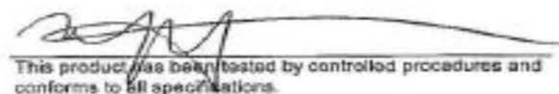
Relevant Donor Demographics, **as reported to In Vitro Technologies

Age/Gender/Race	Medical history: COD	Medications	Serology	Social
66/F/C	Head injury		CMV+	EtOH, Tobacco
55/F/C	CVA	Prevacid, Albuterol, Magnes, Flovent, Trazodone, Doxazosin, Prednisolone, Percocet, Lipitor, Vitamins	CMV+	EtOH
62/F/C	Lupus:ICH			EtOH, Tobacco
39/F/C	Asthma: Anoxia	Asthma Meds		EtOH, Tobacco, Marijuana
56/F/C	Stroke, Skin Cancer:CVA		CMV+	Marijuana
80/F/C	Transient Ischemic Attack, HTN, CVA, GERD, Ulcer: CVA	Lipitor, Atacel, Plavix, Pepcid		Tobacco
62/F/C	High Blood Pressure, Stroke and CEA, Poor Circulation in Leg, Leg Amputation, Stent: CVA	Lipitor, Insulin, Plavix	CMV+	
57/F/C	HTN, A-Fib, Mitro Valve Prolapse: CVA	Nadol, Benecar, Lipitor, Warfin		Tobacco
48/F/C	Depression, MS: Head Trauma	Depression Meds, Effexor, MS Meds	HBsAb +, CMV+	
74/F/B	Angina, HTN, CAD, A-Fib, MI w/ Pacemaker:CVA		CMV+	Tobacco

1) ≥ 5 million cells with at least 70% post-thaw viability as determined by Trypan blue exclusion: 80%

Lot Characterization Results

COUM: total rate of formation of 7-hydroxycoumarin	79	pmole/10^6 cells/min
DEX: rate of formation of dextrorphan	26	pmole/10^6 cells/min
ECOD: Total rate of formation of 7-hydroxycoumarin	46	pmole/10^6 cells/min
7-HCG: rate of formation of 7-hydroxycoumarin glucuronide	319	pmole/10^6 cells/min
7-HCS: rate of formation of 7-hydroxycoumarin sulfate	31	pmole/10^6 cells/min
MEPH: rate of formation of 4'-hydroxymephenytoin	5	pmole/10^6 cells/min
TEST: rate of formation of 6ß-hydroxytestosterone	50	pmole/10^6 cells/min
TOLB: rate of formation of 4'-methylhydroxytolbutamide	38	pmole/10^6 cells/min
PHEN: rate of formation of acetaminophen	31	pmole/10^6 cells/min
CZX: rate of formation of 6-hydroxychlorzoxazone	38	pmole/10^6 cells/min



This product has been tested by controlled procedures and conforms to all specifications.

TOTAL P.001

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Celsis | In Vitro Technologies

LiverPool™* 10-DONOR MALE POOLED CRYOPRESERVED HUMAN HEPATOCYTES
PRODUCT NUMBER: MX008001

Lot Number: GNK

Storage Conditions: below -150°C (vapour phase of liquid nitrogen freezer)

Test Results:

Specification	Result
>70% post-thaw viability by trypan blue exclusion	84%
≥ 5 million viable cells	9.15

Lot Characterization Results:

Assay	Result	
ECOD: total rate of formation of 7-HC and metabolites	77.1	pmol/10^6 cells/min
UGT/ST:		
rate of formation of 7-hydroxycoumarin glucuronide	377	pmol/10^6 cells/min
rate of formation of 7-hydroxycoumarin sulfate	31.1	pmol/10^6 cells/min
CYP1A2: rate of formation of acetaminophen	22.3	pmol/10^6 cells/min
CYP2A6: total rate of formation of 7-HC and metabolites	50.1	pmol/10^6 cells/min
CYP2C9: rate of formation of 4'-methylhydroxytolbutamide	39.6	pmol/10^6 cells/min
CYP2C19: rate of formation of 4'-hydroxymephenytoin	16.5	pmol/10^6 cells/min
CYP2D6: rate of formation of dextrorphan	28.3	pmol/10^6 cells/min
CYP2E1: rate of formation of 6-hydroxychlorzoxazone	53.8	pmol/10^6 cells/min
CYP3A4: rate of formation of 6β-hydroxytestosterone	237	pmol/10^6 cells/min

*The process for producing the LiverPool™ pooled human hepatocyte products is covered by one or more U.S. or foreign patents and patent applications, including U.S. Patent No. 7,604,929.

