Investigation of the pharmacokinetic interactions between *Hypoxis hemerocallidea* and indinavir

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ABSTRACT

It has been established that phytochemicals in herbal medicines can result in pharmacokinetic interactions when co-administrated with allopathic drugs. These herb-drug pharmacokinetic interactions may lead to an increase or decrease in drug bioavailability with consequences of adverse effects or toxicity due to increased drug plasma levels or treatment failure due to decreased plasma drug levels. The relevance of research concerning pharmacokinetic interactions between herbal medicines and anti-retroviral drugs is reflected by the fact that a relatively large portion of human immunodeficiency virus (HIV) infected patients with acquired immunodeficiency syndrome (AIDS) commonly use herbal medicines to complement their highly active anti-retroviral therapy. The aim of this study was to confirm pharmacokinetic interactions that different *Hypoxis hemerocallidea* (African potato) materials may have on indinavir by means of *in vitro* transport studies as well as by means of acute and chronic *in vivo* bioavailability studies.

The selected *H. hemerocallidea* test materials included a dried plant reference material, an aqueous extract and a solid oral commercial product. Bi-directional transport of indinavir across Caco-2 cell monolayers were determined in the absence and presence of the various *H. hemerocallidea* materials. Indinavir alone served as the negative control and the positive control consisted of verapamil, a known P-glycoprotein (P-gp) inhibitor. The transport samples obtained were analysed by a validated high performance liquid chromatography (HPLC) method. The apparent permeability coefficient (P_{app}) values in both transport directions and efflux ratio (ER) values were calculated.

In vivo studies were conducted in male Sprague-Dawley rats which were randomly selected and divided into 9 groups. The negative control consisted of indinavir alone. Verapamil served as the positive control for efflux inhibition and ketoconazole, a known CYP3A4 inhibitor, was used as positive control for metabolism inhibition. The *H. hemerocallidea* materials in combination with indinavir formed the experimental groups. The acute study consisted of a single administration and the chronic study entailed daily administrations over 14 days by means of oral gavage. A validated Liquid Chromatography tandem Mass Spectrometry (LC/MS/MS) method for the analysis of indinavir was used in order to analyse the plasma samples. Relevant pharmacokinetic parameters such as peak plasma concentration (C_{max}) and area under the curve (AUC) were determined

The *H. hemerocallidea* test materials demonstrated an inhibition of efflux of indinavir in the Caco-2 cell model. In agreement with this finding and other published findings on metabolism inhibition, an increase in indinavir bioavailability in the presence of the selected test materials

was shown *in vivo* for both the acute and chronic studies. The commercial product had a similar increasing effect on indinavir bioavailability as the aqueous extract, while the reference plant material exhibited a higher effect on indinavir bioavailability enhancement. A higher effect on indinavir bioavailability was observed for two of the test materials during the chronic study when compared to the acute study.

The selected *H.hemerocallidea* materials interfered with indinavir pharmacokinetics in both the *in vitro* and *in vivo* models and these effects may be attributed to inhibition of efflux transporters and enzymatic metabolism.

Key words: Herb-drug pharmacokinetic interactions, HIV, *Hypoxis hemerocallidea,* indinavir, Caco-2, efflux, Sprague-Dawley

UITTREKSEL

Daar is vasgestel dat fitochemikalieë in kruiemedisyne kan lei tot farmakokinetiese interaksies wanneer dit saam met allopatiese medisyne geadministreer word. Hierdie plant-geneesmiddel farmakokinetiese interaksie kan lei tot 'n toename of afname in die geneesmiddel se biobeskikbaarheid. Dit kan lei tot negatiewe gevolge of toksisiteit as gevolg van verhoogde geneesmiddelplasmavlakke of mislukte behandeling as gevolg van verminderde geneesmiddelplasmavlakke. Die noodsaaklikheid van navorsing met betrekking tot farmakokinetiese interaksies tussen kruiemedisyne en anti-retrovirale middels word weerspieël deur die feit dat 'n relatiewe groot deel van pasiënte wat besmet is met menslike immuniteitsgebreksvirus (MIV) met verworwe immuniteitsgebreksindroom (VIGS), gebruik kruiemedisyne algemeen saam met hulle hoogs aktiewe anti-retrovirale terapie. Die doel van hierdie studie is om die farmakokineties interaksies te bevestig wat verskillende *Hypoxis hemerocallidea* (Afrika-aartappel) materiale op indinavir mag hê deur middel van *in vitro* transport studies sowel as deur middel van akute en chroniese *in vivo* biobeskikbaarheid studies.

Die gekose *H. hemerocallidea* toetsmateriale bestaan uit 'n droë verwysingsplantmateriaal, 'n water-ekstrak en 'n solide orale kommersiële produk. Tweerigting beweging van indinavir oor Caco-2 selmonolae is bepaal in die afwesigheid en teenwoordigheid van die verskillende *H. hemerocallidea* materiale. Indinavir alleen dien as die negatiewe kontrole, terwyl die positiewe kontrole bestaan uit verapamil, wat 'n bekende P-glikoproteïen (P-gp) inhibeerder is. Die ingesamelde monsters is geanaliseer deur middel van ‘n gevaludeerde hoëdruk vloeistofchromatografiese (HDVC) metode. Die oënskynlike deurlaatbaarheidskoëffisiënt (P_{app}) waardes in beide vervoer rigtings en effluksverhouding (EV) waardes is bereken.

Die *in vivo* studies is in manlike Sprague-Dawley rotte uitgevoer wat lukraak gekies is en in 9 groepe verdeel is. Die negatiewe kontrole bestaan uit indinavir alleen. Verapamil dien as die positiewe kontrole vir efluks inhibisie en ketokonasool, 'n bekende CYP3A4 inhibeerder, is gebruik as die positiewe kontrole vir metaboliese inhibisie. Die *H. hemerocallidea* materiale in kombinasie met indinavir vorm die eksperimentele groepe. Die akute studie het bestaan uit 'n enkele toediening en die chroniese studie het daaglikse toedienings behels oor 14 dae by wyse van mondelinge intubering. ‘n Gevaludeerde vloeistofchromatografie gekoppel aan ‘n massaspektrometer (VC/MS/MS) metode vir die analise van indinavir is gebruik om die plasma monsters te ontleed. Relevante farmakokinetiese parameters soos piek plasmakonsentrasie (C_{max}) en area onder die kurwe (AOK) is vasgestel.

Die *H. hemerocallidea* toetsmateriale het efluks inhibisie van indinavir in die Caco-2 sel model gedemonstreer. In ooreenstemming met hierdie bevinding en ander gepubliseerde bevindinge op metaboliese inhibisie, het 'n toename in indinavir biobeskikbaarheid in die teenwoordigheid van die gekose toetsmateriale *in vivo* voorgekom vir beide die akute en chroniese studies. Die kommersiële produk het 'n soortgelyke toenemende effek op indinavir biobeskikbaarheid as die water-ekstrak gehad, terwyl die verwysing plantmateriaal 'n hoër uitwerking op indinavir biobeskikbaarheid verbetering getoon het. 'n Hoër uitwerking op indinavir biobeskikbaarheid was waargeneem vir twee van die toetsmateriale tydens die chroniese studie in vergelyking met die akute studie.

Die gekose *H. hemerocallidea* materiale het met die farmakokinetika van indinavir ingemeng in beide die *in vitro* en *in vivo* modelle en hierdie effekte kan aan inhibisie van efluks transporters en ensiematiese metabolisme toegeskryf word.

Sleutel woorde: Kruie-geneesmiddel farmakokinetiese interaksies, MIV, *Hypoxis hemerocallidea*, indinavir, Caco-2, efluks, Sprague-Dawley

CONGRESS PROCEEDINGS & ARTICLES

1.1 Congress proceedings

Pharmacokinetic interactions (*in vitro* and *in vivo*) between indinavir and *Hypoxis hemerocallidea:* comparing a commercial product with a crude extract and a dried plant material. Presented at the 37th Conference of the Academy of Pharmaceutical Sciences held from 5-8 October 2016 at Misty Hills Hotel and Conference Centre in Muldersdrift, Gauteng. The Congress was hosted by the Department of Pharmaceutical Sciences from the Tshwane University of Technology (on behalf of the Academy of Pharmaceutical Sciences South Africa) and the Department of Pharmacology and Therapeutics at the Sefako Makgatho Health Sciences University (on behalf of the South African Society for Basic and Clinical Pharmacology). (See Appendix A)

1.2 Articles

Havenga, K., Abay, E., Wiesner, L., Viljoen, A., Steyn, D., Hamman, J. 2016. Effect of *Hypoxis hemerocallidea* on indinavir pharmacokinetics (*in vitro* and *in vivo*): comparing a commercial product with plant extract and reference material. In process of submission to The Journal of Ethnopharmacology.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

1.1.1 Herb-drug pharmacokinetic interactions

The use of herbal products as an alternative source of medicinal treatment has become more popular. More than 80% of the population in some developing countries, especially in Africa, use herbal medicines as part of their primary health care (Brijlal *et al*, 2011). A misinterpretation of safety regarding herbal treatment use is evident amongst the general public. Besides potential toxic effects, herbal medicines can cause alterations in drug transport and metabolism due to modulation of Cytochrome P450 (CYP) enzymes and efflux transporters such as P-glycoprotein (P-gp), thereby causing herb-drug pharmacokinetic interactions. These pharmacokinetic interactions involve changes in absorption, distribution, metabolism or elimination of the drug compound, which may result in an increase or decrease in drug plasma concentration (Cordier & Steenkamp, 2011; Lam & Ernst, 2006).

CYP enzymes are important metabolisers of xenobiotics, herbal and endogenous compounds as well as drugs. CYP iso-enzyme 3A4 (CYP3A4) is predominantly found in the liver and small intestinal epithelium and is responsible for about 30% (in the liver) and 70% (in the intestinal epithelium) of the total CYP450 activity. First-pass metabolism plays an important role in the poor and inconstant bioavailability of certain drugs, especially those that are CYP3A4 substrates (Li *et al*., 2002).

Drug absorption is influenced by the induction or inhibition of active transporters. Some drugs are pumped out of the epithelial cells back into the lumen of the gastrointestinal tract by means of active transporters, which is referred to as efflux. Efflux transporters such as P-gp are located on epithelial cell membranes. P-gp is susceptible to modulation, which includes activation, induction or inhibition by chemicals such as herbal medicines (van den Bout-van den Beukel *et al*., 2006). Reports have shown that P-gp and CYP3A4 have the possibility to share substrates and through interplay cause an enhanced effect on drug pharmacokinetics (Hellum *et al*., 2007). Intestinal P-gp efflux demonstrates a reduction in the bioavailability of numerous CYP3A4 substrates (Benet *et al*., 2004).

Some herbal products showed the ability to modulate CYP metabolizing enzymes, either through inhibition or induction, which may modify the bioavailability of drugs as a result. An

increase in plasma drug concentration levels is associated with inhibition of metabolic enzymes and/or efflux drug transporter activity. On the other hand, an increase in enzyme expression and/or efflux drug transporter activity reduces the drug plasma concentration (Liu *et al*., 2011).

The use of antiretroviral drugs together with herbal medicines may produce substantial interactions. Interactions with non-nucleoside reverse transcriptase inhibitors and protease inhibitors can clinically result in adverse effects (Mills *et al*., 2005). *In vitro* studies have suggested that *Echinacea purpurea* extracts can inhibit CYP3A4 and concurrent use with alprazolam, protease inhibitors and calcium channel blockers could cause an increase in plasma drug concentration levels (Scott & Elmer, 2002). Clinical studies have shown that Grapefruit (*Citrus paradisi*) juice is capable of inhibiting CYP3A4 and has reduced plasma concentrations of indinavir by 15%-30%, while an increase in saquinavir concentration was observed (Hernández *et al*., 2009). Chronic intakes of garlic supplements have shown a decrease in the plasma drug concentration of saquinavir (Piscitelli *et al*., 2002). *In vitro* studies showed that *Sutherlandia frutescens* extract inhibited CYP3A4 activity by 70-96% and *Hypoxis hemerocallidea* extract inhibited CYP3A4 activity by 31-79%. Both herbal medicines showed a significant activation of pregnane X receptor (PXR) which modulates expressions of both P-gp and CYP3A4. The concurrent use of these herbs with anti-retroviral drugs may result in adverse effects as a result of inhibition of drug metabolism and transport (Mills *et al*., 2005).

1.1.2 African potato (*Hypoxis hemerocallidea*)

Hypoxis hemerocallidea (also known as *H. rooperi*) is a popular traditional medicinal plant that is broadly distributed in southern Africa. It is characterized by its star-shaped flowers which are bright yellow in colour and its strap like leaves (Van Wyk *et al*., 2002). The plant has a potato shaped tuberous rootstock (the corm), which is referred to as the 'African potato'. These corms are washed, chopped and then administered orally after boiling. 'African potato' powders, extracts, decoctions and infusions are popular amongst traditional healers. For centuries this plant has been used for the treatment or management of a number of human disorders (Owira & Ojewole, 2009). Many African countries' Ministers of Health formulate policies promoting the usage of African traditional medicines for the control, management and treatment of HIV/AIDS related diseases as well as other chronic conditions (Morris, 2002). It has been used for certain types of cancers, heart failures, nervous disorders, immune-related illnesses and urinary tract infections (Singh, 1999). *H. hemerocallidea* extract which contains phytosterols, hypoxoside and its active form, rooperol, has also been used for the treatment of benign prostate hyperplasia, as an anti-inflammatory agent, anti-oxidant, anti-convulsant and as an anti-diabetic agent. Scientific evidence was found that it is active against cancerous and pre-malignant cancer cells (Drewes *et al*., 2008). The hypoxoside is considered to be the most important

phytochemical with regards to the African potato's medicinal value (Nair *et al*., 2007). Although *in vivo* data is lacking, *in vitro* observations seemed to suggest the possibility of pharmacokinetic interactions between anti-retroviral drugs and African potato. Patients taking African potato extracts concurrently with anti-retroviral drugs may possibly develop adverse effects and/or may lead to viral resistance, treatment failure and drug toxicity (Owira & Ojewole, 2009).

Commercialised oral products containing *H. hemerocallidea* extracts or ground plant material are available. For example, Harzol® was released in 1974 and gained a widespread acceptance in Germany. This product contained ß-sitosterols and its glucoside, which were originally obtained from *H. hemerocallidea*. Harzol® was used to treat benign prostate hypertrophy (Drewes *&* Khan, 2004). Moducare® is a commercially available herbal product that is used as an immune system enhancer and contains sitosterol and its glucoside, sitosterolin, in a ratio of 100:1 (Moducare, 2015). These phytochemical substances were originally isolated and prepared from *H. hemerocallidea*, but at a later stage manufactured synthetically or obtained from other plants (Drewes *&* Khan, 2004).

Another over-the-counter *H. hemerocallidea* product is Hypo-Plus™, which is marketed as an energy booster, immune modulator, food supplement and for improving other conditions such as diabetes, impotency, memory loss, gout, arthritis and HIV/AIDS. It is composed of amino acids, vitamins, an anti-oxidant component, plant sterols and 'variable ratios of *Mopanus vermus* and *Hypoxis*' (Drewes *&* Khan, 2004). Found on the KwaZulu- Natal north coast at Isithebe, the firm Impilo Drugs (Pty) Ltd has developed a factory production for indigenous products. 'Impilo African Potato' or '*ilabatheka*' is one of the top sellers. The product is sold in a capsule or tablet form consisting of ground *H. hemerocallidea* plant material (Drewes *&* Khan, 2004).

1.1.3 Indinavir (Crixivan®)

Crixivan® (indinavir sulfate) is used for the treatment of human immunodeficiency virus (HIV) infection in combination with other anti-retroviral drugs. Indinavir is classified as an HIV protease inhibitor. During viral replication, cleavage of the viral polyproteins is inhibited by indinavir and immature non-infectious viral particles are formed. Hard gelatine capsules for oral administration are available in 200 mg and 400 mg dose strengths. The recommended dosage is 800 mg orally every 8 hours and should be taken without food for optimal absorption. Ideally the drug product should be taken 1 hour before or 2 hours after meals. Dose reduction of Crixivan® is considered with concomitant use of delavirdine, didanosine, itraconazole, ketoconazole and rifabutin. Patients with hepatic insufficiency as a result of cirrhosis should

decrease the dose to 600 mg every 8 hours (Merck & Co inc, 2013). Co-administration with hormonal contraceptives, anti-convulsants (e.g. carbamazepine, phenytoin) and anti-tuberculosis drugs are examples of drugs that produce an interaction with indinavir (Cohen *et al*., 2002).

Indinavir is a substrate for both CYP3A4 and P-gp (Hochman *et al*., 2001) and therefore undergoes metabolism by CYP3A4 and is effluxed by P-gp. Co-administration with drugs/herbs that inhibit CYP3A4 may decrease indinavir clearance, which may lead to an increased indinavir plasma concentration. Grapefruit juice and St. John's Wort have, for example, exhibited pharmacokinetic herb-drug interactions if taken in conjunction with Crixivan® (Merck & Co inc, 2013).

1.1.4 *In vitro, ex vivo* and *in vivo* models

1.1.4.1 *In vitro* cell cultures

Cultured cells are commonly used to study the transport and metabolism of compounds. When cells are cultured as a monolayer, polarized behaviour is exhibited and therefore it represents the situation in the intestine (Tukker, 2000). The use of primary cells have been attempted for the intestinal epithelium, but poor viability was produced and it did not form a confluent monolayer where tight junctions were created (Barthe *et al*., 1999). Most cell culture models are based on immortalized cell lines that have been derived from normal cells, induced tumours or human colonic cancers. Human colonic cancer cell lines are widely used for drug absorption studies as they differentiate and readily form confluent and polarized monolayers. The Caco-2 cell line has originated from colon adenocarcinoma cells and is commonly used in pharmacokinetic studies. This cell line has the advantage of being able to form polarized cell monolayers on a porous membrane and differentiate into absorptive intestinal cells containing the typical enterocyte morphology, which includes some brush border enzyme activity and tight junctions. Caco-2 cells express active transport systems such as amino acid transporters, glucose, small peptide, bile acid and the P-gp efflux system (Tukker, 2000). Studies on Caco-2 cells have resulted in gaining valuable information on drug interactions involving both P-gp and CYP3A (Raessi, 1999). Another cultured cell line is the LS180 cell line, which is also derived from human colon adenocarcinoma. LS180 cells can be used to study induction of drug metabolising enzymes. Unlike Caco-2 cells, LS180 cells express PXR (Hartley *et al*., 2006).

1.1.4.2 *Ex vivo* (excised animal tissues)

Permeability screening for drug discovery purposes are usually conducted on different animals species' tissues as animal intestinal tissues are also made up of basically the same type of endothelial cells as in humans. Excised animal tissue models have been used since the 1950s to study the mechanism of intestinal absorption. However, the viability of the excised tissue is challenging to maintain as the tissues are devoid of direct blood supply and constant oxygenation is needed (Krishna & Yu, 2008).

1.1.4.3 *In vivo*

In vivo models refer to the use of living organisms such as vertebrates (e.g. mice or rats) or primates (e.g. vervet monkeys) or humans in pharmacodynamic and pharmacokinetic studies. During *in vivo* pharmacokinetic studies, a compound is administered extravascularly and its permeation into the systemic blood circulation is measured by means of blood sampling (Hidalgo, 2001).

1.2. RESEARCH PROBLEM

In vitro research has demonstrated that a potential herb-drug pharmacokinetic interaction exists between *H. hemerocallidea* and anti-retroviral drugs. The literature indicates alterations in the transport and metabolism of indinavir upon co-administration with extracts of *H. hemerocallidea*. For example, *H. hemerocallidea* extract has been observed to inhibit CYP3A4 enzyme activity and P-gp related efflux within *in vitro* models (Mills *et al*., 2005).

Research on pharmacokinetic interactions between *H. hemerocallidea* and anti-retroviral drugs has primarily been conducted by means of *in vitro* models using crude plant extracts or isolated phytochemicals. *In vivo* data is lacking in this regard (Cordier & Steenkamp, 2011) and this information is needed in order to determine the clinical significance of the pharmacokinetic interaction of commercially available *H. hemerocallidea* products on commercially available indinavir products (e.g. Crixivan®).

1.3. AIM AND OBJECTIVES

The aim of this study was to identify the pharmacokinetic interactions between *H. hemerocallidea* extracts as well as a commercial product and an indinavir commercial product (e.g. Crixivan®) by means of *in vitro* studies and to determine the significance of these interactions *in vivo.*

The objectives of the study were:

- To validate a high performance liquid chromatographic (HPLC) analysis method for indinavir for the *in vitro* transport study.
- To conduct bi-directional *in vitro* pharmacokinetic studies on indinavir (Crixivan®) in the presence of *H. hemerocallidea* extracts and a product across Caco-2 cell monolayers.
- To conduct *in vivo* pharmacokinetic studies on indinavir (Crixivan®) in Sprague-Dawley rats. The acute effect will be measured after a single dose of each *H. hemerocallidea* extract and a product, while the chronic effect will be measured after pre-treatment with each of the *H. hemerocallidea* extracts and product for 2 weeks.
- To use a validated liquid chromatography linked to mass spectrometer (LC/MS/MS) method for analysis of indinavir and its metabolite (M6) in the plasma of the rats.

1.4 STRUCTURE OF DISSERTATION

This dissertation starts off with an introductory chapter (Chapter 1) that provides motivation and justification for the research study as well as the aim and objectives of the study. A literature overview follows in Chapter 2 that focuses on mechanisms of pharmacokinetic interactions, such as efflux transport modulation and alterations in the metabolism of drugs produced by co-administered drug compounds. The scientific methods performed during the *in vitro* transport study and *in vivo* pharmacokinetic study is described in Chapter 3. The results obtained from these study experiments are demonstrated and discussed in Chapter 4. The last chapter, Chapter 5, draws final conclusions from the results attained in this research study and suggests recommendations for future studies.

CHAPTER 2

LITERATURE REVIEW ON THE PHARMACOKINETIC INTERACTIONS BETWEEN HERBAL MEDICINES AND ANTI-RETROVIRAL DRUGS

2.1 INTRODUCTION

Herbal therapy has been used for thousands of years for a broad range of ailments and its use wasn't unique to one specific civilisation, historical or cultural era (Venkataramanan *et al*., 2006). Although there is insufficient information available regarding the safety of some herbal medicines, their use as alternative or complementary medicinal treatment is popular around the world. About 40% of the American adult population makes use of herbal medicine and an exponential increase in the consumption rate has been observed in Canada, Australia and Europe. Medicinal herb consumption is also relatively high in Africa with 60 to 85% of native Africans estimated to use herbal medicines, typically in combination with prescribed medicines (Fasinu *et al.,* 2012).

The use of complementary and alternative medicine is common amongst HIV-infected patients as surveys have shown that 67% of the patients receiving anti-retroviral drugs (ARV) were also using one or several supplementary natural health products (Gore-Felton *et al.,* 2003). Reasons given for the use of complementary and alternative medicine include an increase in quality of life, perceived efficacy, a decrease in the adverse effects of ARVs and a sense of control experienced by the patients (Lee *et al.,* 2006). A survey conducted in Massachusetts showed that 63% of physicians believed complementary and alternative medicine were helpful to patients infected with HIV (Rivera *et al*., 2005).

Studies have shown a habitual pattern concerning the concurrent use of herbal products together with prescription medication. A reported 14 - 16% of the American adult population use herbal medicines with their prescribed medicines (Kaufman *et al.,* 2002). However, many patients do not disclose their use of herbal medicines to their health care providers, while many physicians are not aware of the possible risks of herb-drug interactions (Fasinu *et al.,* 2012). Furthermore, there is a misconception regarding the safety of herbal medicines due to their "natural" origin. Herbal medicines are regarded as supplements or food products and are therefore normally not exposed to the same strict safety and efficacy trials and pre-marketing approval procedures that prescription drugs require (Tarirai *et al.,* 2010).

2.2 PHARMACOKINETIC HERB-DRUG INTERACTIONS

Co-administration of herbal medicines with Western drugs may result in the reduction or increase of the effects of either compound, which represent herb-drug interactions that may in some cases be of clinical importance (Hu *et al.,* 2005). The severity of the herb-drug interaction ranges from being minor to more serious effects that may result in prolonged morbidity, life threatening consequences and even death (Tarirai *et al*., 2010). Interactions between herbal medicines and drugs may involve pharmacokinetic and/or pharmacodynamic mechanisms. Pharmacokinetic interactions include alterations to absorption, metabolism, distribution or excretion of the affected herb or drug. Pharmacodynamic interactions involve alterations to the pharmacological response of the herb or drug (Lam & Ernst, 2006). The pharmacokinetic and pharmacodynamic processes where potential interactions can occur are schematically illustrated in Figure 2.1.

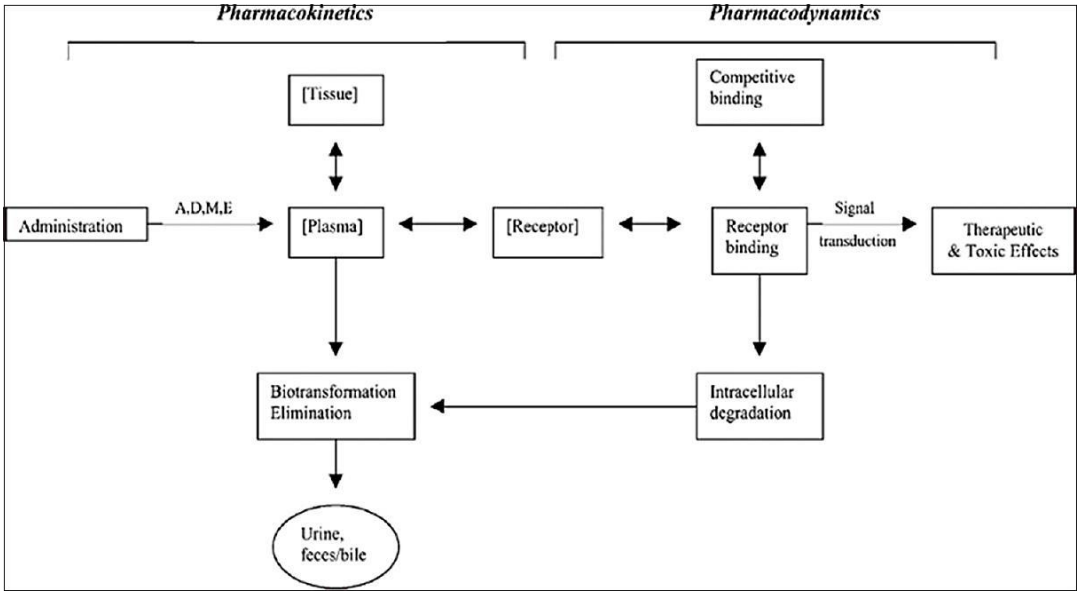

Figure 2.1: Schematic demonstration of the processes of pharmacokinetics and pharmacodynamics that may be involved in herb-drug interactions A: Absorption, D: Distribution, M: Metabolism, E: Elimination (Bounda *&* Feng, 2015)

A pharmacodynamic interaction occurs when the herb synergises, antagonises or enhances the biological activity of the drug (van den Bout-van den Beukel *et al.,* 2006; Fasinu *et al.,* 2012). Not all pharmacodynamic interactions produce an undesirable effect as some interactions may result in a beneficial effect by either increasing the efficacy of the drug or diminishing potential adverse effects (Shi *&* Klotz, 2012).

Clinical pharmacodynamic interactions between herbal medicines and ARV drugs have been observed, for example, some herbal medicines indirectly enhanced ARV therapy by stimulating the immune system (Lee *et al.,* 2006). However, alteration of the pharmacokinetics (i.e. absorption, distribution, metabolism or elimination) of a drug can lead to a situation where the drug plasma concentration are shifted outside of its therapeutic limits, thus causing a potential for sub-therapeutic levels (low activity) or supra-therapeutic levels (toxicity) (Cordier *&* Steenkamp, 2011). The main mechanism underlying pharmacokinetic interactions is either the inhibition or induction of intestinal and hepatic metabolising enzymes (e.g. cytochrome P450 [CYP] enzymes). The effect on drug transporters such as efflux pumps, particularly intestinal p-glycoprotein (P-gp), also plays an important role (Fasinu *et al.,* 2012). Pharmacokinetic interactions can become clinically significant when considerable alterations in pharmacokinetic parameters occur. Examples of these parameters include the area under the plasma concentration-time curve (AUC), the maximum plasma concentration (C_{max}), the time of maximum plasma concentration (t_{max}) and the elimination half-life ($t_{1/2}$) of the drug (Shi *&* Klotz*,* 2012). Severe adverse effects and high risks associated with herb-drug pharmacokinetic interactions usually occur with drugs that exhibit narrow therapeutic indices such as phenytoin, digoxin and warfarin (Tarirai *et al.,* 2010).

2.2.1 Efflux transporters

Drug transporters can be classified into those mediating the uptake of drugs into the cells and those mediating the export of drugs and its metabolites out of the cells. Uptake and efflux transporters are localised in organs such as the liver, small intestine and kidney, which are crucial for drug disposition (especially absorption and elimination). Induction or inhibition of transporters caused by co-administration of herbal medicines can possibly alter pharmacodynamics and pharmacokinetics of the drug of interest (König *et al.,* 2013).

Studies have shown that efflux transporters, that act alone or together with drug metabolising enzymes, may play an important role in oral drug bioavailability. The ATP-binding cassette (ABC) transporters are a group of active transporters that have a significant influence in the absorption, distribution and elimination of some drugs (Fasinu *et al.,* 2012). The ATP-binding cassette transporters mediate the efflux transporters that can actively transport drugs against a steep concentration gradient and are mostly found in the intestinal epithelium, the canaliculi membrane of the kidney, liver cell membranes and the endothelium of blood capillaries in the brain (Hellum *&* Nilsen, 2008: Tarirai *et al*., 2010*).* The efflux transporters limit the influx of xenobiotic compounds into cells and therefore prevent the intracellular accumulation of compounds that are substrates thereof (Chan *et al.,* 2004).

Pharmacokinetic interactions can occur when herbal medicines inhibit or reduce the ordinary activity level of drug transporters through competitive or non-competitive mechanisms (Fasinu *et al.,* 2012). Blood plasma concentrations that are potentially toxic may arise from an inhibited activity of the efflux transporters, while induction of efflux transporter can result in sub-therapeutic plasma drug levels (Tarirai *et al.,* 2010).

2.2.1.1 P-gp

P-gp is the most studied member of the ABC family of transporters and functions as an efflux pump (Shi *&* Klotz*,* 2012). It is also known as multi-drug resistance protein 1 (MDR1) or as ABC subfamily B member 1 (ABCB1). Originally, P-gp was discovered in drug-resistant tumour cells and was only identified later in normal human tissues (Hansten, 2001). P-gp is a 170-kDa plasma glycoprotein consisting of 1 280 amino acids (schematically illustrated in Figure 2.2), which is constitutively expressed in body tissues such as in the apical epithelial surfaces of the liver's bile canaliculi, pancreatic ductal cells, proximal tubules of the kidneys, small intestines columnar mucosal cells, the colon and adrenal glands (Fasinu *et al.,* 2012). P-gp uses ATP as an energy source to actively pump compounds from the epithelial cells back to the intestinal lumen and from the brain's capillary endothelial cells back into the blood (Tarirai *et al.,* 2010*)*.

P-gp plays a significant part in the regulation of absorption, distribution and re-absorption/elimination of many therapeutic compounds. P-gp decreases the bioavailability of a broad range of compounds from the gastro-intestinal lumen (Chan *et al.,* 2004). The oral bioavailability of a drug may therefore be reduced as the net passage of orally administered drugs across the gastro-intestinal epithelium might be limited (Huisman *et al.,* 2000). With regards to the blood-brain barrier, P-gp is important as a defense mechanism against the penetration of drugs and toxins from entering into the central nervous system (CNS) (König *et al.,* 2013).

P-gp is involved in the manifestation of some cell drug resistance, which is mediated by a reduction in the accumulation of the therapeutic drug in the target cells. This P-gp mediated drug resistance has a negative effect on HIV and cancer therapies (Hansten, 2001).

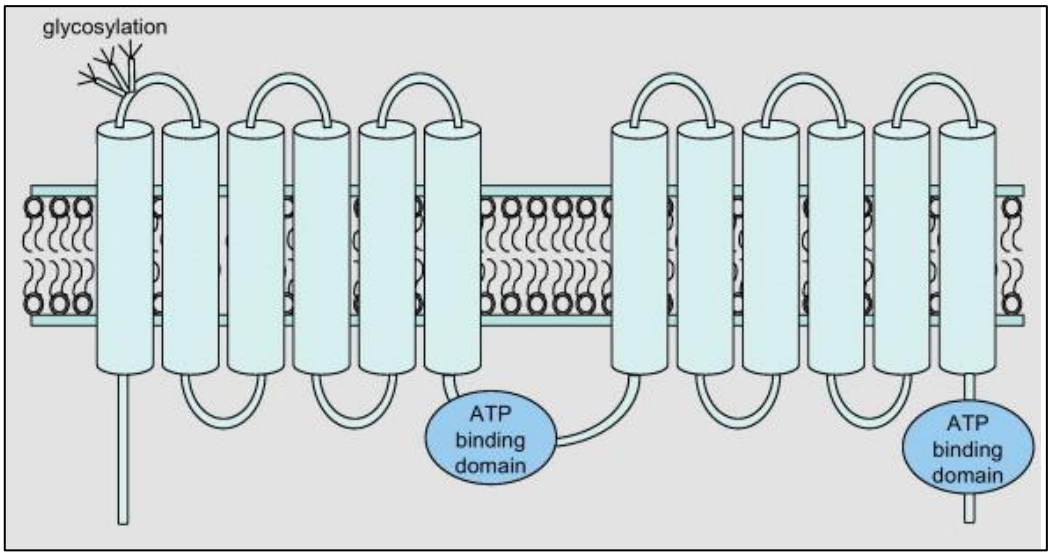

Figure 2.2: A schematic illustration of the structure of P-gp (Bansal *et al*., 2009)

P-gp transports a wide range of moderately hydrophobic and amphipathic drugs out of cells (Huisman *et al*., 2000). As seen in Figure 2.2, P-gp's single chain is divided into two homologous halves and each contains six trans-membrane domains. Furthermore, P-gp contains two ATP-binding regions that are divided by a flexible polypeptide linker (Ambudkar *et al*., 2006).

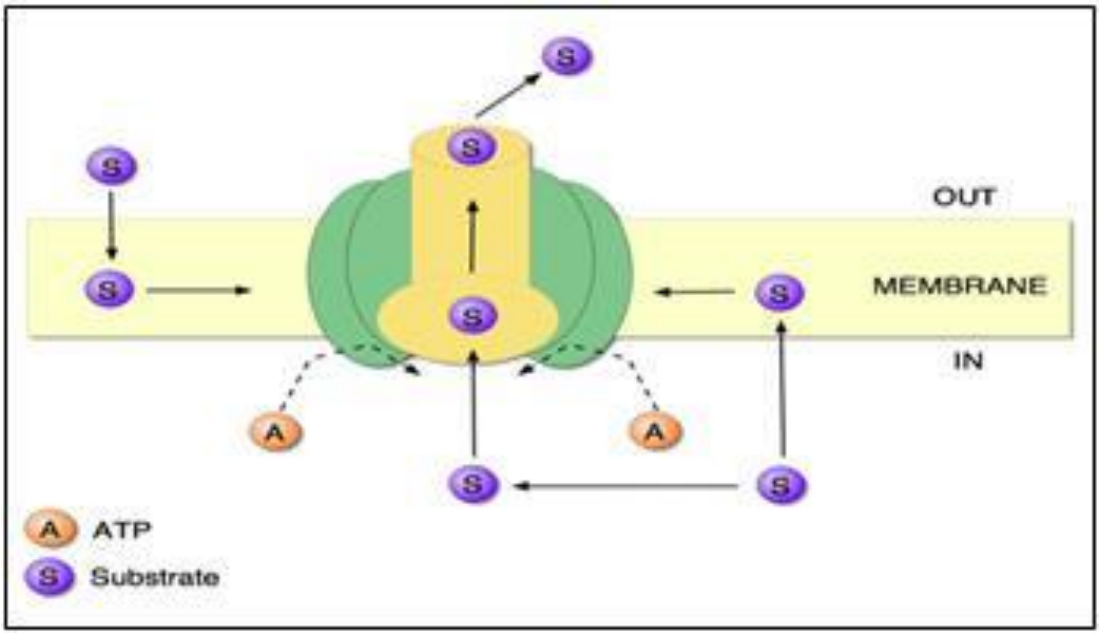

Figure 2.3: Schematic illustration of the P-gp efflux mechanism (Gohel *et al.,* 2011)

The P-gp molecule is expressed over cell membranes and exhibits efflux action both inside and outside the cell. As illustrated in Figure 2.3, a pore or channel is found in the centre of the P-gp

structure and is responsible for the efflux of drugs out to the extracellular region. P-gp efflux action is energy dependent, ATP mediated (Gohel *et al.,* 2011).

2.2.1.2 P-gp modulation

Modulation of P-gp by herbal medicines holds the potential for alterations involving the pharmacokinetic profile of drugs that are substrates. These pharmacokinetic changes can take place when herbal medicines inhibit the drug transporters through either a competitive or non-competitive mechanism. On the other hand, the induction of transporter proteins by means of an increase of the related proteins' mRNA can also produce interactions (Fasinu *et al.,* 2012).

P-gp has a broad substrate spectrum, which range from small molecules (200 Da) to peptides of a larger structure (4000 Da) (Estudante *et al.,* 2013). P-gp substrates are generally hydrophobic but mycophenolic acid, a hydrophilic compound, has been shown to be transported by P-gp (Hansten, 2001). Various drugs that are substrates of P-gp are listed in Table 2.1. It is important to note that some drugs may be both substrates and inhibitors of P-gp.

Table 2.1: Examples of different drug classes that are P-gp substrates, inhibitors or inducers (Adapted from, Calitz, 2014; Hansten, 2001; Haritova, 2008; Hu *et al.,* 2005; König *et al*., 2013; Mercer *&* Coop, 2011)

Antibiotics	Macrolides (Erythromycin), Ketoconazole, Dicloxacillin	• Substrate
	Fluoroquinolones (Ciprofloxacin, Grepafloxacin, Levofloxacin and Sparfloxacin)	• Substrate • Inhibitor
Anti-cancer drugs	Doxorubicin, Idarubicin, Vinblastine, Paclitaxel, Topotecan, Bisantrene, Docetaxel, Etoposide, Vincristine	• Substrate
Opioid Analgesics	Morphine, Methadone, Loperamide	• Substrate
HIV protease inhibitors	Indinavir, Saquinavir,	• Substrate • Inhibitor

	Nelfinavir,	
	Rotinavir	• Substrate • Inhibitor
Antihistamines	Fexofenadine, Terfenadine	• Substrate
H_2-receptor antagonists	Cimetidine	• Substrate
Anti-epileptics	Felbamate, Topiramate	• Substrate
Anti-arrhythmics	Quinidine	• Substrate • Inhibitor
	Amiodarone, Propafenone	• Substrate • Inhibitor
Immunosuppressive drugs	Cyclosporine A	• Substrate • Inhibitor
	Tacrolimus (FK506)	• Substrate
HMG-CoA reductase inhibitors	Lovastatin	• Substrate
	Atorvastatin	• Substrate • Inhibitor
Fluorescent compounds	Rhodamine 123, Calecin-AM	• Substrate
Cardiac glycosides	Digoxin	• Substrate
Calcium channel blockers	Nifedipine	• Substrate
	Verapamil	• Substrate • Inhibitor
Anti-hypertensives	Propanolol, Reserpine	• Substrate • Inhibitor
Anti-emetics	Ondansetron	• Substrate
Anti-helminthics	Ivermectin	• Substrate
Steroids	Cortisol Corticosterone	• Substrate
	Progesterone	• Substrate • Inhibitor
Anti-alcoholism drug	Disulfiram	• Substrate
Psychotrophics	Carbamazepine,	• Substrate

	Amitriptyline, Sertraline	• Inhibitor
Anti-gout drug	Colchicine	• Substrate
Pesticides	Methylparathion, Endosulfan	• Substrate

2.2.1.3 Inhibition and induction of P-gp

P-gp inhibition can either be competitive or non-competitive. Competitive inhibition takes place when two drug substrates are competing for the same drug-binding site. Non-competitive inhibition occurs when a drug substrate inhibits the ATP hydrolysis cycle and/or the conformation of the binding site changes by means of an allosteric mechanism (Fasinu *et al.,* 2012; Marchetti *et al.,* 2007).

As illustrated in Figure 2.4 a and b below, the P-gp transporter is situated on the apical membrane of polarised intestinal epithelial cells. P-gp substrates are pumped out of the cell through the apical membrane back into the intestinal lumen. The absorption of P-gp substrates is therefore reduced. When P-gp is inhibited, it allows for an increase in the movement of P-gp substrate molecules in the absorptive direction as the substrate molecules are now not pumped out of the cells in the secretive direction. This results in an increase in absorption from the intestinal lumen and a decrease in excretion (Hansten, 2001).

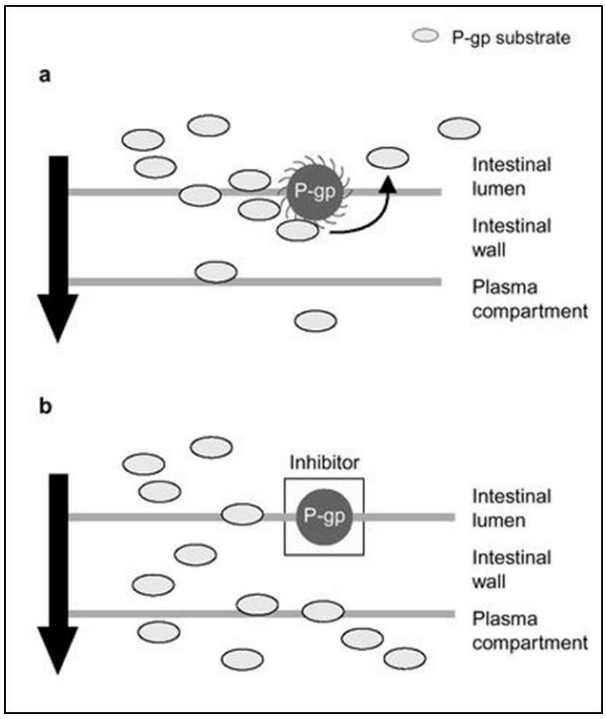

Figure 2.4: Schematic illustration of the effect of inhibition of P-gp on drug absorption (Hansten, 2001)

Up-regulation of the MDR1/ABCB1 gene through pregnane X receptor (PXR) and constitutive androstone receptor (CAR), which act as drug substrate sensors, results in induction of P-gp. Activation creates PXR and CAR to dimerise with the retinoid-X-receptor (RXR) and a heterodimer is formed. This heterodimer is then bound to the response elements that are located on the MDR1/ABCB1 gene. The specific gene transcription is promoted and an increase in messenger ribonucleic acid (mRNA) is produced for the formation of protein (Pal & Mitra, 2006). Induction therefore causes higher expression of P-gp and thereby causes a reduced concentration of the drug reaching the systemic circulation and therefore a decrease in efficacy and oral bioavailability of the co-administrated drugs (Pal & Mitra, 2006).

Studies have shown that an extract of *Hypericum perforatum* (St John's Wort) can induce P-gp and cause a reduction of bioavailability *in vivo* and *in vitro* of warfarin, theophylline, ARVs, oral contraceptives and anti-convulsants (Shi *&* Klotz*,* 2012; Tarirai *et al.,* 2010). Co-administration of *H. perforatum* has also been reported to reduce digoxin plasma concentrations through an increase in P-gp activity (Fasinu *et al.,* 2012).

Grapefruit juice is a well-known inhibitor of P-gp efflux. Cyclosporine is an immunosuppressant drug given during organ transplants to prevent organ rejection. Co-administration of grapefruit juice together with cyclosporine has resulted in a significant increase in cyclosporine bioavailability. On the other hand, *H. perforatum* co-administered with cyclosporine produced a decrease in cyclosporine plasma concentrations to sub-therapeutic levels, possibly resulting in the rejection of the transplanted organ (Hu *et al.,* 2005). Other herbal products containing a P-gp modulator, flavonoid quercetin, such as *Ginkgo biloba* and *Sophora japonica,* have also shown the ability to reduce the bioavailability of cyclosporine (Tarirai *et al.,* 2010).

2.2.2 Interactions involving metabolism

Drug metabolism entails the enzymatic conversion of a therapeutically active drug compound to a new molecule, which can be a pharmacologically active, but usually inactive metabolite. It often involves the conversion of lipophilic drug compounds to highly polar derivatives that are then excreted easily from the human body. The primary drug metabolism site is in the liver cells smooth endoplasmic reticulum. Other organs responsible for metabolism include the lungs, kidney, gastrointestinal tracts and the placenta (Taxak *&* Bharatam, 2014).

The most common cause of drug interactions of clinical significance is inhibition or induction of drug-metabolising enzymes (Lam & Ernst, 2006). Herb-drug metabolic interactions often occur

in the gastrointestinal epithelium and in the liver as CYP450 metabolic enzymes are present in high concentrations in these tissues but are also found in the placenta, lungs and kidneys. Metabolic enzymes mediate two categories of biotransformation reactions namely, Phase I biotransformation reactions (which include oxidation, reduction, hydration and hydrolysis) and Phase II biotransformation reactions (which include methylation, sulfation, acetylation, fatty acid conjugation, glutathione conjugation and glucuronidation) (Tarirai *et al.,* 2010).

2.2.2.1 Cytochrome P450 (CYP) superfamily of enzymes

The cytochrome P450 (CYP) enzymes comprise of a super family of haematoprotein enzymes that are located on the membrane of the endoplasmic reticulum. They are responsible for catalysing the metabolism reactions of many exogenous and endogenous compounds (Singh, 2007). The CYP superfamily of enzymes is divided into families and sub-families based on their nucleotide sequence homology. A high degree of substrate specificity is present among the different families (Fasinu *et al.,* 2012). CYP enzymes belonging to the families 1, 2 and 3 are primarily involved in xenobiotic metabolism, while other CYP enzymes have an essential role in the formation and elimination of endogenous compounds such as bile acids, hormones and fatty acids (Amacher, 2010). Only a portion of the total number of isoforms, belonging to families 1, 2 and 3, are responsible for the biotransformation of foreign compounds including 70-80% of all drugs currently in clinical use (Zanger *&* Schwab, 2013).

The highest expressed CYP iso-enzyme forms present in the liver include CYP3A4, CYP2C9, CYP2C8, CYP2E1 and CYP1A2, while CYP2J2, CYP1A1 and CYP1B1 are mostly expressed extra-hepatically. The expression of CYP enzymes is influenced by a number of factors and usually a combination of mechanisms including the induction by xenobiotics, genetic polymorphism, cytokine regulation, hormones, gender and age amongst others (Zanger *&* Schwab, 2013).

The oxidising site of CYP450 enzymes is located in the heme centre (i.e. an iron-porphyrin unit) and is responsible for catalising oxidation reactions. In a reduced state, the iron can be bound, with a high affinity, to carbon monoxide (CO). CYP enzymes catalyse the transfer of an oxygen atom to a substrate as schematically illustrated in Figure 2.5, resulting in an oxidised substrate together with a water molecule (Taxak *&* Bharatam, 2014).

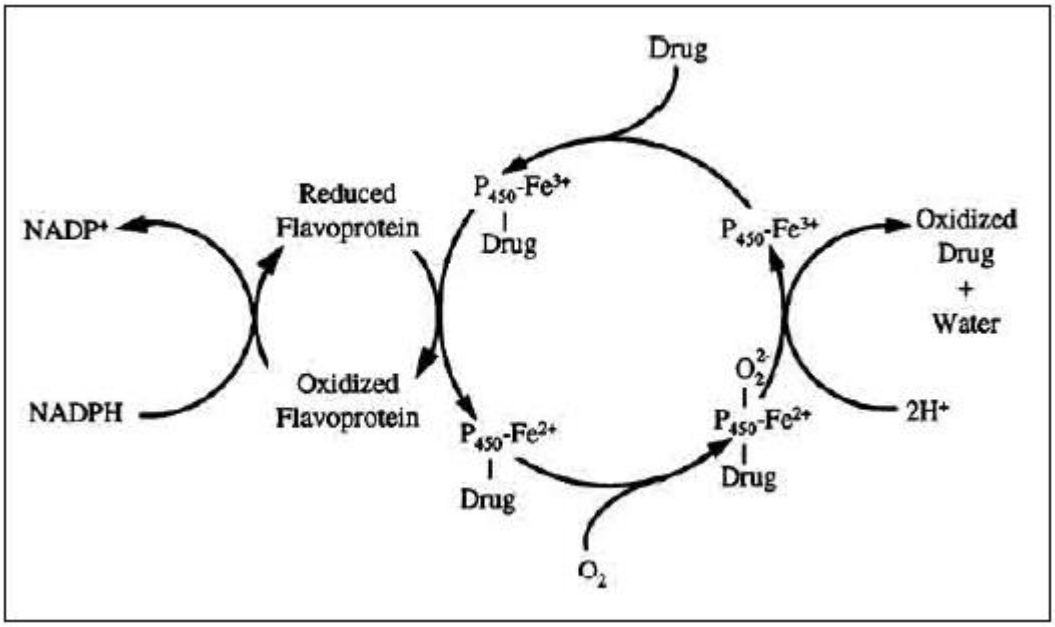

Figure 2.5: Schematic illustration of the catalytic cycle of Cytochrome P450 (Singh, 2007)

The sub-family, CYP3A, constitutes over 40% of the total CYP present in the human body with CYP3A4 found to be the most abundant of all isoforms. CYP3A4 is highly expressed in the liver and intestines and contributes to the metabolism of approximately 50% of all drugs that are currently in use (Singh *et al.,* 2011).

2.2.2.2 Factors influencing cytochrome P450 enzyme expression and function

Genetic polymorphism

Four phenotypic sub-populations based on drug metabolising enzymes exist. These phenotypic populations include poor, intermediate, extensive and ultra-rapid metabolisers (Satoh, 2007).

Factors that may influence some of the CYP450 enzymes are shown in Figure 2.6. CYP450 “loss-of-function” polymorphisms often affect expression and splicing rather than the protein structure or transcription. Variants of “gain-of-function” polymorphisms include copy number variants that involve an increase in the number of functioning gene copies in CYP2A6 and CYP2D6, promotor variants such as CYP2B6 and CYP2C19, and variants of amino acids with an increased substrate turnover. A few polymorphisms have an effect on substrate selectivity or drug metabolising CYP450 induction (Zanger *&* Schwab, 2013).