Donor Demographics, as reported to Celsis | In Vitro Technologies:

Age:	Race:	Cause of death:	Height:	Weight:	Social history:	Medical history:	Serology testing: EBV	RPR	CMV	Hepatitis B	Hepatitis C	HIV
17	H	Head trauma; 2nd to GSW to head	65"	58 Kg	No ETOH use; Tobacco: 1 pk per wk	Exposed to TB and got treatment, but did not have TB	Pos	Neg	Neg	Neg	Neg	Neg
28	C	Head Trauma; 2nd to MVA	70"	134Kg	ETOH: 6 pack per day x 15 yrs; tobacco: 1ppd x 15 yrs; marijuana 1 time in past 5 yrs	Asthma as a child; mild chorditis	Pos	Neg	Neg	Neg	Neg	Neg
40	C	Resp Distress; 2nd to Cardiac Infarct	82"	100Kg	No ETOH, tobacco or drug use	History of sleep apnea and bowel obstruction	Not reported	Neg	Neg	Neg	Neg	Neg

Caution: This product was prepared from fresh human tissue. Treat all products containing human-derived materials as potentially infectious, as no known test methods can offer assurance that products derived from human tissues will not transmit infectious agents.

These products are for research use only. Do not use in animals or humans. These products have not been approved for any diagnostic or clinical procedures.

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52	C	CVA	67"	77 Kg	ETOH: 3 beers daily; No tobacco or drug use	Knee sx, toe broken, hepatitis noted on visual but non-infectious (inflamation related)	Not reported	Neg	Neg	Neg	Neg	Neg
71	C	Stroke	73"	106Kg	No ETOH; Tobacco: 1/2 ppd x 50 yrs on and off	Bladder cancer - removed, HTN x 10yrs -non-compliant, non-cancerous colon polyps removed, pneumonia Meds: Prednisone and Doxycycline for pneumonia	Not reported	Neg	Pos	Neg	Neg	Neg
19	C	Anoxia	53"	134 lb	No ETOH, Tobacco or drug use.	Hx of spinal bifida, replaced J-Tube, HTN x 5 yrs, blood transfusion in past, used ventilator, trach placed, pneumonia previously. Meds: Calcium carbonate, latolose, zantac, tegretol, depakene, lasix, calcitriol, Atrovent, atarax, leveothyrom, singulair, clonidine, albuterol, pulmicort, xopenex, fosamax, prilosec, furadanton, tobi.	Pos	Neg	Neg	HBsAb +	Neg	Neg
80	C	CVA	69"	134 lb	Tobacco: Yes, No ETOH or drug use.	Diabetes - type II-IDDM, Stroke, Femoropopliteal bypass. Meds: Coumadin.	Pos	Neg	Pos	Neg	Neg	Neg
51	C	Stroke (MCA)	67"	89 Kg	ETOH: daily x 20yrs, Tobacco: 1-2 ppd x 30 yrs, marijuana daily x 20 yrs	Spinal elusion, high cholesterol	Not reported	Neg	Neg	HBsAb +	Neg	Neg

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83	C	CVA	70"	236 lb	No ETOH or drug use; Tobacco - unknown amt	IDDM, CVA w/shunt, COPD, HTN, AAA repair, high cholesterol. Meds: diabetes meds, cardiac meds	Pos	Neg	Pos	Neg	Neg	Neg
46	B	Anoxia; 2nd to house fire	6'	68 Kg	ETOH: Cronic panceatitis; Tobacco: 2 ppd x 26yrs; Drugs: Cocaine in past	HTN, Asthma, Pancreatitis, Surgery on abdominal pancreatic problems, 21% burns on body. Meds: Asthma - meds unknown compliance	Not reported	Neg	Pos	Neg	Neg	Neg

Carolyn P. Watt

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