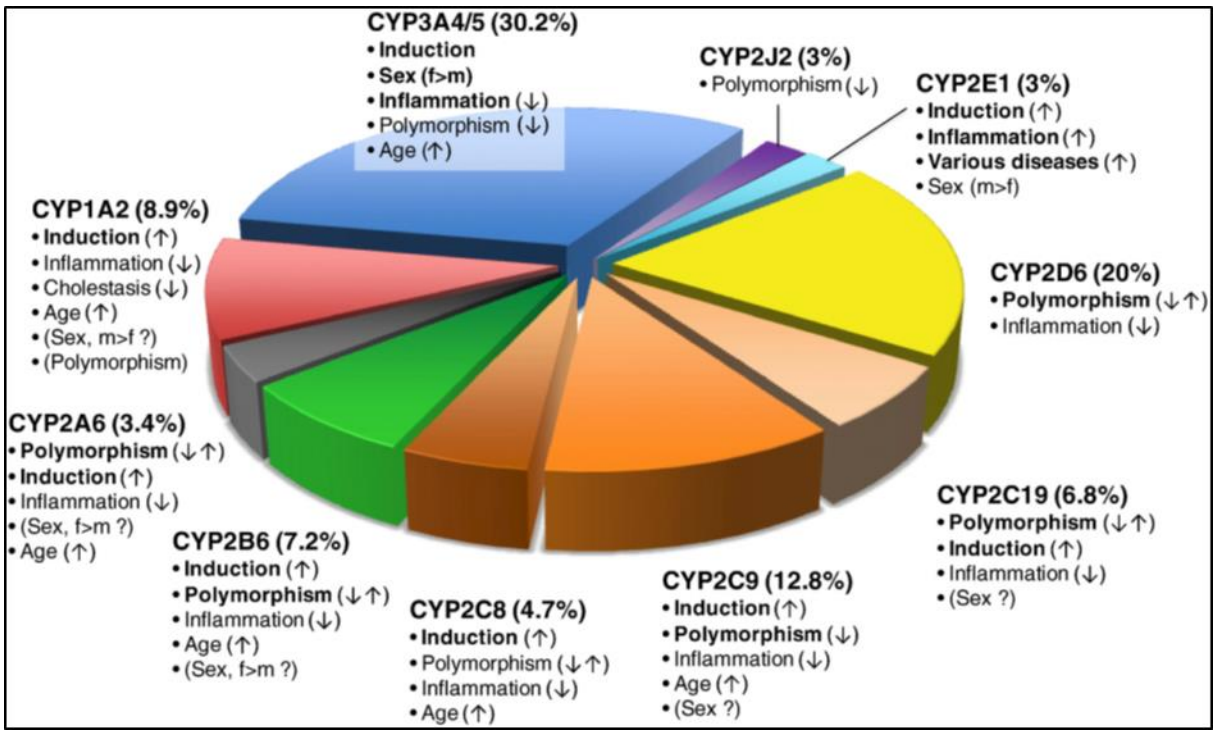

Figure 2.6: Fraction of clinical drugs metabolised by CYP450 isoforms and the factors influencing variability (Zanger *&* Schwab, 2013)

Variants of “loss-of-function” can result in a reduced drug clearance and an increase in drug plasma concentration, while variants of “gain-of-function” can result in an increased drug clearance and a lower drug plasma concentration (Zanger *&* Schwab, 2013).

Epigenetic influence on drug metabolism

Epigenetics describes the heritable changes that may occur in gene function that are not based on sequence variations in DNA. Epigenetics is caused by mechanisms such as DNA methylation and modification of histone protein, while gene regulatory mechanisms by microRNA also exist. Epigenetic patterns are primarily reversible and may possibly be tissue-specific and host factors such as sex and age play an important role. Environmental factors may also have an influence (Zanger *&* Schwab, 2013).

Studies on CYP1 genes have indicated that CYP1A1 promotor methylation in human lung tissue was the lowest amongst heavy tobacco smokers and the highest in non-smokers. This is an example of an environmental influence that can occur in DNA methylation patterns (Zanger *&* Schwab, 2013). Targets of microRNA include nuclear receptors and were also shown in the PXR, as this receptor has demonstrated to be under control of miR-148a. This can have an influence on CYP2B6 and CYP3A4 expression levels and xenobiotic drug substrate metabolism (Takagi *et al.,* 2008).

Non-genetic host factors

Pharmacokinetic parameters such as body weight, blood flow, fat distribution and the expression of drug transporters and metabolising enzymes are influenced by gender (Gandhi *et al.,* 2004). CYP450 sex-specific expression is typical in laboratory animals and was established to be controlled by different secretion profiles of growth hormone in male versus female animals (Waxman & Holloway, 2009). A genome-wide gene expression profiling investigation conducted in 112 female and 112 male livers recognised over 1300 genes whose mRNA expression was considerably affected by gender and 75% showed a higher expression in females (Zhang *et al.,* 2011). Amongst these gene expression differences, there were 40 genes related to pharmacokinetics, which included CYP1A2, CYP3A4 and CYP1A7 displaying female bias, and a male bias displayed by CYP27B1, CYP3A5 and UGT2B15. Most clinical investigations have established that women metabolise drugs quicker than men and is particularly found in CYP3A4 substrates (Zanger *&* Schwab, 2013).

Age is a well-recognised and established factor that influences drug metabolism capacity. Extremes of life are usually involved in cases of a lower drug metabolism capacity. This is represented in neonates due to the fact that several enzymes are still undeveloped. In the elderly, the ability of drug clearance is decreased and therefore drugs with a narrow therapeutic index, including anti-depressants, anti-psychotics, beta-blockers and anti-coagulants, should be taken into consideration (Zanger *&* Schwab, 2013). It has been found in human liver studies that there is a modest increase in activity and expression of most CYPs during life, particularly for CYP2C9. The age influence on CYPs 1A2, 2A6, 2B6, 2C8 and 3A4 moderately interacted with sex (Yang *et al.,* 2010). Limited drug clearance as a function of age can also be associated with the reduced renal function and liver blood flow in the elderly. Furthermore, age-associated modifications in gene expression involved in xenobiotic metabolism have been established in rats and mutant mice (Zanger *&* Schwab, 2013).

Disease conditions normally have a negative impact on drug metabolism. Liver cirrhosis represents liver architecture changes which results in a blood flow reduction, functional hepatocyte loss, and a reduction of drug metabolising enzymes. This all contributes to the decrease in drug metabolism capacity as well as a lower synthesis of serum proteins, leading to a decrease in drug clearance. During inflammation, infection and cancer, pro-inflammatory cytokines circulate and act as signalling molecules to stimulate marked changes in gene expression profiles of the liver. As a result, severe down regulation of numerous drug metabolising enzymes occurs (Zanger *&* Schwab, 2013).

2.2.2.3 Pre-systemic metabolism/first-pass effect

Orally administered drugs are subject to pre-systemic metabolism, also referred to as the first-pass metabolism effect, which can be defined as the metabolism of drug molecules between administration and appearance in the systemic circulation as schematically illustrated in Figure 2.7. The concentration of the drug that reaches the systemic circulation can be reduced by hepatic and intestinal metabolism (Pond *&* Tozer, 1984). The liver was thought to be fully responsible for the pre-systemic elimination until 1997, but it was found that CYP3A sub-family enzymes are expressed at high levels in the premature villous tips of enterocytes in the small intestine (Gavhane *&* Yaday, 2012).

Any molecule that has been absorbed from the gastrointestinal lumen passes through the gastrointestinal epithelial mucosa, intestinal capillaries and is then transferred to the liver via portal circulation before reaching the systemic circulation. It is transported in the blood to the rest of the organs after the systemic circulation has been reached (Gavhane *&* Yaday, 2012). Intestinal first-pass metabolism could result in a low oral drug bioavailability and provides a platform for drug-drug or herb-drug interactions (especially for CYP3A substrates) (Gertz *et al.,* 2010).

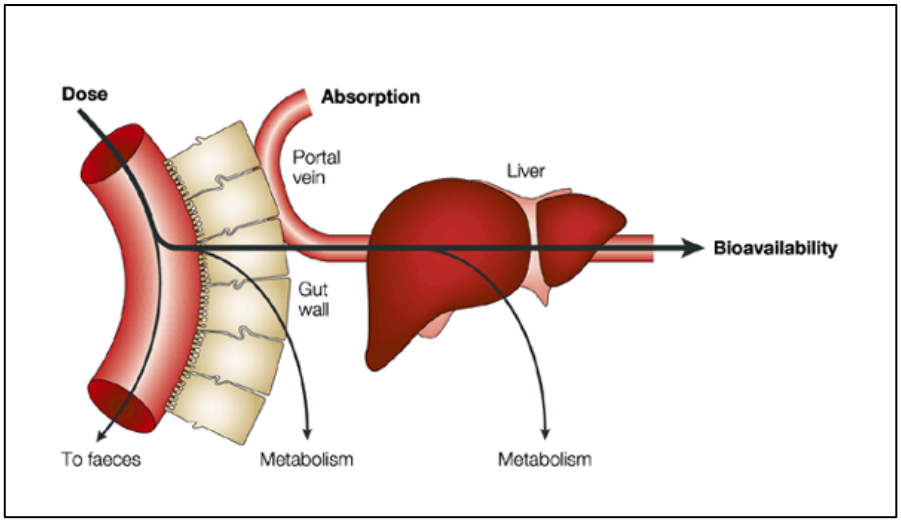

Figure 2.7: Schematic illustration of pre-systemic metabolism (Adapted from Dickens & Van de Waterbeemd, 2004)

Pre-systemic metabolism is clinically important when the fraction of administered drug dose that escapes this metabolism is small (Pond *&* Tozer, 1984).

2.2.2.4 Inhibition and induction of metabolic enzymes

Drug interactions involving the CYP enzymes are generally a result of either an induction or inhibition of these enzymes. The induction of CYP can lead to an increased metabolite production rate, which is associated with decreased plasma levels and drug response. CYP inhibition can be classified into reversible and irreversible inhibition. Reversible inhibition occurs when direct competition for the CYP enzyme binding site takes place between a substrate and an inhibitor. Irreversible inhibition is produced by reactive metabolites that are generated from CYP-catalysed reactions (Mukherjee *et al.,* 2011). In the case of irreversible inhibition, the mechanism-based inhibitors become activated during metabolism and a complex with the CYP3A heme is formed, the metabolite intermediate complex, which result in a covalent modification of the enzyme. This results in irreversible enzyme activity loss. This irreversible mechanism seem to be the inhibition of CYPs that takes place after exposure to bergamottins (grapefruit juice), capsaicin (chili peppers), glabridin (licorice root), isothiocyanates (cruciferous vegetables), oleuropein (olive oil), resveratrol (red wine constituent) and diallyl sulfone (garlic). Irreversible inhibition can take one to two weeks to resolve after the inhibitor has been discontinued, as this is the time that it takes the CYP3A to restore to its pre-drug steady state (Lam & Ernst, 2006).

As demonstrated in Figure 2.8 bellow, CYP inhibition by herbal medicines may cause toxicity as a result of an increased plasma and tissue drug concentration. CYP induction may result in reduced drug concentrations and therefore causing a decrease in drug efficacy and potentially failure in treatment (Mukherjee *et al.,* 2011). Induction may be a result of enhanced gene transcription rates, translational efficiency, increased stability of mRNA or protein stabilisation that have been induced by post-translational modifications or substrate binding. The most common induction mechanism is the binding and activation of nuclear factors that act in protein heteromer forms to increase gene transcription rates. A single nuclear factor has the potential to modulate the expression of several genes. The transcriptional regulation involving drug-metabolising enzymes is usually tissue selective. Tissues with a low concentration expression of nuclear factors do not show significant induction. Liver and intestines both express substantial concentrations of nuclear factors, such as PXR, and a profound induction are experienced (Lam & Ernst, 2006).

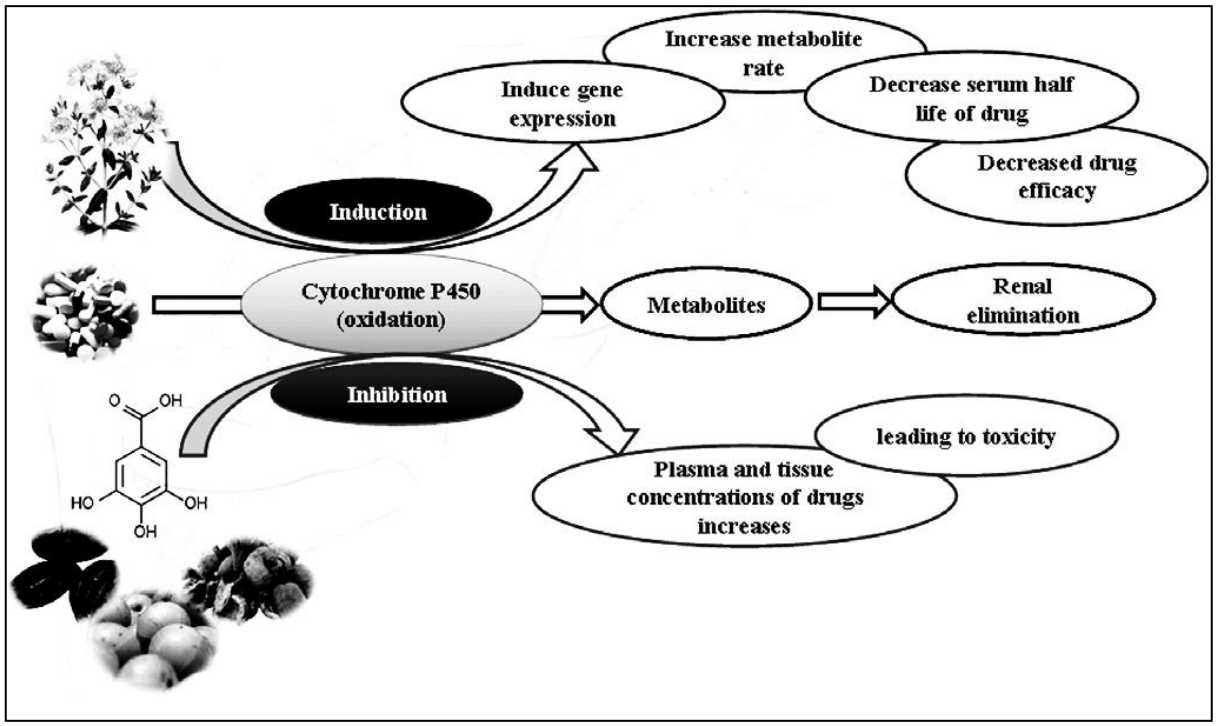

Figure 2.8: Schematic illustration of cytochrome P450 inhibition and induction (Mukherjee *et al.,* 2011)

Examples of enzyme inhibition and induction by herbal products are listed in Table 2.2. Herbal medicines can cause CYP inhibition through competitive inhibition, non-competitive inhibition and mechanism-based inhibition. Induction is usually mediated through the ligand-binding domain of PXR. Studies have demonstrated that PXR is involved in both CYP3A and P-gp induction. The inductive effect caused by herbal medicines and drugs may be mediated through a few sub-mechanisms and not just one single mechanism (Tarirai *et al.,* 2010).

Table 2.2: Enzyme inhibition and induction of herbal medicines (Adapted from Liu *et al.,* 2011)

Scientific plant name	**Chemical Component**	**CYP substrate**	**Study method conducted**	**Effect on enzyme**
Hypericum perforatum (St. John's wort	Hypericin	CYP3A4	*In vivo, in vitro*	Induction
Ginkgo biloba	Quercetin, kaempferol, p-coumarin acid	CYP3A4	*In vivo, in vitro*	Inhibition
Angelica sinensis	Lactones of Artemisa, butane	CYP3A4	*In vitro*	Inhibition

	acid lactones, ferulic acid			
Angelica sinensis	Furanocoumarins, scopoletin, coumarin	CYP2E1, CYP3A4	*In vivo, In vitro*	Inhibition
Glycyrrhiza uralensis (licorice)	Isoflavones, glabridin	CYP3A4	*In vitro*	Induction
Cortex acanthopanacis (Acanthopanax bark)	Eleutheroside	CYP2C9	*In vivo*	Induction
Panax ginseng	Ginsenoside Rd	CYP3A4, CYP2D6, CYP2C19, CYP2C9	*In vitro*	Inhibition
Panax ginseng	Ginsenoside Re, Rf	CYP2C9, CYP3A4	*In vitro*	Induction
Panax ginseng	Ginseng extract	CYP3A4	*In vivo*	Inhibition
Panax ginseng (Red Ginseng)	Red ginseng extract	CYP2E1	*In vivo*	Inhibition
Allium sativum (garlic extract)	Allicin, saponin	CYP3A4	*In vivo*	Induction
Salvia miltiorrhiza (red sage)	Salvia extract	CYP2C9	*In vivo*	Inhibition

2.2.2.5 Herb-drug pharmacokinetic interactions involving anti-retroviral drugs

Many herbal medications used by HIV infected patients may have an influence on the metabolism of their ARV drugs through interaction with CYP450 enzymes (Phase I reactions), uridine diphosphate (UDP)-glucuronosyltransferases (Phase II reactions) and P-gp (Tarirai *et al*., 2010). The ARV treatment therapy usually consists of either a protease inhibitor (PI) or non-nucleoside reverse transcriptase inhibitor (NNRTI) combined with nucleoside reverse transcriptase inhibitors (NRTI). NRTIs are primarily excreted through renal clearance and CYP based interactions are not frequently uncounted. PI and NNRTI are both mainly metabolised by CYP450 enzymes. Higher ARV plasma levels would be as a consequence of CYP inhibition by

herbal medicines and the patient would therefore be at a higher risk of experiencing side effects. CYP induction would result in sub-therapeutic plasma levels, causing therapeutic failure and a greater risk of developing ARV drug resistance (van den Bout-van den Beukel *et al.,* 2006).

Garlic (*Allium sativum*) is popular amongst HIV-infected patients as a dietary supplement in order to improve health and fight opportunistic infections. Pharmacokinetic interactions between garlic and the ARVs saquinavir and ritonavir have been reported and studies have indicated the mechanism is most likely an induction of CYP3A4 and P-gp by garlic constituents (van-den Bout-van den Beukel *et al.,* 2006).

Milk thistle (*Silybum marianum*) is administered by HIV-infected patients for the prevention of ARV treatment related hepatotoxicity and treatment of hepatitis (Lee *et al.,* 2006). Co-administration with indinavir indicated a reduction in indinavir blood plasma concentration and *in vivo* data have suggested this is due to an induction of CYP3A4 or P-gp. Potential clinically important interactions of Millk thistle taken with PIs and NNRTs that are P-gp substrates have been observed (van den Bout-van den Beukel *et al.,* 2006).

Clinically important interactions have been reported with St. John's Wort and ARVs. Co-administration of indinavir and St John's Wort has shown to decrease indinavir plasma concentrations. Concomitant use of St John's Wort increased nevirapine clearance by 35% in HIV-infected patients (Lee *et al.,* 2006; van den Bout-van den Beukel *et al.,* 2006). This study will focus on the pharmacokinetic interactions that occur when *Hypoxis hemerocallidea* is co-administrated with indinavir.

2.3 *HYPOXIS HEMEROCALLIDEA* (AFRICAN POTATO)

The African potato, *Hypoxis hemerocallidea*, also known as *H. rooperi,* is a popular medicinal plant used as a traditional herbal medicine in southern Africa. It is found in the southern Africa sub-region where it usually grows in meanders, grasslands and mountain regions. It has the ability to survive well in high stressed environments (Cordier & Steenkamp, 2011; Owira & Ojewole, 2009).

2.3.1 Botany and uses

H. hemerocallidea is a plant of about 100 to 500 mm in height and is characterised by its strap-like deciduous leaves and star-shaped flowers that are bright yellow in colour as illustrated in Figure 2.9 (Van Wyk *et al.,* 2002). The plant has an unbranched stem and a rootstock growing underground. This tuberous rootstock is also called the corm and has a potato-like shape. The

plant grows well in warm and cold sub-tropical areas as it prefers full sunlight with well-drained soil (Owira & Ojewole, 2009).

Traditionally, the corms of the plant are chopped into tiny pieces and boiled for approximately 20 min. The decoction is then administered orally. The daily oral dose administered is estimated to correspond to 250 ml derived from about 20 g of the corms (Nair *et al.,* 2007). The corm and roots have been used to treat a wide range of diseases, which include colds and flu, hypertension, diabetes mellitus, psoriasis, urinary tract infections, testicular tumours, prostate hypertrophy and internal cancers, central nervous system disorders and HIV/AIDS. The South African Ministry of Health recommended that HIV-infected patients use this herbal plant because it contains immune system stimulating properties (van den Bout-van den Beukel *et al.,* 2006). A recommended daily dose of 2 400 mg raw plant is claimed to be therapeutically effective (Mills *et al.,* 2005).

Figure 2.9: Photograph of the African potato (*Hypoxis hemerocallidea)* plant showing its characteristic yellow flower (Germishuizen *et al.,* 2003)

2.3.2 Biological activity

In vivo studies have indicated that aqueous and alcohol extracts of *H. hemerocallidea* hold pharmacological properties, which include anti-nociceptive, anti-inflammatory and anti-diabetic properties (Ojewole, 2006). The extracts could inhibit inflammatory cytokine synthesis, production and release mediators such as prostaglandins. Aqueous extracts of *H. hemerocallidea* corms have also shown anti-oxidant activity *in vitro* by scavenging free radicals

and both extracts, aqueous and alcohol, have indicated the ability to inhibit the cyclooxygenase (COX) enzyme that mediates the synthesis of prostaglandin (Owira & Ojewole, 2009).

Lectin-like proteins derived from *H. hemerocallidea* extracts have been reported to inhibit *Staphylococcus aureus* growth *in vitro.* Other laboratory reports have shown the extracts to inhibit *Escherichia coli* growth (Owira & Ojewole, 2009). This observation may explain the traditional use of the plant in the treatment of urinary tract infections and benign prostate hyperplasia. It has been suggested that the anti-bacterial properties of *H. hemerocallidea* might not be the only reason for the treatment of benign prostate hyperplasia as anti-inflammatory and anti-oxidant properties could also be responsible for treatment of this condition. This data supports the plant's use in the treatment of certain cancers (Owira & Ojewole, 2009; Nair *et al*., 2007).

Certain healthcare providers (e.g. traditional healers) are using *H. hemerocallidea* extracts/plant materials in the treatment of patients infected with HIV/AIDS because it has been said to boost the human's immune system. The plant's usage has been extended to other immune-related conditions such as flu, the common cold and arthritis (Mills *et al.,* 2005).

2.3.3 Phytochemistry

H. hemerocallidea has been chemically characterised in the attempt to determine which chemical constituents are responsible for its medicinal properties (Owira & Ojewole, 2009). A norlignan-diglucoside, hypoxoside, has been found to be one of the most important chemical constituents in terms of medicinal properties. Hypoxoside is a biologically inactive pro-drug, which has an aglycone structure that consists of a diphenyl-1-en-4-yne-pentane skeleton (Figure 2.10) (Nair *et al.,* 2007). It is converted by β-glucosidase enzyme in the human gastrointestinal tract to a biologically active compound, rooperol (Figure 2.10) (Owira & Ojewole, 2009). Rooperol exhibited both anti-cancer and anti-inflammatory activity and most of *H. hemerocallidea's* therapeutic properties have been scientifically, clinically and pharmacologically attributed to rooperol. Sterols and stanols, such as stigmastanol, stigmasterol and β–sitosterol (Figure 2.10), are also found in *H. hemerocallidea* extracts but their medicinal significance has not been proven yet (Cordier & Steenkamp, 2011).

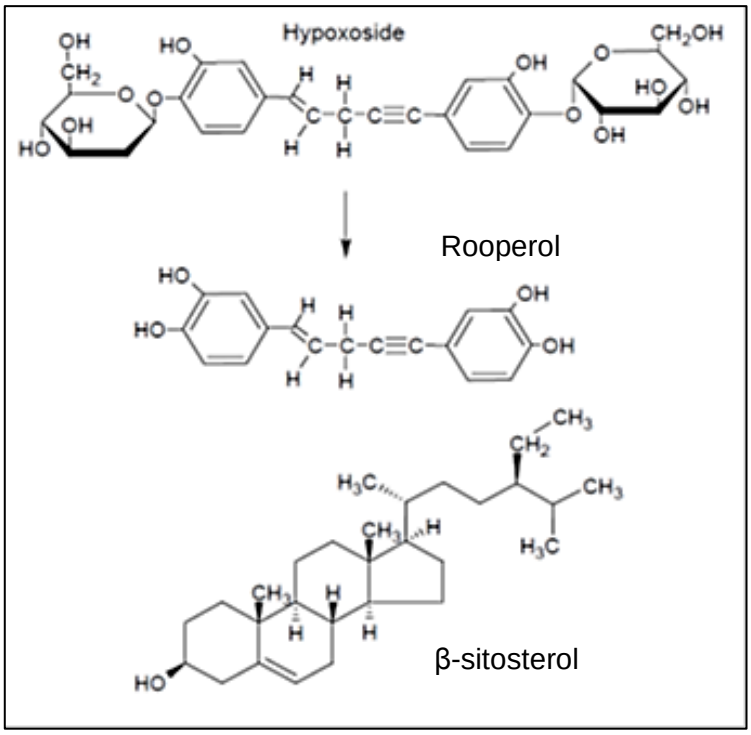

Figure 2.10: Chemical structures of hypoxoside, rooperol and β-sitosterol (Adapted from Owira & Ojewole, 2009)

Rooperol and hypoxoside have been found to undergo phase I hepatic metabolism by CYP450. A product containing rooperol from *H. hemerocallidea* extract with the trade name, 'Harzol™', is registered in Germany for the treatment of prostate cancer. Many other commercial herbal medicines containing rooperol or *H. hemerocallidea* extracts are found on the market (Nair *et al.,* 2007).

2.3.4 Interactions

Administration of *H. hemerocallidea* extracts may possibly affect enzymes and transporters and therefore consequently have an effect on the metabolism and absorption of concurrently administered therapeutic drugs (Mogatle *et al.,* 2008). Possible herb-drug interactions between extracts of *H. hemerocallidea* and ARV drugs have been identified (Mills *et al.,* 2005). Studies have shown that *H. hemerocallidea* extract can inhibit CYP3A4 and P-gp. The extracts have also been claimed to activate PXR, which will modulate the expressions of P-gp and CYP3A4. As mentioned before, some ARV drugs are CYP3A4 substrates (Mills *et al.,* 2005; Tarirai *et al.,* 2010).

H. hemerocallidea extracts are likely to affect cellular drug transport systems since an up-regulation of PXR has been reported (Tarirai *et al.,* 2010). From *in vitro* results it was postulated that patients administering *H. hemerocallidea* extracts simultaneously with ARV

treatment therapy may be at risk to experience pharmacokinetic interactions that may result in treatment failure, viral resistance and drug toxicity (Owira & Ojewole, 2009). Findings suggested co-administration may lead to early drug metabolism and transport inhibition (van-den Bout-van den Beukel *et al.,* 2006).

In vitro studies in human liver microsomes have shown that *H. hemerocallidea* is capable of inhibiting CYP3A4 activity by 86%. *H. hemerocallidea* also showed a P-gp inhibition activity of 42% - 51% of that of verapamil, a known P-gp inhibitor. Exposure of *H. hemerocallidae* resulted in a two-fold activation of PXR (van-den Bout-van den Beukel *et al.,* 2006).

Another *in vitro* study demonstrated that an aqueous extract of *H. hemerocallidea* presented a substantial inhibition (i.e. 33.9% – 85.6%) of CYP3A4 activity at an initial concentration of 100 mg/ml. An increase in inhibition to 56.1% - 79.4% was observed with the use of a methanol extract (Mills *et al.,* 2005). A study revealed that stigmasterol and rooperol inhibited CYP3A4, CYP3A5 and CYP19 (Nair *et al.,* 2007; Mogatle *et al.,* 2008).

It is clear from previous studies that more research needs to be conducted in order to investigate the potential for clinically important interactions of *H. hemerocallidea* with drugs, especially ARVs (van-den Bout-van den Beukel *et al.,* 2006).

2.4 MODELS TO EVALUATE PHARMACOKINETIC INTERACTIONS

Intestinal absorption and hepatic metabolism of orally administered drugs are essential factors in the determination of the drug's systemic bioavailability and therapeutic effect. Pre-clinical examination of drug metabolism and transport plays an important role in the identification of potential bioavailability problems or pharmacokinetic interactions between drugs and other compounds. Several well-established cell-based and animal models are in use for the study of hepatic clearance and intestinal absorption (Alqahtani *et al.,* 2013).

Permeability studies have shown that the *in vitro* Caco-2 cell culture model as well as the *in vitro* mucosal sheets of animals, such as rats and pigs, can predict human intestinal drug absorption to an acceptable extent (Versantvoort *et al.,* 2000). Other cell culture models that are commonly used in drug transport and metabolism studies include the MDCK and LS180 cell lines. HepG2 is a well-known liver cell line used in metabolism studies, while microsomes and precision cut liver slices are also used for this application. A summary of commonly used models for drug permeation and metabolism studies is given in Table 2.3.

Table 2.3: Commonly used models to study metabolism and transport of drugs (Adapted from Alqahtani *et al.,* 2013)

INTESTINAL ABSORPTION MODELS	HEPATIC METABOLISM MODELS	SIMULATION MODELS
***In vitro* models** • Caco-2 cells • MDCK cells ***Ex vivo* models** • Ussing chamber (rat/pig) • Everted intestinal sac (rat/pig) ***In situ* perfusion models** • Small intestine segment of rats • Human intestine ***In vivo* models** • Animals	**Primary hepatocytes model** SCHs of human, rat and mice origin **Hepatoma cell lines** • HepG2 cells • Huh7 cells • HepaRG • LS180 cells **Microsomes** **Precision-cut liver slices**	**Compartment models** • CAT model • ACAT model • ADAN model **Dispersion model**

2.4.1 *In vitro* study models

Most research studies on metabolic herb-drug interactions have been focused on *in vitro* models such as microsomal systems, cytosols, expressed enzymes or cell cultures. These cell culture systems include primary cultures of human hepatocytes, transfected cell lines and tumour derived cells (Venkataramanan *et al.,* 2006). Immortalised cell cultures are continually being used to investigate drug permeability. The reason for this is because primary cultures of enterocyte cells are not able to form polarised monolayers containing specified apical and basolateral surfaces (Alqahtani *et al.,* 2013). However, primary cultures of human hepatocytes can result in better predictions of metabolic herb-drug interactions. In addition, primary cultures of human hepatocytes are viable for a period of up to two weeks and maintain all the co-substrates and co-factors needed for Phase I and Phase II metabolic pathways as well as

transporter function. This makes these models useful to study inhibition and induction of drug metabolising enzymes (Venkataramanan *et al.,* 2006).

In vitro models are popular as they have the potential for high throughput, cost-effectiveness and can display an adequate predictability of the *in vivo* situation. Furthermore, the use of human derived cell lines avoids problems that occur with animal tissues such as inter-species differences. Financial and ethical considerations are among many reasons why the cell culture model has been pursued (Sarmento *et al.,* 2012).

However, the influence of physiological factors such as age, diseases, renal or hepatic dysfunctions and environment conditions cannot be included in the interpretation of the results from the *in vitro* model (Sarmento *et al.,* 2012). The success of the *in vitro* model depends on how strictly the model mimics the conditions presented *in vivo* (Versantvoort *et al.,* 2000).

2.4.1.1 Caco-2 cell line

Caco-2 cells are derived from human colon adenocarcinoma and are one of the most frequently used cell lines in the investigation of drug transport and permeation (Alqahtani *et al.,* 2013). These cells hold many functional and morphological characteristics of normal, differentiated enterocytes when they are cultured as confluent monolayers. Caco-2 cells are cultured in cell-culture flasks and then seeded out onto filters in Transwell® plates, which have been designed specifically for drug uptake studies. Transwell® plates contain an inner well placed in a larger outer well. A semi-permeable membrane is found at the bottom of the inner well on which the cells are grown to confluence (Li, 2001).

Caco-2 cells grow to form a polarized monolayer on the porous membrane and tight junctions are formed and several enzymes and transporters are expressed. A well-defined brush border is formed on the apical surface which expresses digestive enzymes such as lactase, sucrase, isomaltase and alkaline phosphatase (Versantvoort *et al.,* 2000). A schematic illustration of a Caco-2 cell monolayer is illustrated in Figure 2.11 bellow. Caco-2 cells express active transport systems which include carrier-mediated transport systems for peptides, glucose, vitamins, amino acids and bile acids (Alqahtani *et al.,* 2013). Caco-2 cells also express efflux transporter systems (P-gp, MRP1-3), Phase I metabolic enzymes (CYP450) and Phase II conjugating enzymes (UDP-glucuronyltransferase, sulfotransferase, glutathione S-transferase). Caco-2 monolayers polarise more on permeable filter supports than on plastic when cultured for a period of 2-4 weeks and consequently provide a good *in vitro* study model for intestinal drug transport studies (Versantvoort *et al.,* 2000).

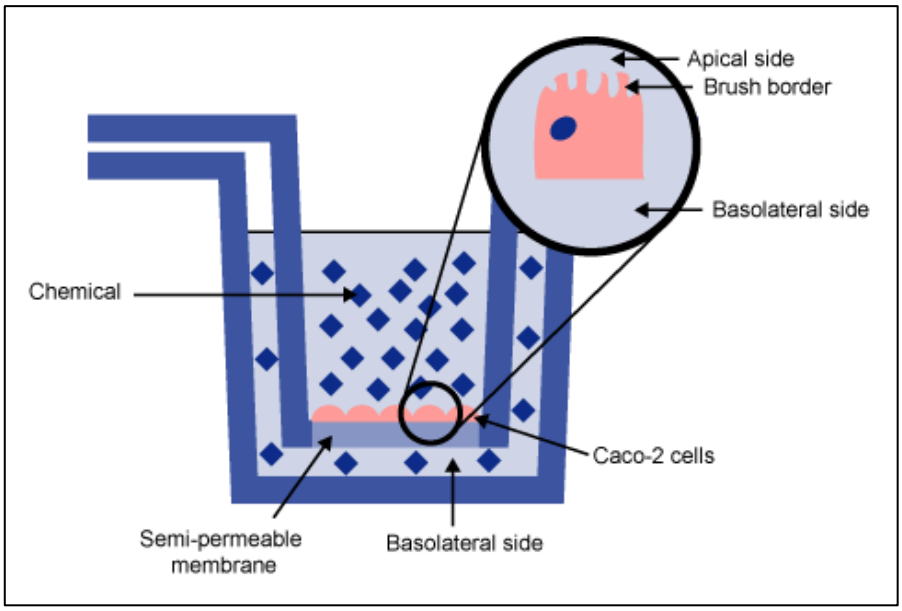

Figure 2.11: Schematic illustration of a Caco-2 cell monolayer on a membrane in a Transwell plate (Li, 2001)

Transport studies are conducted by placing the test compounds in the inner well (apical side) and observing the amount of test compound in the outer well (basolateral side) over a period of time (Li, 2001). There are two methods used to measure the epithelial tight junctions' integrity before transport studies are conducted. First by measuring the transepithelial electrical resistance (TEER) (should be more than 150 ohm/cm^2) or by making use of small hydrophilic molecules such as sucrose or mannitol, which can only pass through the tight junctions via paracellular transport. Caco-2 cells are also included as a model in several studies to test formulations as delivery systems (Alqahtani *et al.,* 2013).

Caco-2 cells have potential for high-throughput screening as culturing the cells in large quantities is relatively simple and reproducible, and the permeability investigation results are also highly reproducible (Tukker, 2000).

The Caco-2 cell line also holds certain disadvantages. Because the cell line has been established on a colonic cell line that may differentiate into small intestinal-like epithelial tissue, it may have tight junctions tighter than that found in the small intestine. The under-prediction of hydrophilic compounds permeabilities with a low *in vivo* uptake could arise. Another disadvantage is that the expression of active transport systems is quantitatively different in numerous transporters when compared to excised intestinal tissue. The less expressed glucose transporter in cultured cells is an example. Expression variation between-laboratory or batch-to-batch can occur as the third disadvantage. A poor prediction of paracellular transport

and carrier mediated compounds with limited cell usefulness thereof may be a consequence of the above (Tukker, 2000).

2.4.1.2 LS180 cell line

The LS180 cell line is also derived from human colon adenocarcinoma. The expression characterization of drug metabolising enzymes and drug transporters in LS180 cells has not been done as extensively as for Caco-2 cells. The level of expression of CYP3A4 and MDR1 is comparable in LS180 and Caco-2 cells and in both cell cultures CYP3A4 can be up-regulated by adding 1α, 25-dihydroxyvitamin D3 to the growth medium. However, the induction of MDR1 has been observed in LS180 cells because PXR is expressed in LS180 cells and not in Caco-2 cells (van de Kerkhof *et al.,* 2007).

The LS180 cell line can be used to investigate the induction of drug metabolising enzymes. It has been shown to be responsive to 1α, 25-dihydroxyvitamin D3, dexamethasone, 3-methylcholantrene, rifampicin, 2,3,7,8-tetrachlorodibenzo-p-dioxin and phenobarbital (van de Kerkhof *et al.,* 2007).

2.4.2 *In vivo* models

Ultimate information regarding oral bioavailability of a compound is derived from *in vivo* models. A wide range of methods is available for determining the bioavailability or intestinal absorption of compounds in both humans and experimental animals. The methods can be classified into three groups, namely intestinal perfusion (in humans), mass balance studies (in humans and animals) and blood kinetics (in humans and animals) (Versantvoort *et al.,* 2000). *In vivo* studies conducted in an appropriate animal model can be of significant value when used in combination with *in vitro* models as it helps to verify *in vivo* relevance (Tang *&* Prueksaritanont, 2010).

After a drug has been orally administrated to an animal model, blood samples are withdrawn at pre-determined time intervals and then analysed to determine the drug concentration at each time point. The plasma level-time profile is then plotted and the area under the curve (AUC) is established as an indication of the bioavailability. *In vivo* animal models that are generally used for the prediction of drug pharmacokinetics include rats, monkeys, dogs and pigs (Alqahtani *et al.,* 2013).

Concerning pharmacokinetics, a crucial consideration for the selection of an animal model is established on the extensive similarities to humans in important biochemical and physiological parameters that govern drug absorption, distribution, metabolism and elimination (ADME) processes (Tang *&* Prueksaritanont, 2010). The presence of the intact system including

lymphatic absorption, mesenteric blood circulation and intestinal membrane is the main advantage of the *in vivo* animal model as it results in a good prediction. Anatomical and physiological differences do, however exist between human and animal models, such as expression of metabolising enzymes, transport proteins, pH, gastrointestinal motility (GI) and gastric emptying rate. Other limitations of the animal *in vivo* model is the requirement of a large amount of resources, labour intensive, time-consuming, not practical for high-throughput screening and the difficulty studying individual drug absorption mechanisms (Alqahtani *et al.,* 2013).

Ethical issues should be considered in terms of the animal sacrifice, pain and discomfort. The principle of 3Rs (Replacement, Reduction and Refinement) acknowledges these aspects. In essence this principle strives to replace animal usage with non-animal models where possible, reduce the amount of animals required by enhanced study designs and decrease pain and stress by refining methodologies (Sjögren *et al.,* 2014).

2.4.2.1 The rat model

The rat is the most common species used for animal studies (Butterweck *&* Derendorf, 2008). There are differences between the rodent and human physiology, for instance rodents are nocturnal animals which warrants considerations concerning dose timing and the possibility of disrupting their natural circadian rhythm. Rodents are also susceptible to co-prophagy and so re-uptake of faecal excreted drugs can occur. A higher metabolism is generally observed in rodents when compared to humans (Sjögren *et al.,* 2014).

The absorption rate of Biopharmaceutics Classification System (BCS) class 1 drugs in rats is similar to that in humans. The rat's gastric pH is often higher than that of humans and has an increased gastric secretion. The small intestines pH increases from the duodenum to the terminal ileum within a range similar as found in humans (pH 4.5-7.5). As far as epithelial permeability is concerned in the small and large intestine, the rat has been used as a model in numerous *in situ* perfusion experimental studies and is considered to be the most appropriate animal model to predict human permeability and intestinal absorption. It has been observed that the jejunal permeability of the rat correlates strongly with human jejunal permeability (Sjögren *et al.,* 2014).

GeneChip techniques have been used to determine rat and human jejunal permeability and examine expression levels of metabolic enzymes and transporters. The investigations have found a good correlation between the human and rat permeability (R^2 = 0.8), moderate correlation for expression levels of transporter in the duodenum (R^2 > 0.56), but no correlation

with regards to levels of metabolising enzymes (Sjögren *et al.,* 2014). Based on data from the small and large intestine, it has also been reported that the Ussing chamber with rat tissue may be a useful model for the prediction of human intestinal absorption (Sjögren *et al.,* 2014).

2.4.2.1.1 Herb-drug interaction studies involving the rat model

Grapefruit juice has been shown to inhibit CYP3A4 *in vitro* and this inhibition may be attributed to high concentrations of bioflavonoids and furanocoumarins. Data has suggested that cyclosporin-A metabolism can be inhibited by grapefruit juice. The main mechanism to identify grapefruit juice inhibitory compounds has been conducted in rat microsomes. Findings suggest that rats may be a good model for the investigation of herb-drug interactions (Mangano *et al.,* 2001). Garlic oil and its organosulfur compounds, diallyl sulfide, diallyl disulfide and diallyl trisulfide, were demonstrated to potentially increase the protein content and levels of mRNA of CYP1A1, CYP2B1 and CYP3A1 in rats. Diallyl sulphide has the potential to inhibit the expression and activity of CYP2E1. Red garlic extracts have been shown, in rat liver slices, to cause a significant inhibition of saquinavir efflux and a substantial increase in the activity of darunavir efflux transporters (Shi *&* Klotz*,* 2012).

Ginkgo biloba extracts have been demonstrated in rats to inhibit or induce various CYP enzymes depending on the constituents or compositions of the extracts. At a concentration of 100-2500 ng/mL in rat primary hepatocytes, *G. biloba* extracts showed (in a dose-dependent manner) a significant induction of activity, protein expression and mRNA expression of CYP3A4 (Shi *&* Klotz*,* 2012). In the rat model, *Panax ginseng* (150 mg/kg/day) was administrated for 14 days and a decreased AUC of oral fexofenadine by 51% ($p<0.005$) was observed. The C_{max} was decreased by 75% ($p<0.001$) and the ratio concentrations of brain to plasma were also significantly decreased ($p<0.05$). This indicated that *P. ginseng* administered over a long term may induce the expression of brain endothelium and intestinal P-gp (Zhang *et al.,* 2009).

Pharmacokinetic interactions between indinavir and St John's Wort that was given at a concentration of 150 or 300 mg/day for 15 days have been observed in rats. This was established to be as a result of CYP3A4 induction. Administration of St John's Wort, 100 mg/kg/day for a period of 10 days, in rats demonstrated a substantial induction of CYP3A2 and CYP2D2, as well as CYP2C6 inhibition. St John's Wort extracts administered in rats for 14 days displayed an increase in P-gp/MDRI expression 3.8-fold and a hepatic CYP3A2 2.5-fold expression (Shi *&* Klotz*,* 2012).

2.5 SUMMARY

The use of herbal medicines has gained popularity over the past few decades. Herbal medicine usage is common amongst HIV-infected patients who are receiving ARV therapy and often without the approval or knowledge of their health care provider. The interactions between herbal medicine and prescribed drugs remain a safety concern. Pharmacokinetic interactions include various mechanisms that have an influence on drug absorption and metabolism such as the inhibition or induction of CYP enzymes and drug transporters. Many herbs and drugs are substrates for both CYP3A4 and P-gp and their effect on these metabolic and efflux pathways have been studied. The possible pharmacokinetic interactions occurring as a result of the concomitant use of herbal medicines and prescribed drugs should be further investigated for clinical importance.

CHAPTER 3

METHODS AND MATERIALS

3.1 INTRODUCTION

The results obtained from *in vitro* studies can predict potential herb-drug interactions that will be experienced *in vivo.* Model systems that are commonly used to investigate metabolism include microsomes, recombinant enzymes and hepatocytes. Epithelial cell cultures such as Caco-2 or MDCK cell lines are typically used to investigate bi-directional transport activity (Brantley *et al.,* 2014).

The Caco-2 cell line is derived from human colon adenocarcinoma, which has been the most popular model used to simulate intestinal epithelium monolayers and to predict drug transport. Caco-2 epithelial cells form tight junctions, differentiate to distinguish between the apical and basolateral sides and express several appropriate efflux transporters such as P-gp (Sarmento *et al.,* 2012). The Caco-2 cell line was selected as the *in vitro* model for the transport experiments in this study.

In vivo models are essential to determine drug bioavailability and to demonstrate an estimate of exposure to metabolites after parent drug administration as well as the influence of co-administered compounds on drug bioavailability. Furthermore, the contribution of an enzymatic pathway to the overall elimination can be predicted (Brantley *et al.,* 2014). *In vivo* animal models are typically used to predict drug pharmacokinetics. After oral administration, the plasma concentration-time profiles are plotted and the area under the curve (AUC) is calculated in order to indicate drug bioavailability. *In vivo* animal models that are commonly used for pharmacokinetic studies include dogs, monkeys, pigs and rats (Alqahtani *et al.,* 2013). The Sprague-Dawley rat model was selected as the *in vivo* model for bioavailability experiments in this study.

3.2 MATERIALS

The materials used in this study were indinavir (Crixivan®, Merck, Kenilworth, NJ, USA) (negative control), and verapamil (donated by Novartis, Kemptonpark, South Africa) and ketoconazole (Aspen, Woodmead, South Africa) which served as the two positive control groups for the *in vitro* bi-directional transport study and pharmacokinetic *in vivo* study. The *Hypoxis hemerocallidea* test materials consisted of a solid oral commercial product (product name available on request) purchased from a local health shop (Potchefstroom, South Africa),

dried plant reference material (ChromaDex®, Cape Town, South Africa) and an aqueous extract. The aqueous extract was prepared by weighing approximately 5 g of dried *H. hemerocallidea* plant material accurately in a 50 ml Erlenmeyer flask. A volume of 10 ml of deionized water was added and the mixture was sonicated at 45 °C for 30 min. After filtering the mixture through a filter paper (No 4, Whatman Ltd, England), the filtrate was kept aside and the residue returned to the flask. This process was repeated twice, where after the filtrates were combined and the residue discarded. The water filtrate was frozen and subsequently freeze-dried overnight and the extract (0.17 g) was stored in a desiccator.

3.3 HIGH PERFORMACE LIQUID CHROMATOGRAPHY ANALYTICAL METHOD FOR INDINAVIR SAMPLES FROM THE *IN VITRO* TRANSPORT STUDIES

A high performance liquid chromatography (HPLC) analysis method that has been previously developed was used to analyse indinavir concentrations in the *in vitro* transport samples (Roos, 2012; Calitz, 2014). Linearity, accuracy, precision and ruggedness were established to confirm that the method was valid, suitable and applicable for the current study.

3.3.1 Chromatographic conditions

The analysis of indinavir was performed on an HP1100n chromatograph which was equipped with a UV detector and Chemstation Rev.A.10.01 Agilent® Technologies data acquisition and analysis software (Hewllet-Packard Paulo Alto, California, USA). The indinavir separation was performed on a Venusil XBP C18(2) column, 150 x 4.6 mm, 5 µm, 100 Å (Agela technologies, Wilmington, Delaware, USA). A mixture of 50% acetonitrile and 0.1% ammonium formate (NH_4HCO_2) in water that has been adjusted to pH 7 was used as mobile phase in isocratic elution mode with an injection volume of 25 µL and a flow rate of 1 ml/min. The UV detector was set on a wavelength of 210 nm and the indinavir retention time was 4.6 min.

3.3.2 Linearity

Linearity is defined as the ability of the analytical method to produce test results (i.e. response values) that are directly proportional to the analyte concentration within a given range. The linear regression curve y-intercept and the correlation coefficient (R^2) establish an indication of the acceptability of linearity. A value of R^2 that is higher than 0.998 is considered as evidence of an acceptable fit of the data to the line of regression (Shabir, 2003; Singh, 2013). A standard solution was prepared by dissolving 400 mg indinavir in 500 ml Dulbecco's Modified Eagle's Medium (DMEM) (800 µg/ml), which then was further diluted to samples of 80 µg/ml, 8 µg/ml and 0.8 µg/ml. Different volumes (5, 10, 15, 20, 25 µl) of the diluted sample solutions were

injected into the HPLC in order to construct a standard curve by plotting the peak area as a function of concentration.

3.3.3 Accuracy

Accuracy is defined as the closeness of the test result of the analytical method to the true value (Shabir, 2003). It is presented by an inverse relation to systematic as well as random errors, where a higher accuracy means a lower error (Singh, 2013). An assay method criteria for accuracy is that 100 ± 2 % at each concentration over the range of 80 – 120 % of the target concentration will be the mean recovery (Shabir, 2003). A solution was made by dissolving 400 mg indinavir in 500 ml ultra-pure water. The standard solution consisted of 10 ml of this solution which was then diluted to 100 ml (80 µg/ml). Sample solutions were obtained by diluting 4 ml, 6 ml and 8 ml of the solution to a volume of 100 ml. Concentrations of 32 µg/ml, 48 µg/ml and 64 µg/ml were obtained. The solutions were withdrawn and transferred in vials and placed into the chromatograph auto sampler for analysis.

3.3.4 Precision

Precision can be defined as the measurement of the degree of repeatability of an analytical method conducted under normal operation conditions. Precision is usually expressed as the percentage relative standard deviation (%RSD) for a number of samples (Shabir, 2003). Precision is sub-divided into intra-day variation (the evaluation of the same batch on the same day) and inter-day variability. Intra-day precision, which can also be referred to as repeatability, is the results of the method operating under the same conditions over a relatively short period of time (Shabir, 2003; Singh, 2013). Inter-day precision is the results obtained within-laboratory variations which can be due to factors such as different day, equipment and analysts. The acceptance criteria for precision have limits of the RSD for intra-day repeatability of 5% and the inter-day precision of 10% (Shabir, 2003).

Intra-day precision was determined by producing a solution made of 400 mg indinavir dissolved in 500 ml ultra-pure water. The standard solution consisted of 10 ml of this solution which was then diluted to 100 ml (80 µg/ml). Sample solutions were obtained by diluting 4 ml, 6 ml and 8 ml of the solution to a volume of 100 ml. Concentrations of 32 µg/ml, 48 µg/ml and 64 µg/ml were obtained. The solutions were withdrawn at different time points on one day and transferred in vials and placed into the chromatograph auto sampler. Multiple injections were made on one day under the prescribed conditions. Inter-day precision was determined in the same way, but over three consecutive days. A standard solution (80 µg/ml) and three sample solutions (48 µg/ml) were prepared. Volumes of 5, 10, 15, 20 and 25 µl of the standard solution

were injected in duplicate to obtain a calibration curve. Precision was determined by conducting a HPLC analysis on the samples prepared over three consecutive days. A volume of 20 µl of the sample was injected eight times over one day and this procedure took place for three consecutive days.

3.3.5 Ruggedness

Ruggedness is defined by the USP (2015) as the degree of reproducibility of the results obtained by analysis of the same samples under diverse conditions such as different laboratories, analysts and instruments. Ruggedness is expressed as %RSD. The ruggedness acceptance criteria for stability are that sample solutions should not be used for a time period longer than it takes to degrade by 2%. The peak area and retention time with a %RSD of 2% or less is regarded as acceptable for system repeatability. A solution consisting of 400 mg indinavir dissolved in 500 ml ultra-pure water was made. The sample consisted of 5 ml of this solution which was then diluted to 50 ml. The sample was left on the autosampler tray and re-analysed over several time intervals (24 h) to determine the sample stability.

3.4 LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY ANALYTICAL METHOD FOR INDINAVIR SAMPLES FROM THE *IN VIVO* STUDY

A liquid chromatography tandem mass spectrometry (LC/MS/MS) method was used for the analysis of indinavir in the plasma samples. A stock solution of indinavir was prepared in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml. Blank Sprague-Dawley plasma was spiked with the stock solution to attain standard 1 (STD 1) at a concentration of 2000 ng/ml. STD 2 (500 ng/ml), STD 3 (125 ng/ml), STD 4 (32 ng/ml), STD 5 (8 ng/ml) and STD 6 (2 ng/ml) were produced by diluting blank plasma. Quality control samples were prepared in the same pool of rat plasma at concentrations of 1600 ng/ml, 400 ng/ml, 50 ng/ml, 10 ng/ml and 2 ng/ml. The quality control samples and calibration standards were vortexed briefly, aliquotted into labelled polypropylene tubes and then stored at -80°C. Samples above the upper limit of quantification were diluted 4 times with blank plasma and re-analysed in a repeat batch.

3.4.1 Chromatography

The liquid chromatography was performed with a Kinetex F5 (4.6 x 100 mm, 2.6 µm) column using an Agilent 1100 series high performance liquid chromatograph. The mobile phase consisted of a mixture of A and B at 50:50 v/v; where mobile phase A was 0.1% v/v formic acid in water and B was 0.1% v/v formic acid in acetonitrile delivered at a flow rate of 500 µl/min. The column was kept in a column compartment at 40 °C. An auto-sampler injected 10 µl onto

the HPLC column. The injection needle was rinsed with mobile phase before each injection for 30 s using the flush port wash station. The samples were cooled to 4 °C while awaiting injection.

3.4.2 Detection

The detection of indinavir and the internal standard (indinavir-d6) was performed on an AB Sciex API 3200 mass spectrometer (ESI in the positive ion mode, MRM) and the settings on the apparatus are summarised in Tables 3.1 and 3.2. The mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions at m/z 614.4, m/z 620.5 to the product ions at m/z 421.3 and m/z 421.2 for indinavir and the internal standard, respectively.

Table 3.1: Ionisation source setting

Electro Spray Ionisation Settings	Value
Nebulizer gas (Gas 1) (arbitrary unit)	50
Turbo gas (Gas 2) (arbitrary unit)	40
CUR (curtain gas) (arbitrary unit)	20
CAD (collision gas) (arbitrary unit)	3
TEM (source temperature) (°C)	500
IS (Ion Spray Voltage) (V)	3500

Table 3.2: MS/MS detector setting

MS/MS Settings	Indinavir	ISTD
Protonated molecular mass (*m/z*)	614	620
Product ion molecular mass (*m/z*)	421	421
Dwell time (ms)	200	200
DP (declustering potential) (V)	45	40
EP (entrance potential) (V)	12	12
CEP(collision cell entrance potential) (V)	35	25
CE (collision energy) (eV)	50	45
CXP (collision cell exit potential) (V)	11	8
Scan Type	MRM	
Polarity	Positive	

Pause time	5 ms

3.4.3 Extraction from plasma

A volume of 200 µl of ice cold 0.1% v/v formic acid in methanol containing 20 ng/ml indinavir-d6 (ISTD) was added to a 20 µl of plasma sample and vortex mixed for 60 s. This was followed by ultra-sonication for 10 min and centrifugation at 10000 rpm for 10 min. Then 180 µl of the supernatant was transferred into a clean culture tube and evaporated to dryness under nitrogen gas at 40 ^{o}C. The residue was reconstituted with 100 µl of 0.1% v/v formic acid in water solution, vortex mixed for 60 s and transferred into a 96-well plate for injection. A volume of 10 µl of the reconstituted solutions were then injected onto the column.

3.5 LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY ANALYTICAL METHOD FOR PHYTOCHEMICAL CHARACTERISATION OF THE *HYPOXIS HEMEROCALLIDEA* MATERIALS

Ultra-Performance Liquid Chromatography (UPLC) analysis was used to determine the hypoxoside concentration in the selected *Hypoxis hemerocallidea* materials. The analysis was done with a Waters Acquity Chromatographic system with PDA detector (Waters, Milford, MA, USA). UPLC separation was achieved on an Acquity UPLC BEH C18 column (150 mm × 2.1 mm, i.d., 1.7 µm particle size, Waters) maintained at 40 °C. The mobile phase consisted of 0.1% v/v formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 ml/min. A gradient elution was used as follows: 85% A: 15% B to 65% A: 35% B in 7 min, changed to 50% A: 50% B in 1 min, keeping for 0.5 min and back to initial ratio in 0.5 min. The running time was 11 min. The standard and samples were injected in the mobile phase with an injection volume of 1.0 µl (full-loop injection). Mass spectrometry was operated in negative ion electrospray mode. Nitrogen (N_2) was used as the desolvation gas. The desolvation temperature was set to 350 °C at a flow rate of 500 L/Hr and the source temperature was 100 °C. The capillary and cone voltages were 2500 and 45 V, respectively. The data was collected between 100 and 1000 m/z.

3.6 PREPARATION OF SOLUTIONS FOR THE *IN VITRO* TRANSPORT STUDY

3.6.1 Preparation of the indinavir solution (negative control group)

The negative control group for the transport studies consisted of a 200 µM indinavir solution. In order to prepare this solution for the transport study, a Crixivan® capsule (containing 400 mg indinavir) was opened and 2.02 mg of the capsule contents was weighed and added to 10 ml

DMEM (for the permeation study in the A-B direction) and to 10 ml DMEM and HEPES solution (for the permeation study in the B-A direction).

3.6.2 Preparation of verapamil solution (positive control group)

Verapamil is a known P-gp inhibitor and was therefore used as the positive control group for the transport studies. A 100 µM verapamil solution was prepared by dissolving 0.45 mg verapamil in 5 ml DMEM containing 200 µM indinavir for the permeation study in the A-B direction (and 5 ml DMEM and HEPES solution containing 200 µM indinavir for the permeation study in the B-A direction).

3.6.3 Preparation of the test solutions for the transport studies

A final concentration of 500 µg/ml of each of the selected *H. hemerocallidea* materials were used for the transport studies (Fasinu *et al.,* 2013). A double strength of this concentration was prepared as it was diluted when combined with the indinavir solution. For the commercial product, this was obtained by weighing 6.729 mg of the capsule contents (to compensate for additives in the capsule formulation according to the label) and adding it to 5 ml DMEM for the permeation study in the A-B direction and to 5 ml DMEM and HEPES solution for the permeation study in the B-A direction. For the *H. hemerocallidea* aqueous extract and dried plant reference material, 5 mg of each of these materials were added to 5 ml DMEM for the permeation study in the A-B direction and 5 mg were added to 5 ml DMEM and HEPES solution for the permeation study in the B-A direction. These materials were dissolved in combination with 200 µM indinavir which was applied to the donor chamber of the Transwell® plates containing Caco-2 cell monolayers during the bi-directional transport study.

3.7 TRANSPORT STUDIES

3.7.1 Caco-2 cell culturing

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC), by Sigma Aldrich, Johannesburg, South Africa. The Caco-2 cells were subsequently cultured in high-glucose Dulbecco's Modified Eagles Medium (DMEM) (Separations, Randburg, South Africa) supplemented with 10% v/v foetal bovine serum (The Scientific Group, Johannesburg, South Africa), 1% v/v non-essential amino acids (NEAA) (Whitehead Scientific, Cape Town, South Africa), 1% v/v penicillin/streptomycin (Separations, Johannesburg, South Africa), 1% v/v of 2 mM L-glutamine (Whitehead Scientific, Cape Town, South Africa) and 1% v/v amphotericin B (250 µg/ml) (The Scientific Group, Randburg, South Africa).

The Caco-2 cells were cultured at a temperature of 37°C with 5% carbon dioxide and 95% humidified air in a Galaxy 170R incubator (Eppendorf Company, Stevenage, UK). The growth medium was exchanged every second day under sterile conditions in a laminar flow hood. The cells were examined by means of a light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan) prior to exchange of the growth medium. The percentage confluency was estimated and the absence of any contamination was ensured by macroscopic visual evaluation. The growth medium was decanted and removed from the cell culture flask and thereafter replaced with 10 ml pre-warmed growth medium by means of a serological pipette and pipettor.

3.7.2 Sub-culturing of the Caco-2 cells

The Caco-2 cells were sub-cultured by means of trypsinisation upon reaching 50% confluency. The growth medium and phosphate buffered saline (PBS) (Sigma Aldrich, Johannesburg, South Africa) solutions were pre-warmed to 37°C in a circulating water bath. The growth medium was decanted and removed from the cell culturing flask and the cells were rinsed twice with 10 ml PBS by using a pipettor and serological pipettes. A volume of 3 ml Typsin-Versene mixture (Whitehead scientific, Cape Town, South Africa) was supplemented to the cell culturing flask and the mixture was evenly distributed on the cell layer. The cell flask was placed in the CO_2 incubator and incubated for 5 min at 37°C. After the 5 min incubation time, the flask was removed and tapped. After ensuring complete detachment of the cells, 6 ml pre-warmed growth medium was added to the trypsin mixture to inactivate its action. The cell suspension was gently agitated by means of a pipette to ensure that all the cells were rinsed from the bottom of the flask. The cell suspension was then divided to a ratio of 1:4 and hereafter 10 ml pre-warmed growth medium was added to each flask. The flasks were returned back to the CO_2 incubator to grow under normal cell culturing conditions.

3.7.3 Seeding of Caco-2 cells

Caco-2 cells were seeded onto Transwell® 6-well membrane filters (Corning Costar® Corporation, Tewksbury, USA) with a surface area of 4.67 cm^2 and a pore diameter of 0.4 µm. As described previously, the cell suspension was obtained by trypsinisation with Trypsin-Versene. After cell detachment, 6 ml of pre-warmed growth medium was added to the flask. The cell suspension was extensively agitated with a pipette to ensure complete cell detachment and de-agglomeration in order to form a suspension consisting of single cells. This single cell suspension was then transferred to a 50 ml tube. A Pasteur pipette was used to agitate the cell suspension to make sure that a homogenous cell distribution is present. A haemocytometer was used to count the cells in the suspension after Trypan blue was added. Using a pipette,

10 µl cell suspension together with 15 µl PBS and 25 µl Trypan blue (Sigma Aldrich, Johannesburg, South-Africa) were mixed in a 2.5 ml Eppendorf tube in order to count the cells. The mixture was left for 3 min and thereafter 10 µl of the mixture was placed on either side of the cover slip of the haemocytometer. A light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan) was used to count the cells. The cells were counted on 5 of the 9 squares on the grid of the haemocytometer on each side. The average number of cells per square was calculated and this number was then multiplied with the dilution factor (5×10^4). This established the total number of cells per ml in the cell suspension. The cell suspension was diluted to a concentration of 20 000 cells per ml for seeding onto the Transwell® filter membranes.

Seeding of the Caco-2 cells onto Transwell® filter membranes took place under sterile conditions in a laminar flow hood by pipetting a volume of 2.5 ml final cell suspension into each apical chamber of the Transwell® plates. In order to produce intact epithelial monolayers, the Caco-2 cells were grown for 21 to 24 days on the membrane filters. The growth medium was replaced every second day while under sterile conditions in a laminar flow hood.

3.7.4 Bi-directional transport studies

The transepithelial electrical resistance (TEER) of each cell monolayer was measured with a Millcell ERS II meter (Millipore, Billerica, Massachusetts, USA) at the start of the transport study, which served as an indication of the integrity of the cell monolayers. A TEER reading higher than 250 Ω (equivalent to 1167.5 Ω/cm^2) was required before conducting the transport study.

3.7.4.1 Transport in the apical-to-basolateral direction

For the transport study in the apical to basolateral (AP-BL) direction, the growth medium was removed from the basolateral chambers of the Transwell® plates using an aspirator. A volume of 2.5 ml pre-heated transport medium (DMEM) buffered with 25 mM 2-4-2-hydroxyethyl piperazin-1-yl-ethanesulfonic acid (HEPES) was added to the basolateral compartments and then the plate was placed in the CO_2 incubator at 37°C for 30 min. The growth medium was hereafter removed from the apical chambers and replaced with 2.5 ml test solutions (i.e. indinavir with or without the selected *H. hemerocallidea* material) in DMEM. The negative control group consisted of indinavir alone (200 µM) and indinavir combined with verapamil (100 µM) (a known P-gp inhibitor) as the positive control group. A sample volume of 200 µl of each test solution was withdrawn at time 0 min. Samples (200 µl) were withdrawn from the basolateral chambers at time intervals of 20, 40, 60, 80, 100, and 120 min for the transport study in the A-B direction. Each sample withdrawn was replaced with an equal volume of pre-

warmed DMEM buffered with HEPES. The plates were incubated in a CO_2 incubator at 37°C between the withdrawals. The TEER was measured again at the end of the AP-BL transport study to confirm that the cell monolayer integrity was still intact after the cells have been exposed to the test and control solutions. Samples were analysed by a validated high performance liquid chromatography (HPLC) analysis method to determine the indinvair concentration.

3.7.4.2 Transport in the basolateral-to-apical direction

For the transport in the basolateral to apical (BL-AP) direction, the growth medium was removed from the apical chambers of the Transwell® plates using an aspirator. A volume of 2.5 ml pre-warmed DMEM was added to the apical chambers of the Transwell® plates and the plate was thereafter incubated in a CO_2 incubator for 30 min at 37°C. The growth medium was removed from the basolateral chambers and replaced with 2.5 ml of each test solution (i.e. indinavir with or without the selected *H. hemerocallidea* material) in DMEM buffered with HEPES. Indinavir alone served as the negative control group, while indinavir combined with verapamil served as the positive control group. A test solution sample (200 µl) was withdrawn at time 0 min, while samples (200 µl) were withdrawn from the apical chambers at the predetermined time intervals of 20, 40, 60, 80, 100, 120 min for the transport study in the B-A direction and immediately replaced with an equal volume of pre-warmed DMEM. The TEER was measured again at the end of the BL-AP transport study, which provided an indication of the cell monolayers integrity. The samples were analysed by a validated high performance liquid chromatography (HPLC) analysis method to determine the indinavir concentration.

3.8 *IN VIVO* PHARMACOKINETIC STUDY

3.8.1 Animal selection and study design

A total of 45 male Sprague-Dawley rats weighing 250 - 300 g were randomly selected and divided into 9 different groups (i.e. 3 control groups, 3 acute experimental groups and 3 chronic experimental groups), which consisted of 5 animals per group (as schematically illustrated in Figure 3.1). During the acute herb-drug pharmacokinetic interaction study, a single administration of each test solution was given to each rat in concentrations outlined in Figure 3.1 and described in section 3.8.2 below. During the chronic herb-drug pharmacokinetic interaction study, daily administrations were given over a total period of 14 days in concentrations outlined in Figure 3.1 and described in section 3.8.2 below (de Peyster *et al.,* 2003; Husna *et al.,* 2013; Robertsa *et al.,* 2016).

This *in vivo* study in Sprague-Dawley rats was approved by the Animal Ethics Committee of the Faculty of Health Sciences of the University of Cape Town (FHC AEC Ref no: 015/041).

Control groups:	**Group 1** Indinavir alone (40 mg/kg) (Negative control) n = 5	**Group 2** Indinavir (40 mg/kg) with ketoconazole (40 mg/kg) (Positive control - Metabolism) n = 5	**Group 3** Indinavir (40 mg/kg) with verapamil (9 mg/kg) (Positive control - Efflux) n = 5
Experimental groups:	**Group 4 & 5** Indinavir (40 mg/kg) with *H. hemerocallidea* aqueous extract (15 mg/kg) **4:** n = 5 (acute, single administration) **5:** n = 5 (chronic, 14 days)	**Group 6 & 7** Indinavir (40 mg/kg) with *H. hemerocallidea* commercial product (15 mg/kg) **6:** n = 5 (acute, single administration) **7:** n = 5 (chronic, 14 days)	**Group 8 & 9** Indinavir (40 mg/kg) with *H. hemerocallidea* reference material (15 mg/kg) **8:** n = 5 (acute, single administration) **9:** n = 5 (chronic, 14 days)

Figure 3.1: Schematic illustration of the layout of the *in vivo* study design in Sprague-Dawley rats. Dosing concentrations were selected based on previous studies (Van Wauwe *et al*., 1990; Cools *et al*., 1992; Mogatle *et al*., 2008; Choi *et al.,* 2009; Ho *et al*., 2009)

3.8.2 Administration of test solutions to rats

The test solutions were administered by means of an oral gavage technique at a volume of 500 µl per animal. The test solutions consisted of *H. hemerocallidea* materials (i.e. *H. hemerocallidea* reference plant material, an aqueous extract and commercial oral product) at a concentration of 15 mg/kg (Mogatle *et al.,* 2008), together with indinavir (40 mg/kg) in distilled water. All *H. hemerocallidea* materials were administrated in the same concentration to the animals in order to measure the effects of the difference in chemical composition (e.g. hypoxoside content) of the selected materials on indinavir bioavailability. Indinavir (40 mg/kg) administered alone served as the negative control (Ho *et al*., 2009). The positive control for metabolism inhibition consisted of indinavir (40 mg/kg) with ketoconazole (40 mg/kg) (Van

Wauwe *et al.,* 1990; Cools *et al.,* 1992) and the positive control for transport (efflux inhibition) was indinavir (40 mg/kg) with verapamil (9 mg/kg) (Choi *et al.,* 2009).

3.8.3 Blood sampling

Blood samples (200 µl) were collected from the tail veins of the animals at the predetermined time intervals of 0, 0.5, 1, 2, 4, 8 and 24 h after each experimental and control test solution. Sample tubes were sprayed with heparin beforehand, which served as an anti-coagulant. The blood samples were then centrifuged for 8 min at 14 000 rpm, after which the plasma was recovered from each blood sample. The plasma samples were kept at -80°C until the indinavir analysis was done with LC/MS/MS (as describe in section 3.3).

3.9 DATA ANALYSIS

3.9.1 Transport data for indinavir

The apparent permeability coefficient (P_{app} x 10^{-6}cm/s) values for indinavir transport in the AP-BL and BL-AP direction across the Caco-2 monolayers were calculated according to the following equation (Tarirai *et al.,* 2012).

$$P_{app} = \frac{dQ}{dt}\left(\frac{1}{A \cdot C_{o} \cdot 60}\right) \qquad \text{(Eq. 1)}$$

Where P_{app} is the apparent permeability coefficient ($cm.s^{-1}$), dQ/dt is the permeability rate (amount permeated per minute), A is the diffusion area of the membrane (cm^2) and C_0 is the initial concentration of the model drug.

The efflux ratio (ER) values demonstrate any asymmetry regarding indinavir directional transport in combination with the *H. hemerocallidea* materials. The ER was calculated according to the following equation (Tarirai *et al*., 2012):

$$ER = P_{app}(BL\text{-}AP)/P_{app}(AP\text{-}BL) \qquad \text{(Eq. 2)}$$

Where P_{app} (BL-AP) is the permeability coefficient for the permeation in the basolateral to the apical direction and P_{app} (AP-BL) is the permeability coefficient for the permeation in the apical to basolateral direction.

3.9.2 Pharmacokinetic data analysis for *in vivo* study model

The bioavailability profiles were created by using WinNonlin software (Pharsight Corporation, California USA) to gather the pharmacokinetic parameters of relevance (i.e. peak plasma

concentration (C_{max}) and area under the curve (AUC)). The relative bioavailability (F_{rel}) of indinavir was calculated by the following equation:

$$F_{rel} = \frac{[AUC]A}{[AUC]B} \qquad \text{Eq. (4)}$$

Where [AUC]A represents the area under the curve for indinavir in the presence of the experimental material (i.e. *H. hemerocallidea* materials) and [AUC]B represents the area under the curve for indinavir alone.

3.9.3. Statistical Data Analysis

Data analyses were performed by using STATISTICA Ver 12. Analysis of variances (ANOVA's) were conducted with Tukey's Honest significant post-hoc tests and statistically significant differences were accepted when $p < 0.05$. All results obtained were verified with nonparametric Kruskall-Wallis and Dunn's post-hoc tests.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

In this study, both *in vitro* and *in vivo* pharmacokinetic herb-drug interactions between selected *Hypoxis hemerocallidea* materials (i.e. an aqueous extract, dried plant reference material and commercial oral product) and indinavir were investigated. The Caco-2 cell line was employed as the *in vitro* model to investigate the potential modulation of indinavir transport by the selected *H. hemerocallidea* materials, specifically efflux modulation concerning P-glycoprotein (P-gp) inhibition. The Sprague-Dawley rat model was employed as the *in vivo* model to study the possible influence of the selected *H. hemerocallidea* materials on the bioavailability of indinavir after oral administration.

The *in vivo* bioavailability study was divided into acute and chronic sub-studies. During the acute study, each rat received a single administration of each *H. hemerocallidea* experimental test solution together with indinavir, while multiple administrations were given daily over a total period of 14 days during the chronic study.

METHOD VALIDATION RESULTS

4.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Linearity, accuracy, precision and ruggedness for the indinavir analysis method were confirmed even though a previously validated HPLC analytical method was used.

4.2.1 Linearity

Linearity is defined as the method's ability to produce a response that is directly proportional to the analyte concentration within a given range (Shabir, 2003; Singh, 2013). A correlation coefficient (R^2) that is higher than 0.998 is indicated as an acceptable fit of the data to the line of response plotted as a function of concentration. An R^2 value of 0.999 was obtained for the calibration graph (Figure 4.1) that was constructed for indinavir with the HPLC method employed in this study. It can be concluded that the HPLC method used for analysis of indinavir in the *in vitro* transport samples complies with the criteria for linearity.

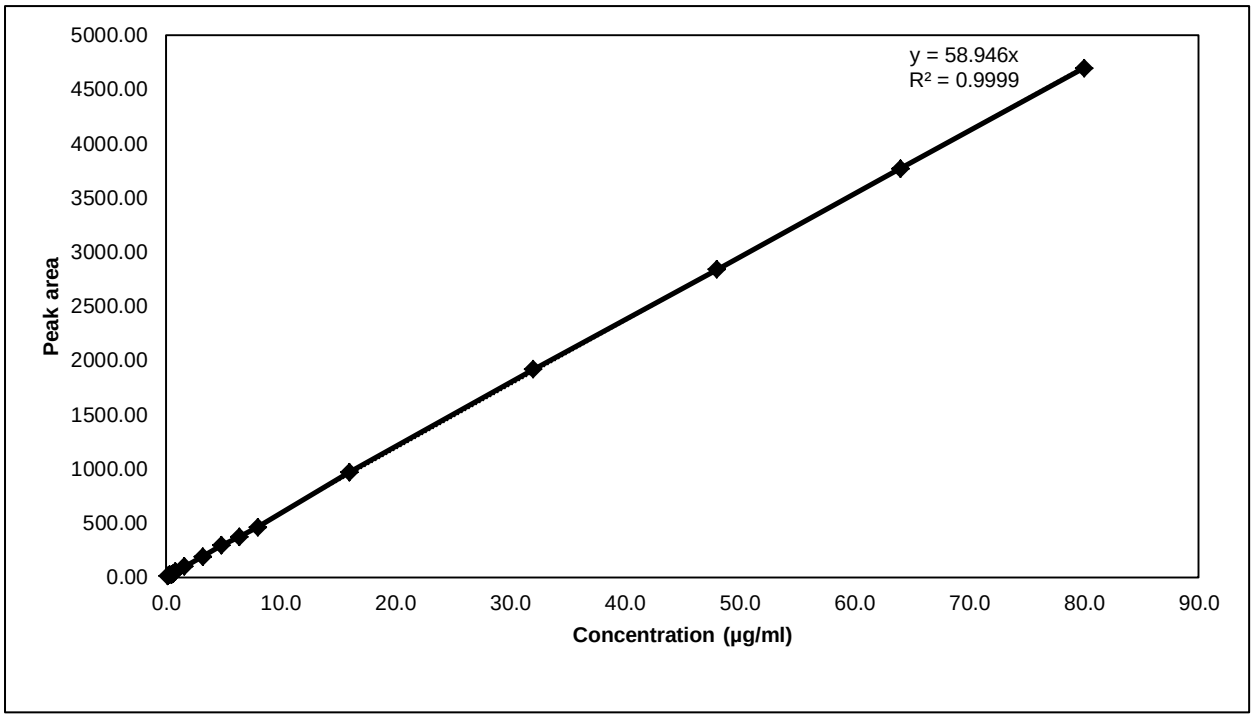

Figure 4.1: Calibration curve obtained for indinavir with high performance liquid chromatography where peak area is plotted as a function of concentration

4.2.2 Accuracy

Accuracy is defined as the closeness of the obtained test results by the analytical method to the true value (Shabir, 2003). Recovery must be between 98% to 102% and over the range of 80% to 120% of the sample concentration. The HPLC method employed for indinavir analysis yielded a mean recovery of 101.4% with a %RSD of 1.2% as indicated in Table 4.1.

Table 4.1: Data obtained for accuracy of indinavir analysis with high performance liquid chromatography

Concentration spiked (µg/ml)	Area 1	Area 2	Mean area	Recovery (µg/ml)	Recovery (%)
32.0	1490.8	1489.4	1490.1	31.9	99.8
32.0	1487.6	1490.0	1488.8	31.9	99.7
32.0	1485.7	1487.0	1486.4	31.9	99.6

48.0	2273.4	2266.8	2270.1	48.9	101.9
48.0	2270.1	2265.0	2267.6	48.9	101.8
48.0	2265.9	2263.2	2264.6	48.8	101.6
64.0	3042.7	3043.3	3043.0	65.7	102.7
64.0	3040.9	3038.9	3039.9	65.7	102.6
64.0	3040.2	3036.5	3038.4	65.6	102.5
Mean					101.4
***SD**					1.2
****% RSD**					1.2

*SD refers to the standard deviation, **% RSD refers to the percentage relative standard deviation

4.2.3 Precision

Precision can be defined as the measurement of the degree of repeatability of an analytical method conducted under normal operation and is usually expressed in terms of the %RSD (Shabir, 2003). The acceptance criterion for inter-day precision is given by the USP (2015) as a % RSD ≤ 5%.

Table 4.2: Percentage recovery (%) obtained for inter-day precision of indinavir analysis by high performance liquid chromatography

	Day 1	**Day 2**	**Day 3**	**Between days**
	101.90	99.48	99.43	
	101.78	99.59	99.16	
	101.64	99.43	99.21	
Mean	101.77	99.50	99.27	100.18
SD	0.10	0.07	0.12	1.13

%RSD	0.10	0.07	0.12	1.13

Table 4.3: Statistical analysis of data for inter-day precision

ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between days	11.503	2.0	5.751	405.843	8.552E-05
Within days	0.085	6.0	0.014		
Total	11.588	8.0			

The % RSD of the inter-day analysis of indinavir was 1.13%, which means the repeatability is within the specified limits. Furthermore, the statistical analysis of the inter-day precision data revealed that there is no statistical significant difference between the values obtained on different days.

4.2.4 Ruggedness

Ruggedness is defined by the USP (2015) as the degree of reproducibility of the results obtained by analysis of the same samples under diverse conditions such as different laboratories, analysts and instruments. Ruggedness is expressed in terms of %RSD, which should be ≤ 2%, between measurements made under diverse conditions. The ruggedness acceptance criteria for stability are that sample solutions should not be used for a time period longer than it takes to degrade by 2%. The sample solution proved to be stable over a period of 24 h with a %RSD value of 1.57 % when measured under different conditions.

4.3 LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY ANALYTICAL METHOD FOR PHYTOCHEMICAL CHARACTERISATION OF THE *HYPOXIS HEMEROCALLIDEA* MATERIALS

TIC and UV chromatograms obtained for the various selected *H. hemerocallidea* materials are shown in the Figures 4.2 to 4.5 below.

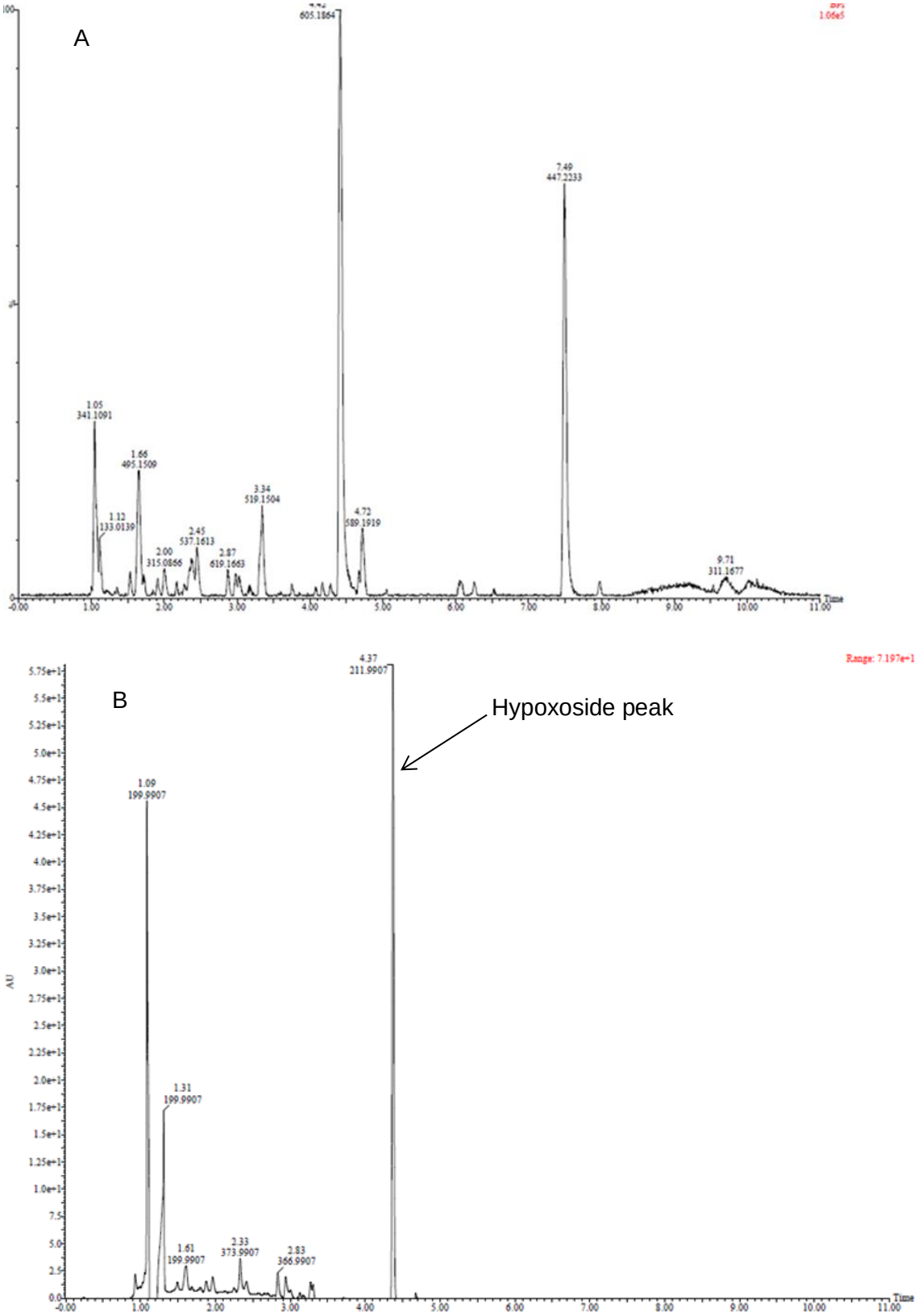

Figure 4.2: TIC (A) and UV (B) chromatograms of *Hypoxis hemerocallidea* reference plant material

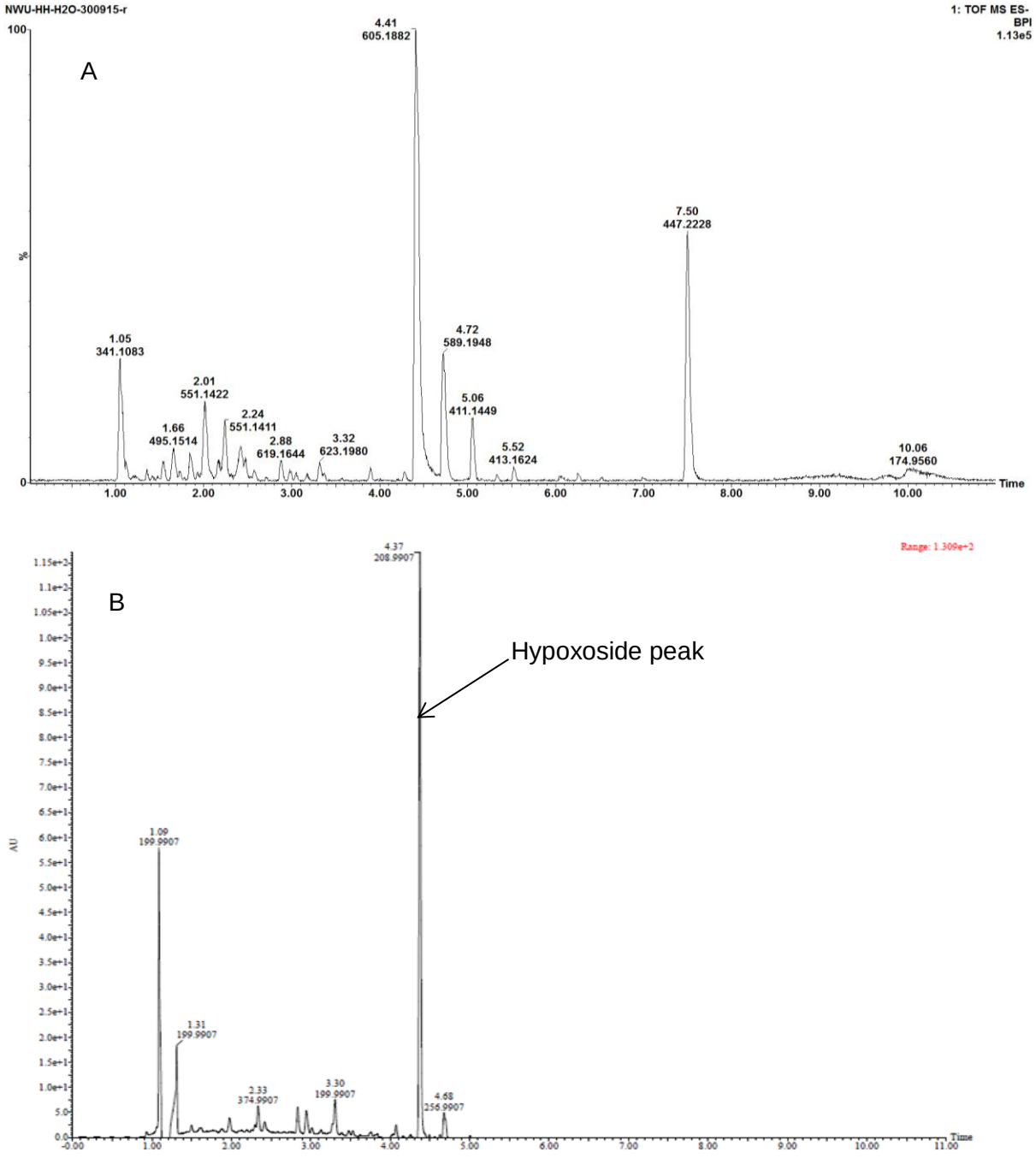

Figure 4.3: TIC (A) and UV (B) chromatograms of *Hypoxis hemerocallidea* aqueous extract

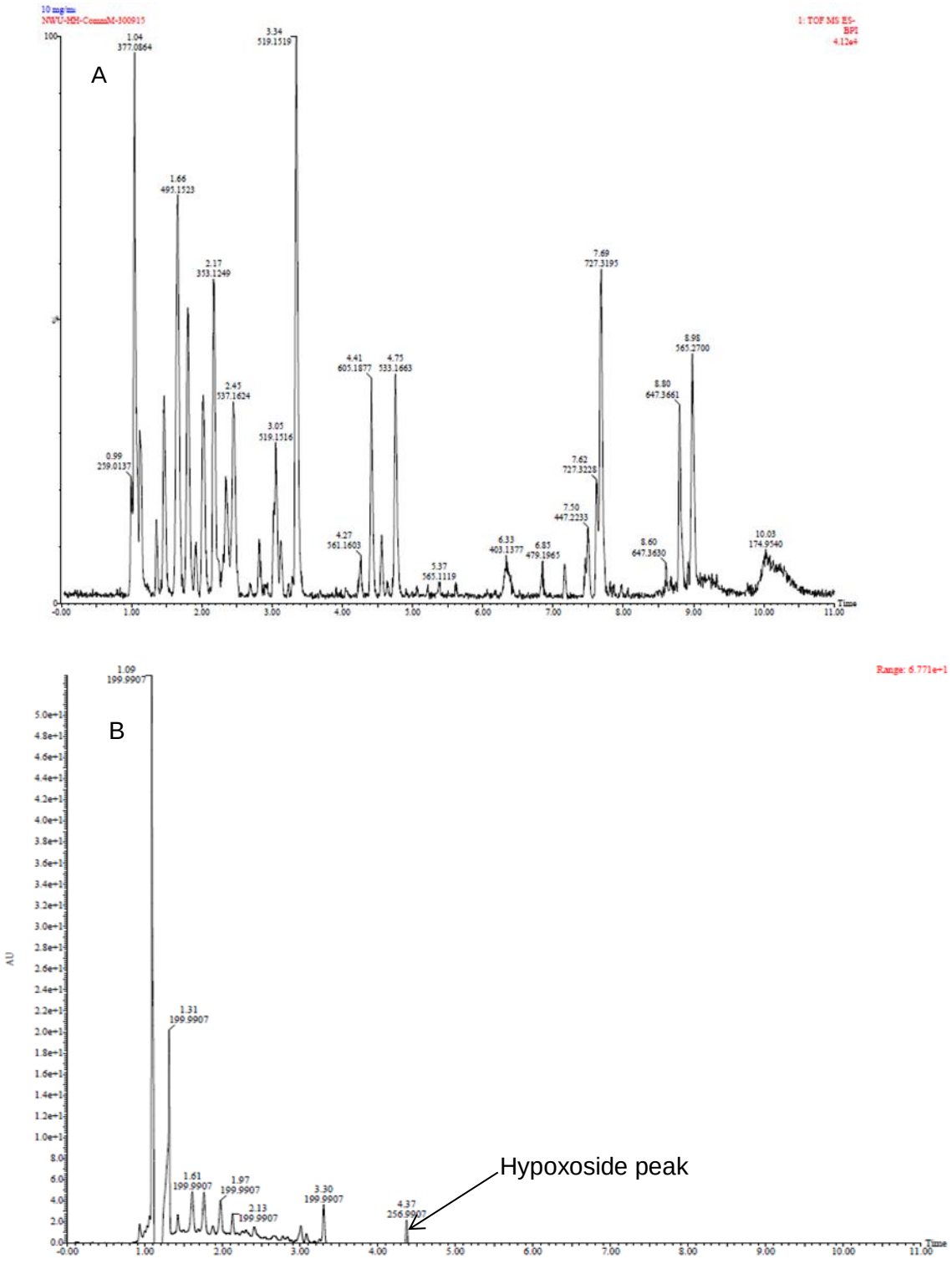

Figure 4.4: TIC (A) and UV (B) chromatograms of *Hypoxis hemerocallidea* commercial product

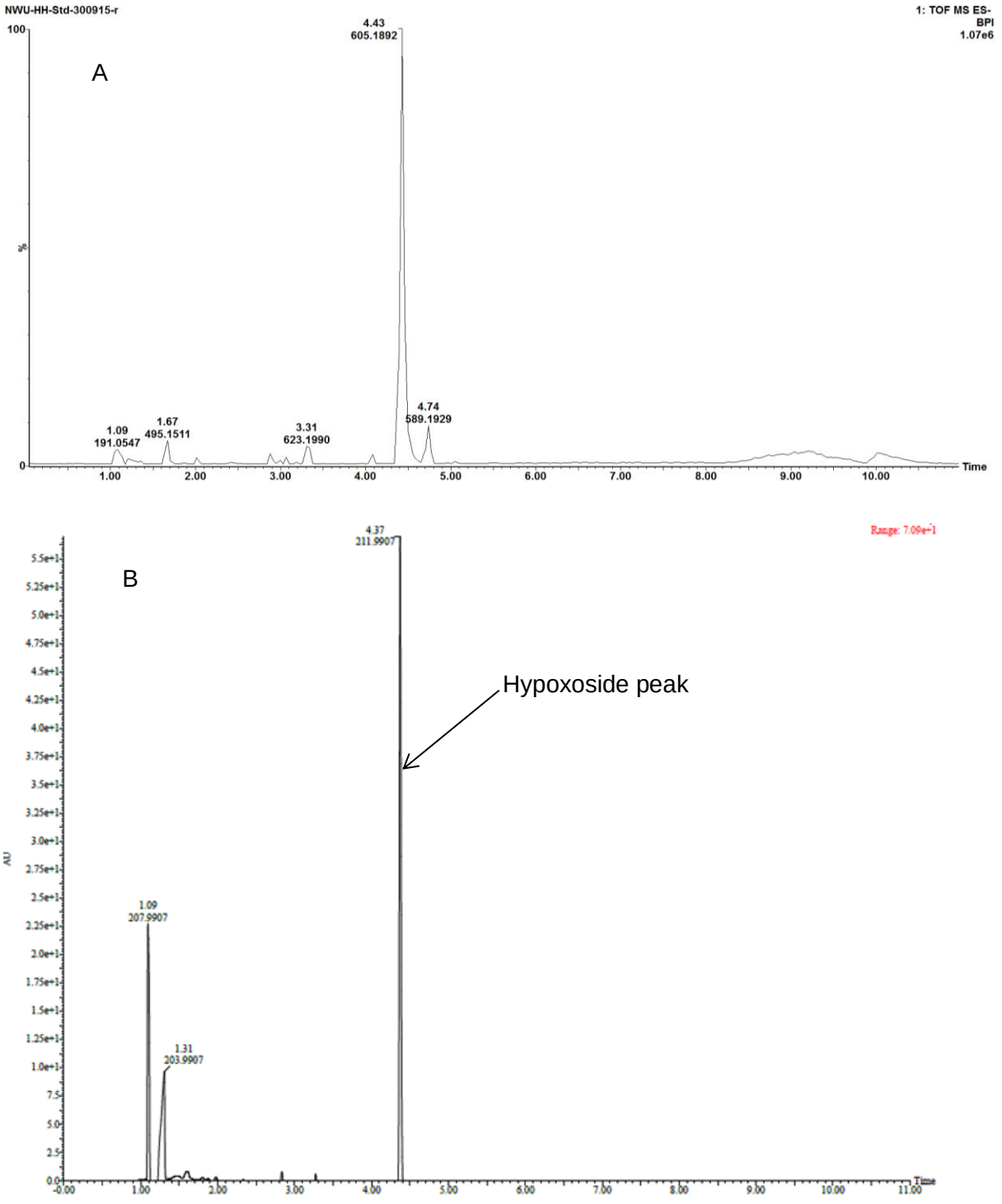

Figure 4.5: TIC (A) and UV (B) chromatograms of the marker molecule, hypoxoside (retention time = 4.37 min)

The quantities of the marker molecule, hypoxoside, as determined by UHPLC analysis in each of the selected *H. hemerocallidea* materials are shown in Table 4.4.

Table 4.4: Quantity of hypoxoside in each of the selected *Hypoxis hemerocallidea* materials

Selected *H. hemerocallidea* material	**Hypoxoside (mg/g)**
Reference plant material	13.3
Commercial product	0.6
Aqueous extract	151.7

UHPLC analysis demonstrated that all three the selected *H. hemerocallidea* materials contained the marker molecule, hypoxoside, although in different quantities. As expected, the aqueous extract contained a higher quantity of hypoxoside compared to that of the dried plant reference material and commercial product. The relatively low hypoxoside content of the commercial product can possibly be explained by a low content in the original plant material used or degradation of this phytochemical during production and/or storage of the product.

EXPERIMENTAL RESULTS

4.4 BI-DIRECTIONAL TRANSPORT STUDIES

The TEER of Caco-2 cell monolayers on Transwell® 6-well plates was measured at the beginning, before commencing the transport study, and a TEER value higher than 250 Ω (i.e. equal to 1167.5 Ω/cm^2) served as an indication of cell-monolayer integrity. The average TEER values were all above this prescribed value, which can be seen in Table 4.5. The TEER values obtained at the end of the transport study (120 min) were in some instances slightly lower, but remained constant or even increased slightly. These changes in TEER values could be attributed to possible modulation effects on the tight junctions between adjacent epithelial cells when the Caco-2 cell monolayers were exposed to the test solutions.

Table 4.5: TEER values of Caco-2 cell monolayers taken at the beginning (0 min) and end (120 min) of the bi-directional transport study of indinavir in the presence of the selected *H. hemerocallidea* materials

Experimental groups	Average TEER value (Ω)			
	AP-BL direction		BL-AP direction	
	0 min	120 min	0 min	120 min
Indinavir alone (negative control)	252.7	251.7	250.7	252.3
Indinavir with verapamil (positive control)	279.0	253.3	278.7	251.0
Indinavir with *H. hemerocallidea* commercial product	258.0	280.7	340.3	386.3
Indinavir with *H. hemerocallidea* aqueous extract	261.7	255.0	265.3	256.3
Indinavir with *H. hemerocallidea* plant reference material	284.0	253.3	281.0	264.0

The percentage transport of indinavir was calculated in both directions, apical to basolateral (AP-BL) and basolateral to apical (BL-AP) and these values were plotted as a function of time as shown in Figures 4.6 and 4.7. This percentage transport is an indication of the amount of indinavir crossing the cell monolayer into the acceptor chamber in relation to the indinavir concentration applied to the donor chamber. Active efflux of the investigated compound is demonstrated when the transport percentage is higher in the BL-AP direction compared to that in the AP-BL direction.

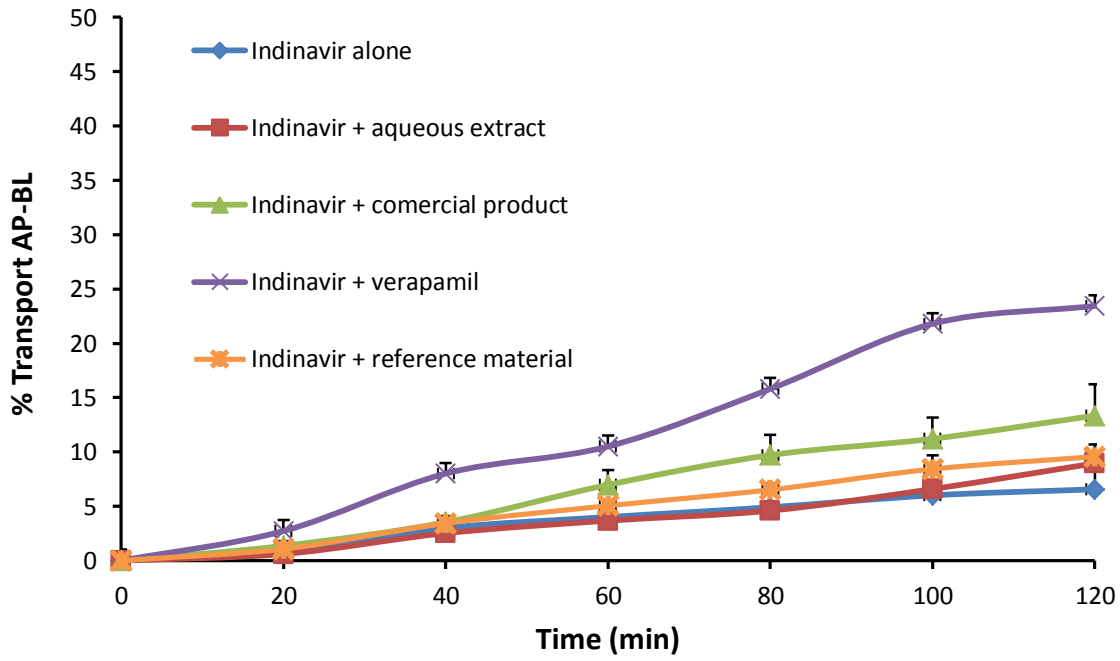

Figure 4.6: Percentage of indinavir transport across the monolayers of Caco-2 cells in the apical to basolateral (AP-BL) direction plotted as a function of time (n = 3, error bars indicate standard deviation)

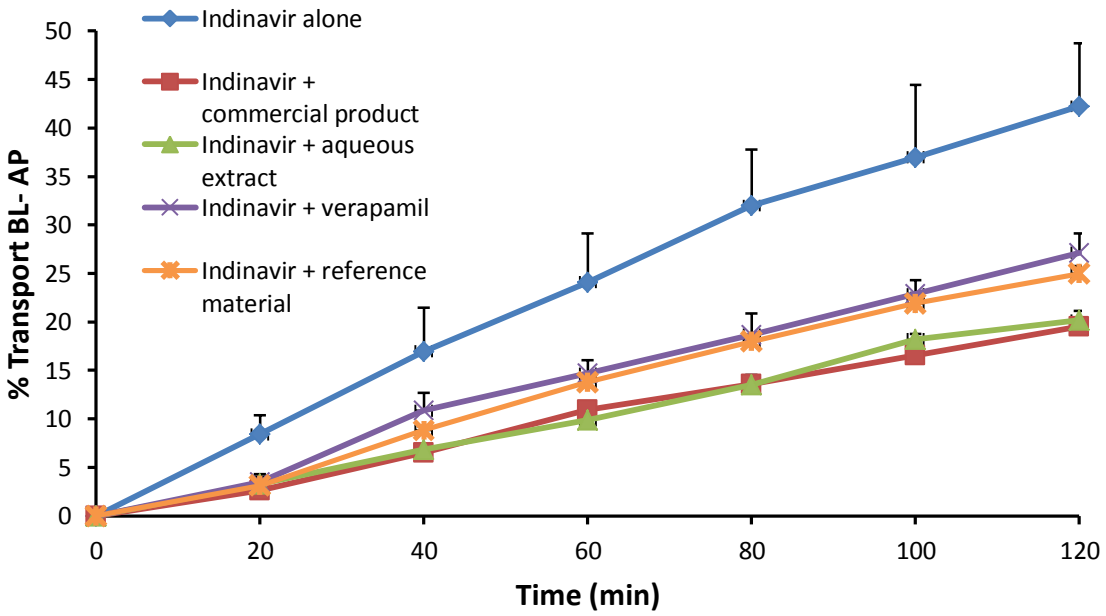

Figure 4.7: Percentage of indinavir transport across the monolayers of Caco-2 cells in the basolateral to apical (BL-AP) direction plotted as a function of time (n = 3, error bars indicate standard deviation)

It is clear from Figures 4.6 and 4.7 that verapamil (positive control group), increased the uptake of indinavir in the AP-BL direction compared to the indinavir alone (negative control group) and resulted in the highest percentage indinavir transport in the AP-BL direction. Verapamil is a known P-gp inhibitor and therefore decreased the efflux of indinavir in the BL-AP direction. Furthermore, the transport results in the AP-BL direction demonstrated an overall increase in the uptake of indinavir in the presence of the *H. hemerocallidea* materials (i.e. oral commercial product, an aqueous extract and dried plant reference material) when compared to that of the negative control group. Out of the selected *H. hemerocallidea* materials, the commercial product produced the most pronounced effect regarding indinavir uptake. The transport results in the BL-AP direction (Figure 4.7) demonstrated a prominent decrease of efflux of indinavir in the presence of all the *H. hemerocallidea* materials. The commercial product produced the most pronounced effect on indinavir efflux, followed by the reference plant material and aqueous extract.

The P_{app} values for indinavir in both directions across Caco-2 cell monolayers in the presence of each selected *H. hemerocallidea* material as well as the control groups are demonstrated in Figure 4.8.

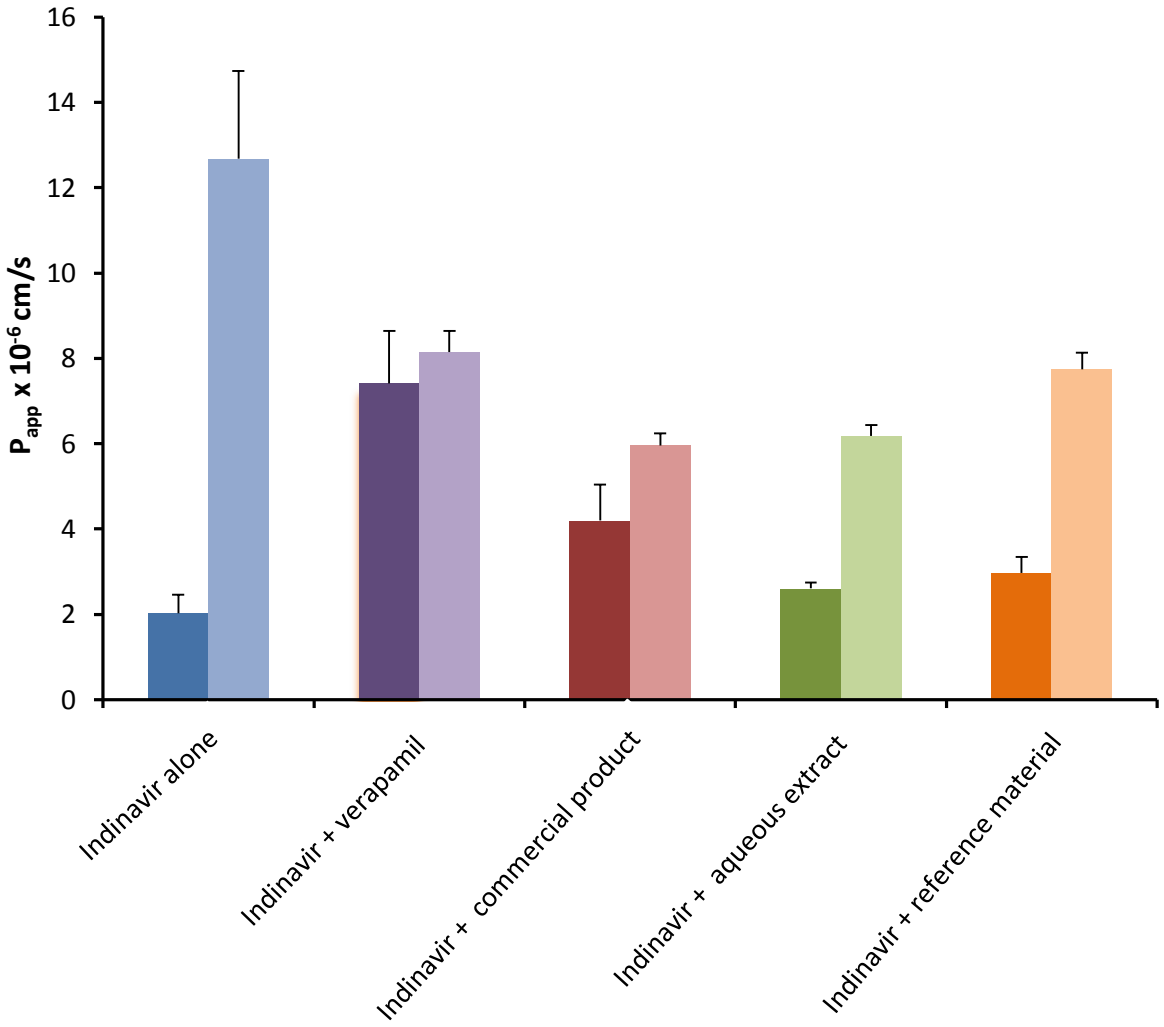

Figure 4.8: P_{app} values for indinavir in both directions across Caco-2 cell monolayers alone (negative control group) and in combination with the selected *Hypoxis hemerocallidea* materials as well as the positive control group (indinavir with verapamil). P_{app} bar graphs for A-B direction are indicated by dark colours and P_{app} bar graphs for B-A direction are indicated by light colours. (n = 3, error bars indicate standard deviation)

The P_{app} values represent the transport (diffusion rate, cm/s) of the compound across the Caco-2 cell monolayer normalised for absorption surface and initial concentration applied (Tarirai *et al*., 2012). Figure 4.8 confirmed an increase in the uptake of indinavir in the presence of all the *H. hemerocallidea* materials with the commercial product exhibiting the highest effect. An obvious decrease in the P_{app} value of indinavir in the BL-AP direction demonstrates inhibition of efflux produced by the positive control group (i.e. verapamil), as well as the experimental groups (i.e. selected *H. hemerocallidea* materials).

The efflux ratio (ER) values for indinavir for all the experimental and control groups are shown in Table 4.6.

Table 4.6: Efflux ratio (ER) values for indinavir in the absence (negative control) and presence of the selected *Hypoxis hemerocallidea* materials as well as verapamil (positive control)

Experimental group	ER ± SD*
Indinavir alone (negative control)	6.27 ± 4.75
Indinavir with verapamil (positive control)	1.10 ± 0.40
Indinavir with *H. hemerocallidea* commercial product	1.42 ± 0.33
Indinavir with *H. hemerocallidea* aqueous extract	2.37 ± 1.70
Indinavir with *H. hemerocallidea* reference material	2.61 ± 1.02

*SD = standard deviation

The ER values establish any asymmetry regarding indinavir directional transport in combination with the *H. hemerocallidea* materials. An ER value >> 1 indicates that a compound undergoes active efflux transport in the secretory direction. It is clear from Table 4.6 that indinavir is a substrate for P-gp related efflux due to the relatively high ER value (6.27) obtained for the negative control group, which was reduced to just above unity (1.10) by verapamil, a known P-gp inhibitor. Higher uptake transport of a drug is expected when the efflux of a drug compound from the epithelium is decreased. A higher drug bioavailability is consequently expected as a higher drug concentration will most probably reach the systemic circulation due to the result of efflux inhibition.

The pronounced reduction in ER values clearly indicate that active efflux transport of indinavir was inhibited by the selected *H. hemerocallidea* materials, although not to the same extent of efflux inhibition observed for verapamil.

From the *in vitro* transport results obtained in this current study and metabolism results obtained in previous *in vitro* studies, it can be expected that co-administration of indinavir (as well as other anti-retroviral drugs that are substrates for P-gp) with *H. hemerocallidea* extracts and plant materials will most probably lead to enhanced blood plasma levels due to efflux inhibition.

4.5 *IN VIVO* PHARMACOKINETIC STUDIES

The plasma concentration time curves for indinavir in Sprague-Dawley rats in the absence (control group) and presence of the selected *H. hemerocallidea* materials administered after a single administration (acute study) and after multiple administrations over 14 days (chronic study) are shown in Figures 4.9 and 4.10, respectively.

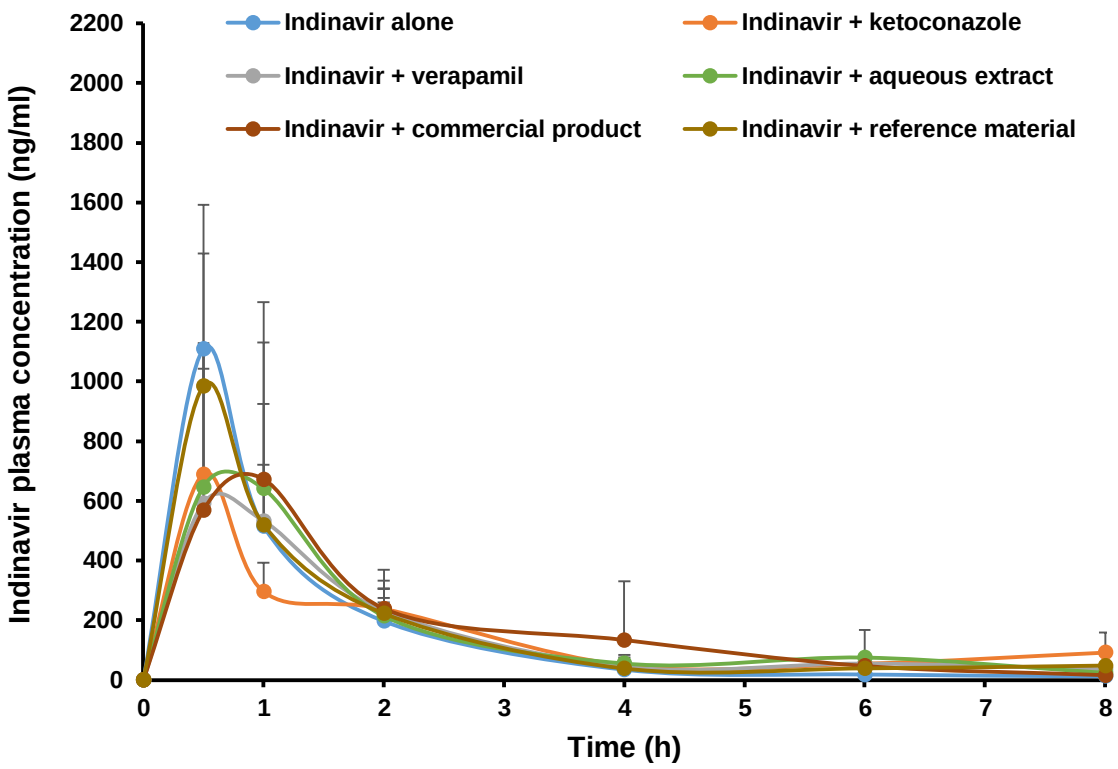

Figure 4.9: Plasma concentration time curves of indinavir in Sprague-Dawley rats in the absence and presence of the various *Hypoxis hemerocallidea* materials for the acute study (single administration). n = 5, error bars indicate standard deviation.

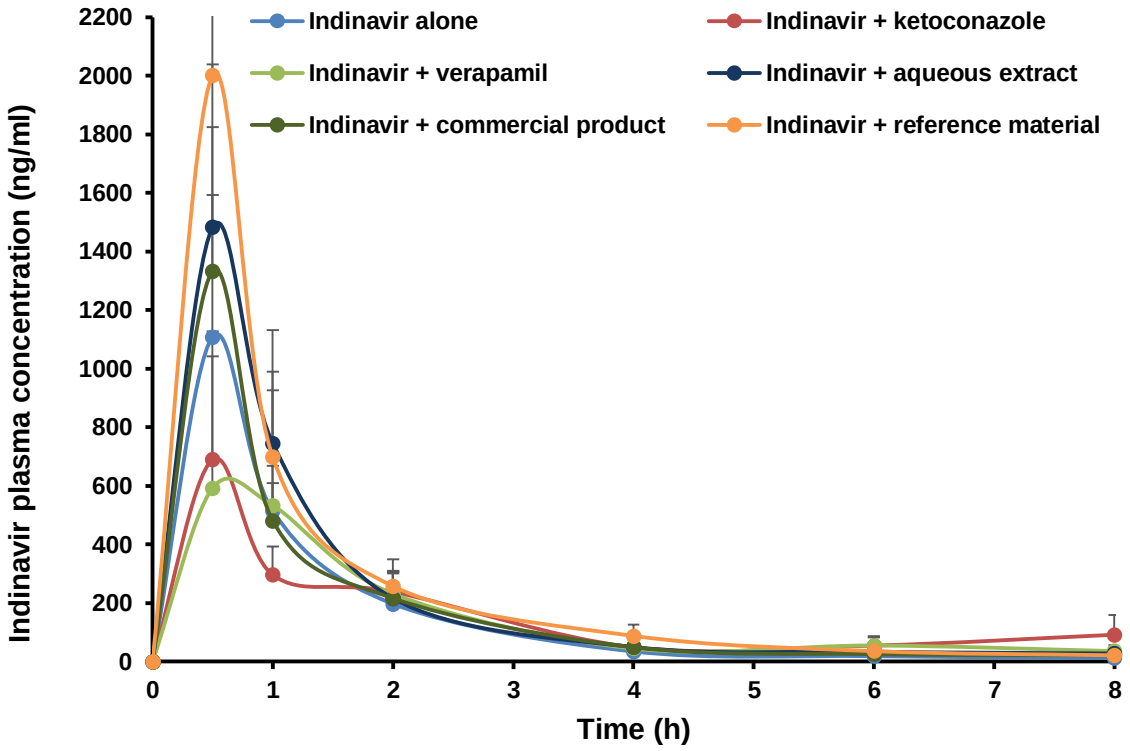

Figure 4.10: Plasma concentration time curves of indinavir in Sprague-Dawley rats in the absence and presence of the various *Hypoxis hemerocallidea* materials for the chronic study (14 days). n = 5, error bars indicate standard deviation.

The bioavailability parameters for indinavir in the absence and presence of the selected *H. hemerocallidea* test materials after a single administration (acute study) and multiple administrations over 14 days (chronic study) are listed in Table 4.7. The bioavailability parameters include the area under the curve extrapolated to infinity ($AUC_{0-\infty}$) as well as maximum plasma concentration (C_{max}) and relative bioavailability (F_{rel}) values.

Table 4.7: Biopharmaceutical parameters for indinavir administrated to rats in the absence and presence of the selected *H. hemerocallidea* materials

Experimental Group	$AUC_{0-\infty}$ (ng.min/ml)	C_{max} (ng/ml)	F_{rel}
Indinavir alone (negative control)	1371.0 ± 389.8	1108.6 ± 484.3	1.00
Indinavir with ketoconazole (positive control)	1428.6 ± 303.4*	689.0 ± 354.0*	1.04
Indinavir with verapamil (positive control)	1501.3 ± 462.2	644.1 ± 488.8	1.10
Indinavir with aqueous extract (acute)	1458.3 ± 512.6	813.6 ± 563.5	1.06
Indinavir with aqueous extract (chronic)	1847.2 ± 517.2	1483 ± 555	1.35
Indinavir with commercial product (acute)	1564.4 ± 638.2	928 ± 493	1.14
Indinavir with commercial product (chronic)	1530.7 ± 375.7	1333 ± 492	1.12
Indinavir with reference plant material (acute)	1456.6 ± 316.3	1022 ± 412	1.06
Indinavir with reference plant material (chronic)	2226.3 ± 930.6	2001 ± 955	1.62

* average and standard deviation values after removal of an outlier

From Table 4.7, it is clear that co-administration of the selected *H. hemerocallidea* test materials increased the bioavailability ($AUC_{0-\infty}$) of indinavir in rats in both the acute and chronic studies compared to the negative control group (indinavir alone), albeit not statistically significantly ($p \geq 0.05$). In accordance with the *in vitro* transport results, where the *H. hemerocallidea* commercial product increased the AP-BL transport the highest of all three the selected materials, it also exhibited the highest enhancement of indinavir bioavailability ($AUC_{0-\infty}$) in the rats during the acute study when compared to the other two *H. Hemerocallidea* materials. This acute effect correlates well with the *in vitro* transport results in terms of the relatively high efflux inhibition as expressed by the efflux ratio results (i.e. ER = 1.42 for the commercial product compared to ER = 2.37 and ER = 2.61 for the aqueous extract and reference material, respectively). On the

other hand, the aqueous extract and reference plant material showed higher bioavailability enhancement effects during the chronic study when compared to that of the commercial product during the chronic study.

In addition, a higher effect on indinavir bioavailability enhancement was obtained during the chronic study for the aqueous extract and reference plant material compared to the bioavailability enhancement effect during the acute study, which was not observed for the commercial product. This can possibly be explained by the hypoxoside content of the different materials investigated in this study (Table 4.4). CYP3A4 can be inhibited by both hypoxoside and rooperol *in vitro*, but since only rooperol is absorbed into the systemic circulation it is this compound that will cause enzyme inhibition *in vivo* rather than hypoxoside. Hypoxoside is converted to rooperol in the gastrointestinal tract after oral ingestion (Mogatle et al., 2008). Multiple administrations during the chronic study of the aqueous extract and reference plant material provided potentially higher cumulative levels of rooperol compared to that after a single administration during the acute study, which led to an increased effect on indinavir bioavailability. Since the commercial product contained a relatively low concentration of hypoxoside (0.6 mg/g), a relatively low level of hypoxoside was available for conversion to rooperol. This low level may be eliminated before the next dose and therefore would not lead to a cumulative increase during the chronic study, which may explain why there is a negligible difference between the acute and chronic study in terms of the effect on indinavir bioavailability. These results therefore indicate that longer term exposure to *H. hemerocallidea* materials may have a higher effect on the pharmacokinetics of indinavir and also potentially on other drugs that are substrates of CYP450 enzymes and P-gp efflux transporters, depending on the concentration of hypoxoside in the product that is consumed concomitantly with the drug. On the other hand, *H. hemerocallidea* contains other bioactive chemical compounds such as phytosterols and sterolins which could have affected the outcome (Owira & Ojewole, 2009).

The expression of CYP450 enzymes is influenced by a combination of different factors and mechanisms. A change in CYP450 enzyme expression would possibly alter the metabolism of drug compounds (Zanger *&* Schwab, 2013). Long term exposure to CYP450 inhibition (as in this study) could lead to changes in the expression of these enzymes therefore influencing metabolism.

4.6 CONCLUSIONS

The selected *H. hemerocallidea* materials inhibited *in vitro* efflux of indinavir and enhanced *in vivo* bioavailability of indinavir. The effect *in vivo* was more prominent after multiple administrations (i.e. chronic study) compared to a single administration (i.e. acute study) for the

H. hemerocallidea materials. This could possibly be related to the differences found in hypoxoside concentration of the *H. hemerocallidea* materials. Concomitant administration of *H. hemerocallidea* with indinavir causes pharmacokinetic interactions, especially during long term use depending on the concentration of hypoxoside in the material.

CHAPTER 5

FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 INTRODUCTION

Concomitant use of herbal medicines and other drugs have the potential to produce herb-drug pharmacokinetic and pharmacodynamic interactions. Phytochemicals in herbal medicines can cause these pharmacokinetic interactions with other drugs when co-administrated. Pharmacokinetic interactions occur by means of induction and/or inhibition of intestinal efflux proteins such as P-gp or multiple resistance proteins, as well as modulation of intestinal and hepatic metabolising enzymes, specifically the CYP450 super family. These pharmacokinetic interactions may lead to increased or decreased bioavailability of the drug. Consequences of these bioavailability changes may either be adverse effects due to increased drug plasma levels or lack of pharmacological responses due to decreased drug plasma levels.

Hypoxis hemerocallidea is a popular traditional herbal medicine in the southern Africa region. Traditionally, the corms of this plant were administered orally after boiling it in water. For many years, this plant has been used for the treatment or management of HIV/AIDS related diseases as well as for the treatment of cancers, heart failures, nervous disorders, immune-related illnesses and urinary tract infections. Furthermore, *H. hemerocallidea* has also been used for the treatment of benign prostate hyperplasia, as an anti-inflammatory agent, anti-oxidant, anti-convulsant and as an anti-diabetic agent. Hypoxoside is considered to be one of the most important phytochemicals with regards to the medicinal value of this plant. Although *in vivo* data is lacking, *in vitro* observations seemed to suggest the possibility of pharmacokinetic interactions between anti-retroviral drugs and African potato.

Obtaining new knowledge with respect to pharmacokinetic interactions between *H. hemerocallidea* and anti-retroviral drugs can contribute to better patient management and to give the correct advice to patients that use herbal medicines together with prescribed chronic medication. This study aimed at identifying pharmacokinetic interactions between different *H. hemerocallidea* materials and indinavir in both *in vitro* and *in vivo* studies. The study specifically measured the effect of a commercial product containing *H. hemerocallidea* plant material on indinavir pharmacokinetics, which was compared to the effects of an aqueous extract and a reference dried plant material.

5.2 FINAL CONCLUSIONS

The results from this study indicate that the selected *H. hemerocallidea* materials caused inhibition of efflux of indinavir *in vitro*, with the commercial product demonstrating the most pronounced effect (although it contained the lowest concentration of hypoxoside). This finding indicated that other components present in the commercial product may also have contributed to efflux inhibition of indinavir in the Caco-2 cell model. The *in vitro* result of a potential pharmacokinetic interaction with respect to efflux inhibition, indicated an increase in the bioavailability of indinavir can be expected when co-administrated with *H. hemerocallidea* materials. In addition, previous *in vitro* metabolism studies have also demonstrated the possibility of *H. hemerocallidea* inhibiting CYP450 enzymes (Nair et al., 2007).

Indeed, as predicted from the results of the *in vitro* study, an increase in indinavir bioavailability in the presence of the selected *H. hemerocallidea* materials was observed *in vivo* in Sprague-Dawley rats for both the acute and chronic studies. The commercial product exhibited the highest enhancement of indinavir bioavailability in the rats during the acute study when compared to the other two *H. Hemerocallidea* materials. This acute effect correlates well with the results obtained during the *in vitro* bi-directional transport study. On the other hand, the aqueous extract and reference plant material displayed higher bioavailability enhancement effects of indinavir during the chronic study when compared to the commercial product. This can possibly be explained by the hypoxoside content of the different materials investigated in this study. Hypoxoside is converted to rooperol in the gastrointestinal tract after oral ingestion and absorbed into the system circulation, which possibly causes CYP3A4 enzyme inhibition. Multiple administrations during the chronic study of the aqueous extract and reference plant material provided potentially higher cumulative levels of rooperol compared to that after a single administration during the acute study. Furthermore, longer exposure of the active ingredients at higher levels most probably modulated the metabolism to a larger extent. This resulted in an increased effect on indinavir bioavailability. The commercial product contained a relatively low hypoxoside concentration, which resulted in a lower level of hypoxoside converted to rooperol.

The *in vivo* results indicated that a longer term exposure to *H. hemerocallidea* materials may have a higher effect on the pharmacokinetics of indinavir (which also depended on the hypoxoside concentration). This pharmacokinetic interaction may also potentially be relevant to other drugs that are substrates of CYP450 enzymes and P-gp efflux transporters. Patients are therefore advised to avoid long term use of this herbal medicine together with anti-retroviral treatment.

5.3 FUTURE RECOMMENDATIONS

This study mainly focused on the hypoxoside concentration present in the selected *H. hemerocallidea* materials. *H. hemerocallidea* contains other bioactive chemical compounds and it is suggested that these other chemical compounds be extracted to evaluate the effect of each of these compounds individually on indinavir pharmacokinetics. More *H. hemerocallidea* materials should also be investigated, such as a methanol extract as well as other commercial products available on the market that may have different chemical compositions when compared to those investigated in this study. It is further recommended that the half-life of hypoxoside should be taken into account when investigating its cumulative effect during chronic administration. Chronic studies that are longer than 14 days can be conducted. *In vitro* metabolism studies involving the various *H. hemerocallidea* materials can also be examined.

The pharmacokinetic interactions should also be investigated on other ARV drugs that are substrates for P-gp and CYP3A4 than the protease inhibitor, indinavir. This could lead to the identification of other probable herb-drug interactions. Inter-individual variability regarding to polymorphisms of CYP3A4 and P-gp, in various ethnic groups, could be studied in order to determine how these factors influence the metabolism of indinavir and other ARVs during the co-administration of *H. hemerocallidea* materials.

Numerous herbal medicines are available commercially and by investigating possible herb-drug interactions, awareness will be raised and knowledge broadened with regards to the issue. The overall treatment and welfare of HIV/AIDS infected patients can be improved when these herb-drug pharmacokinetic interactions are acknowledged.

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APPENDIX A

CONGRESS PROCEEDINGS & ARTICLES

Pharmacokinetic interactions (*in vitro* and *in vivo*) between indinavir and *Hypoxis hemerocallidea*: comparing a commercial product with a crude extract and a dried plant material

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Purpose: It is known that phytochemicals in herbal medicines can cause pharmacokinetic interactions with allopathic drugs when taken concomitantly. These interactions may lead to an increase or decrease in drug bioavailability with consequences of adverse effects or treatment failure. The relevance of research concerning pharmacokinetic interactions between herbal medicines and anti-retroviral drugs is reflected by the fact that a relatively large portion of human immunodeficiency virus infected patients with acquired immunodeficiency syndrome commonly use herbal medicines to complement their anti-retroviral therapy.

Methods: *Hypoxis hemerocallidea* (African potato) test materials investigated in this study included a commercial solid oral product, a dried reference plant material and an aqueous extract. Bi-directional transport studies of indinavir across Caco-2 cell monolayers were conducted in the absence and presence of the selected African potato test materials. The bioavailability of indinavir in the absence and presence of the selected African potato test materials was determined in Sprague-Dawley rats in acute (single administration) and chronic (daily administrations over 14 days) studies. Ketoconazole was used as positive control for metabolism inhibition and verapamil as positive control for efflux inhibition.

Results: The selected African potato test materials demonstrated an inhibition of efflux of indinavir in the Caco-2 cell model. In agreement with this finding and other published

findings on metabolism inhibition, an increase in indinavir bioavailability in the presence of the selected African potato test materials was shown *in vivo* for both the acute and chronic studies. The commercial product had a similar increasing effect on indinavir bioavailability as the aqueous extract, while the reference plant material exhibited a higher effect on indinavir bioavailability enhancement. A higher effect on indinavir bioavailability was observed for two of the test materials during the chronic study when compared to the acute study.

Conclusion: All the selected African potato test materials interfered with indinavir pharmacokinetics in both the *in vitro* and *in vivo* models and these effects may be attributed to inhibition of efflux transporters and enzymatic metabolism.

Pharmacokinetic interactions (*in vitro* and *in vivo*) between indinavir and *Hypoxis hemerocallidea*: comparing a commercial product with a crude extract and a dried plant material

Kaylee Havenga[1], Josias Hamman[1], Dewald Steyn[1], Carlemi Calitz[1], Efrem Abay[2], Lubbe Wiesner[2] and Alvaro Viljoen[3]

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1. Background

The use of herbal medicines is popular as an alternative treatment source and in some developing countries, especially in Africa, more than 80% of the population still use traditional medicines on a regular basis (Brijlal et al, 2011). A misinterpretation of safety regarding herbal treatment use is evident amongst the general public. Besides potential toxic effects, herbal medicines can cause alterations in drug transport and metabolism due to modulation of Cytochrome P450 (CYP) enzymes and efflux transporters such as P-glycoprotein (P-gp), causing herb-drug pharmacokinetic interactions (Cordier & Steenkamp, 2011).

In vitro research has demonstrated that a potential herb-drug pharmacokinetic interaction exists between *Hypoxis hemerocallidea* Fisch., C.A.Mey. & Avé-Lall. (African potato) and anti-retroviral drugs. Literature indicates alterations in the transport and metabolism of indinavir upon co-administration with extracts of *H. hemerocallidea* such as inhibition of CYP3A4 enzyme activity and P-gp related efflux within *in vitro* models (Mills et al., 2005).

2. Aim

The aim of this study was to confirm pharmacokinetic interactions that different *H. hemerocallidea* materials including a dried plant reference material, an aqueous extract as well as a solid oral commercial product (Afrigetics®) may have on indinavir by means of *in vitro* bi-directional transport studies as well as by means of acute and chronic *in vivo* bioavailability studies.

3. Methods

3.1 Chemical composition of the selected *H. hemerocallidea* materials

The concentration of hypoxoside, a marker molecule, in all the selected *H. hemerocallidea* materials was determined by means of liquid chromatography linked to mass spectrometry (LC/MS).

3.2 *In vitro* bi-directional transport studies

Caco-2 cells were seeded and grown onto Transwell® 6-well membranes with a surface area of 4.67 cm^2 for a period of 21-24 days. Indinavir alone (200 µM) served as the negative control group, while indinavir in combination with 100 µM verapamil served as the positive control group. A final concentration of 500 µg/ml of each selected *H. hemerocallidea* material in combination with indinavir formed the experimental groups. For the transport studies in the apical-to-basolateral (AP-BL) direction, the growth medium in the apical chambers was replaced with test solutions in DMEM, while the growth medium in the basolateral chambers was replaced with test solutions in DMEM and HEPES for the transport studies in the basolateral-to-apical (BL-AP) direction. Samples (200 µl) were withdrawn from the acceptor chambers at time intervals of 20, 40, 60, 80, 100, 120 min and analysed by means of a validated high performance liquid chromatograph (HPLC) method.

The apparent permeability coefficient (P_{app}) values as well as efflux ratio (ER) values were calculated as previously described (Calitz *et al.,* 2015).

3.3. *In vivo* pharmacokinetic study

A total of 45 male Sprague-Dawley rats weighing 250-300 g were randomly selected and divided into 9 groups (i.e. 3 control groups, 3 acute experimental groups and 3 chronic experimental groups) which consisted of 5 animals per group. The acute study consisted of a single administration per rat and the chronic study entailed daily administrations over 14 days by means of oral gavage at a volume of 500 µl per animal (Table 1).

Blood samples (200 µl) were collected from the tail veins of the animals at pre-determined time intervals of 0, 0.5, 1, 2, 4, 8 and 24 h after administration of each experimental test solution. A validated Liquid Chromatography tandem Mass Spectrometry (LC/MS/MS) method for the analysis of indinavir was used in order to analyse the plasma samples.

Table 1: Experimental groups for the *in vivo* studies (both acute and chronic)

Composition of the experimental solutions
Group 1: Indinavir alone at a concentration of 40 mg/kg. (IND alone) (Negative control)
Group 2: Indinavir (40 mg/kg) with ketoconazole (40 mg/kg) in water. (IND with ketoconazole) (Positive control for enzyme inhibition)
Group 3: Indinavir (40 mg/kg) with verapamil (9 mg/kg) in water. (IND with verapamil) (Positive control foe efflux inhibition)
Group 4 & 5: Indinavir (40 mg/kg) with *H. hemerocallidea* aqueous extract at a concentration of 15 mg/kg. 4: acute, 5: chronic. (IND with HH aq extract)
Group 6 & 7: Indinavir (40 mg/kg) with *H. hemerocallidea* commercial product at a concentration of 15 mg/kg. 6: acute, 7: chronic. (IND with HH CP)
Group 8 & 9: Indinavir (40 mg/kg) with *H. hemerocallidea* reference dried plant material at a concentration of 15 mg/kg. 8: acute, 9: chronic. (IND with HH RM)

4. Results and discussion

4.1 Chemical composition of selected *H. hemerocallidea* materials

The hypoxoside contained in each of the selected *H. hemerocallidea* materials is presented in Table 2.

Table 2: Hypoxoside contained in the *H. hemerocallidea* test materials

Test Material	Hypoxoside
Reference dried plant material (RM HH)	13.3 mg/g
Commercial product (HH CP)	0.6 mg/g
Aqueous extract (HH aq extract)	151.7 mg/g

4.2 *In vitro* transport study

The overall transport of indinavir in the AP-BL direction increased when applied with the selected *H. hemerocallidea* materials, while the transport decreased in the BL-AP direction.

This indicates that the selected *H. hemerocallidea* test materials caused inhibition of efflux of indinavir in the Caco-2 cell model.

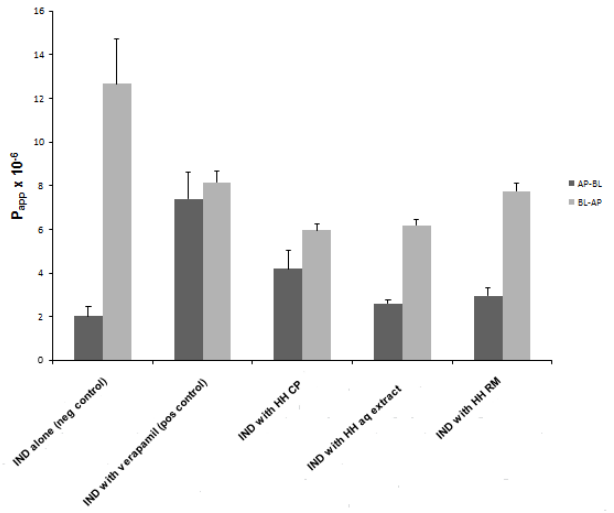

Figure 1: Bi-directional P_{app} values for indinavir obtained from the experimental and control groups. n = 3, error bars indicate standard deviation.

4.3 *In vivo* pharmacokinetic study

An increase in indinavir bioavailability in the presence of the selected *H. hemerocallidea* test materials was observed *in vivo* for both the acute and chronic studies (Table 3). Of the selected *H. hemerocallidea* materials investigated, the dried plant material exhibited the highest enhancement of indinavir bioavailability. In general, a higher effect on indinavir bioavailability enhancement was observed during the chronic study.

Table 3: Area under the curve ($AUC_{0-\infty}$) and maximum plasma concentration (C_{max}) values for indinavir administrated to rats with and without selected *H. hemerocallidea* materials

Experimental group	**$AUC_{0-\infty}$ (min.µmol/L)**	**C_{max} (ng/ml)**
IND alone (negative control)	1371.0 ± 389.8	1109 ± 484
IND with ketoconazole (positive control)	1428.6 ± 303.4*	689 ± 354*
IND with verapamil (positive control)	1501.3 ± 462.2	644 ± 489
IND with HH aq extract (acute)	1458.3 ± 512.6	814 ± 564
IND with HH aq extract (chronic)	1847.2 ± 517.2	1483 ± 555
IND with HH CP (acute)	1564.4 ± 638.2	928 ± 493

IND with HH CP (chronic)	1530.7 ± 375.7	1333 ± 492
IND with HH RM (acute)	1456.6 ± 316.3	1022 ± 412
IND with HH RM (chronic)	2226.3 ± 930.6	2001 ± 955

* average and standard deviation after removal of an outlier

5. Conclusions

The *in vitro* permeation studies showed that the selected *H. hemerocallidea* test materials inhibited efflux of indinavir, while the *in vivo* studies indicated enhanced bioavailability of indinavir. Concurrent administration of *H. hemerocallidea* with indinavir may result in possible adverse effects such as drug toxicity, which can possibly be overcome by taking them at different time periods (Owira et al., 2009).

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ARTICLE

Effect of *Hypoxis hemerocallidea* on indinavir pharmacokinetics (*in vitro* and *in vivo*): comparing a commercial product with plant extract and reference material

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(In process of submission to The Journal of Ethnopharmacology)

APPENDIX B

PACKAGE INSERT CRIXIVAN®

SCHEDULING STATUS S4

PROPRIETARY NAME AND DOSAGE FORM

CRIXIVAN® 200 mg Capsules
CRIXIVAN® 400 mg Capsules

COMPOSITION

CRIXIVAN 200 mg: Each capsule contains 200 mg of indinavir.
CRIXIVAN 400 mg: Each capsule contains 400 mg of indinavir.

CRIXIVAN contains sugar.

PHARMACOLOGICAL CLASSIFICATION

A.20.2.8 Antiviral agents

PHARMACOLOGICAL ACTION

MECHANISM OF ACTION

HIV protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV. Indinavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. The relationship between *in vitro* susceptibility of HIV to indinavir and inhibition of HIV replication in humans has not been established. The *in vitro* activity of indinavir was assessed in cell lines of lymphoblastic and monocytic origin and in peripheral blood lymphocytes. HIV variants used to infect the different cell types include laboratory-adapted variants, primary clinical isolates and clinical isolates resistant to nucleoside analogues and non-nucleoside inhibitors of the HIV reverse transcriptase. The IC_{95} (95 % inhibitory concentration) of indinavir in these test systems was in the range of 25 to 100 nM. In drug combination studies with the nucleoside analogues zidovudine and didanosine, as well as with an investigational non-nucleoside, indinavir showed synergistic activity in cell culture.

PHARMACOKINETICS

Absorption

Indinavir is rapidly absorbed in the fasted state with a time to peak plasma concentration of 0,8 hours. Over the 200 to 1 000 mg dose range administered in both volunteers and HIV-1 infected patients, there was a slightly greater than dose-proportional increase in plasma concentrations. As a result of the short half-life, only a minimal increase in plasma concentrations occurred after multiple dosing.

Administration of indinavir with a meal high in calories, fat and protein resulted in a blunted and reduced absorption with an approximate 80 % reduction in AUC and an 85 % reduction in C_{max}. Administration with light meals (e.g. dry toast with jelly, apple juice, and coffee with skim milk and sugar or corn flakes, skim milk and sugar) resulted in a 2 to 8 % reduction in AUC and C_{max}. The plasma concentrations six and eight hours after administration of indinavir with these light meals were comparable to the corresponding fasted values at 800 mg every 8 hours.

Distribution

Indinavir was ± 60 % bound to human plasma proteins.

Biotransformation

Indinavir is metabolised in the liver and excreted mainly in the faeces. *In vitro* studies with human liver microsomes indicated that cytochrome CYP3A4 is the only P450 isozyme that plays a major role in the oxidative metabolism of indinavir. Analysis of plasma and urine samples from subjects who received indinavir, indicated that indinavir metabolites contribute little to the overall *in vitro* protease inhibitory activity.

Elimination

Over the 200 to 1 000 mg dose range administration in both volunteers and HIV infected patients, there was a slightly greater than dose-proportional increase in urinary recovery of indinavir. Renal clearance (116 ml/min) of indinavir is concentration-independent over the clinical dose range. Less than 20 % of indinavir is excreted unchanged renally. Mean urinary excretion of unchanged medicine following single dose administration in the fasted state was 10,4 % following a 700 mg dose, and 12,0 % following a 1 000 mg dose. Indinavir was rapidly eliminated with a half-life of 1,8 hours.

Characteristics in Patients

Pharmacokinetics of indinavir do not appear to be affected by gender or by race.

Patients with mild-to-moderate hepatic insufficiency and clinical evidence of cirrhosis had evidence of decreased metabolism of indinavir resulting in approximately 60 % higher mean AUC following a single 400 mg dose. The mean half-life of indinavir increased to approximately 2,8 hours. Patients with severe hepatic insufficiency have not been studied.

PHARMACODYNAMICS

The combination of indinavir and one or more nucleoside analogues was associated with somewhat greater median declines in serum viral RNA, and a higher proportion of patients in whom RNA levels fell to below the limit of detection.

Loss of suppression of viral RNA levels occurred in some patients; however, CD4 cell counts were often sustained above pre-treatment levels. When loss of viral RNA suppression occurred, it was typically associated with replacement of circulating susceptible virus with resistant viral variants. Resistance was correlated with the accumulation of mutations in the viral genome that resulted in the expression of amino-acid substitutions in the viral protease enzyme.

At least eleven HIV-1 protease amino-acid residue positions, at which substitutions are associated with resistance, have been identified. No single substitution was capable of engendering measurable resistance to the inhibitor; resistance was mediated by the co-expression of multiple and variable substitutions. In general, higher levels of resistance result from the co-expression of greater numbers of substitutions at the eleven identified positions. Substitutions at these positions appeared to accumulate sequentially, probably as the result of ongoing viral replication.

It should be noted that the decrease in suppression of viral RNA levels was seen more frequently when therapy with indinavir was initiated at doses lower than the recommended oral dose of 2,4 g/day. **Therefore, therapy with indinavir should be initiated at the recommended dose to increase suppression of viral replication and therefore inhibit the emergence of resistant virus.**

INDICATIONS

CRIXIVAN is indicated for the treatment of HIV infection in adults, in combination with other antiretroviral agents.
Clinical studies evaluating patients who received CRIXIVAN in combination with other antiretroviral agents demonstrated a reduced risk of progression to an AIDS-defining illness or death, increased overall survival, a durable reduction in serum viral RNA and a durable increase in CD4 cell counts.

CONTRA-INDICATIONS

CRIXIVAN is contra-indicated in:

1. Patients with clinically significant hypersensitivity to any component of this product.
2. Pregnancy and lactation as safety has not been established.
3. Children under the age of 18 years, as safety and efficacy have not been established.
4. Safety and efficacy in patients with concomitant liver disease (e.g. Hepatitis B) has not been established.
5. Safety and efficacy have not been established in the elderly.
6. Because rifampicin is a potent inducer of P450 3A4 which could markedly diminish plasma concentrations of CRIXIVAN, co-administration with rifampicin is not recommended.

WARNINGS

Nephrolithiasis may occur with CRIXIVAN. If signs and symptoms of nephrolithiasis, including flank pain with or without haematuria (including microscopic haematuria), occur, temporary interruption of therapy (e.g. 1 to 3 days) during the acute episode of nephrolithiasis may be considered. **Adequate hydration is recommended in all patients treated with CRIXIVAN** (see **DOSAGE AND DIRECTIONS FOR USE** Nephrolithiasis).

CRIXIVAN should not be administered concurrently with terfenadine, astemizole, cisapride, triazolam, midazolam, pimozide and ergot derivatives because competition for CYP3A4 by indinavir could result in inhibition of the metabolism of these medicines and create the potential for serious and/or life-threatening events (i.e. cardiac arrhythmias, prolonged sedation).

Rifabutin

The co-administration of CRIXIVAN at a dose of 800 mg every 8 hours with rifabutin at a dose of 300 mg once daily for 10 days resulted in a 34 % decrease in AUC and a 25 % decrease in C_{12h} of CRIXIVAN. In contrast, the AUC and C_{12h} of rifabutin increased by about 173 % and 134 % respectively, vs rifabutin 300 mg once daily alone (see **DOSAGE AND DIRECTIONS FOR USE**). This increase in rifabutin plasma concentrations is likely related to inhibition of CYP3A4-mediated metabolism of rifabutin by CRIXIVAN. A dosage increase of CRIXIVAN and a dosage reduction of rifabutin are necessary when CRIXIVAN and rifabutin are co-administered.

INTERACTIONS

Specific interaction studies were performed with CRIXIVAN and the following medicines, including: zidovudine, zidovudine/lamivudine, stavudine, trimethoprim/sulfamethoxazole, fluconazole, isoniazid, clarithromycin, cimetidine, methadone or an oral contraceptive (norethindrone/ethinyl estradiol 1/35). No clinically significant interactions were observed with these agents. However, clinically significant interactions with other agents are described below.

Pimozide

Pimozide should not be used together with CRIXIVAN. Inhibition of CYP3A4 by CRIXIVAN could result in elevated plasma concentrations of pimozide which could potentially result in QT prolongation and associated ventricular arrhythmias (see **WARNINGS**).

Rifampin

Rifampin is a potent inducer of P450 3A4 which markedly diminishes plasma concentrations of CRIXIVAN. Therefore, CRIXIVAN and rifampin should not be co-administered.

Rifabutin

Due to an increase in the plasma concentrations of rifabutin and a decrease in the plasma concentrations of CRIXIVAN, a dosage reduction of rifabutin and a dosage increase of CRIXIVAN are necessary when rifabutin is co-administered with CRIXIVAN (see **DOSAGE AND DIRECTIONS FOR USE**).

Ketoconazole

Due to an increase in the plasma concentrations of CRIXIVAN, a dosage reduction of CRIXIVAN should be considered when CRIXIVAN and ketoconazole are co-administered.

Itraconazole

Itraconazole is an inhibitor of P450 3A4 that increases plasma concentrations of CRIXIVAN. Therefore a dosage reduction of CRIXIVAN is recommended when CRIXIVAN and itraconazole are co-administered (see **DOSAGE AND DIRECTIONS FOR USE**).

Delavirdine

Due to an increase in CRIXIVAN plasma concentration (preliminary results), a dosage reduction of CRIXIVAN should be considered when CRIXIVAN and delavirdine are co-administered (see **DOSAGE AND DIRECTIONS FOR USE**).

Efavirenz

Due to a decrease in plasma concentrations of CRIXIVAN, a dosage increase of CRIXIVAN is recommended when CRIXIVAN and efavirenz are co-administered. No dosage adjustment of efavirenz is necessary when given with indinavir (see **DOSAGE AND DIRECTIONS FOR USE**).

HMG-CoA Reductase Inhibitors

Concomitant use of CRIXIVAN with lovastatin or simvastatin is not recommended. Caution should be exercised if protease inhibitors, including CRIXIVAN, are also used concurrently with other HMG-CoA reductase inhibitors that are metabolised by the CYP3A4 pathway (e.g. atorvastatin or cerivastatin).

Side effects occurring less frequently (less than 2 %) of patients receiving CRIXIVAN in all Phase II/Phase III studies and considered at least possibly related or of unknown relationship to treatment and of at least moderate intensity are listed below by body system:

Body as a Whole/Site Unspecified
Abdominal distention; Chest pain; Chills; Fever; Flank pain; Flu-like illness; Fungal infection; Malaise; Pain; Syncope

Cardiovascular System
Cardiovascular disorder; Palpitations

Digestive System
Acid regurgitation; Anorexia; Aphthous stomatitis; Cheilitis; Cholestasis; Constipation; Dry mouth; Dyspepsia; Eructation; Flatulence; Gastritis; Gingivitis; Glossodynia; Gingival haemorrhage; Increased appetite; Infectious gastroenteritis; Jaundice; Liver cirrhosis

Haemic and Lymphatic System
Anaemia; Lymphadenopathy; Spleen disorder

Metabolic/Nutritional/Immune
Food allergy

Musculoskeletal System
Arthralgia; Back pain; Leg pain; Myalgia; Muscle cramps; Muscle weakness; Musculoskeletal pain; Shoulder pain; Stiffness

Nervous System and Psychiatric
Agitation; Anxiety; Anxiety disorder; Bruxism; Decreased mental acuity; Depression; Dizziness; Dream abnormality; Dysesthesia; Excitement; Fasciculation; Hypaesthesia; Nervousness; Neuralgia; Neurotic disorder; Paraesthesia; Peripheral neuropathy; Sleep disorder; Somnolence; Tremor; Vertigo

Respiratory System
Cough; Dyspnoea; Halitosis; Pharyngeal hyperaemia; Pharyngitis; Pneumonia; Rales/rhonchi; Respiratory failure; Sinus disorder; Sinusitis; Upper respiratory infection

Skin and Skin Appendage
Body odour; Contact dermatitis; Dermatitis; Dry skin; Flushing; Folliculitis; Herpes simplex; Herpes zoster; Night sweats; Pruritus; Seborrhoea; Skin disorder; Skin infection; Sweating; Urticaria

Special Senses
Accommodation disorder; Blurred vision; Eye pain; Eye swelling; Orbital oedema; Taste disorder

Urogenital System
Dysuria; Haematuria; Hydronephrosis; Nocturia; Premenstrual syndrome; Proteinuria; Renal colic; Urinary frequency; Urinary tract infection; Urine abnormality; Urine sediment abnormality; Urolithiasis

Laboratory Test Findings
The most frequently occurring selected laboratory side effects (incidence greater than or equal to 5 %) considered to be possibly, probably, or definitely drug related by the study investigator in the group treated with CRIXIVAN alone were changes in ALT, AST, indirect serum bilirubin, total serum bilirubin, and urine protein. Only 1 % of patients discontinued treatment due to these laboratory adverse experiences when treated with CRIXIVAN alone or in combination with other antiretroviral agents.

Isolated asymptomatic hyperbilirubinemia (total bilirubin greater than or equal to 42,75 umol/litre), reported predominantly as elevated indirect bilirubin and rarely associated with elevations in ALT, AST, or alkaline phosphatase, has occurred in patients treated with CRIXIVAN alone or in combination with other antiretroviral agents. Most patients continued treatment with CRIXIVAN without dosage reduction and bilirubin values gradually declined toward baseline.

Post-Marketed Experience
The following additional laboratory experiences have been reported and the frequencies are unknown:
Increased serum triglycerides; Increased serum cholesterol.

SPECIAL PRECAUTIONS
Nephrolithiasis
Nephrolithiasis has occurred with CRIXIVAN. In some cases, nephrolithiasis has been associated with renal insufficiency or acute renal failure; in the majority of cases, renal insufficiency and acute renal failure were reversible. Signs and symptoms of nephrolithiasis, including flank pain with or without haematuria (including microscopic haematuria), have been reported in patients receiving the recommended dose of CRIXIVAN (see **WARNINGS** and **DOSAGE AND DIRECTIONS FOR USE**).

During post-marketing surveillance of patients treated with CRIXIVAN, rare reports of interstitial nephritis with medullary calcification and cortical atrophy have been observed in patients with asymptomatic severe leukocyturia (greater than 100 cells/high power field). In patients with asymptomatic severe leukocyturia, further evaluation may be warranted.

Acute Haemolytic Anaemia
Acute haemolytic anaemia has been reported which, in some cases, was severe and progressed rapidly. Once a diagnosis is apparent, appropriate measures for the treatment of haemolytic anaemia should be instituted. It may be necessary to discontinue CRIXIVAN.

Hepatitis
Hepatitis including rare cases of hepatic failure have been reported in patients treated with CRIXIVAN. Because the majority of these patients had confounding medical conditions and/or were receiving concomitant therapy(ies), a causal relationship between CRIXIVAN and these events has not been established.

Diabetes and Hyperglycaemia
There have been reports of new onset diabetes mellitus or hyperglycaemia, or exacerbation of pre-existing diabetes mellitus occurring in HIV-infected patients receiving protease inhibitor therapy. Some patients required either initiation or dose adjustments of insulin or oral hypoglycaemic agents for treatment of these events. In some cases diabetic ketoacidosis has occurred.

In majority of cases, treatment with protease inhibitors was continued, while in some cases treatment was either discontinued or interrupted. In some patients, hyperglycaemia persisted after the protease inhibitor was withdrawn, whether or not diabetes was reported at baseline. A causal relationship between protease inhibitor therapy and these events has not been established.

Immune Reconstitution Syndrome
Immune reconstitution syndrome has been reported in patients treated with combination antiretroviral therapy (CART), including CRIXIVAN. During the initial phase of treatment, a patient whose immune system responds to CART may mount an inflammatory response to indolent or residual opportunistic infections, which may necessitate further evaluation and treatment.

Concomitant use of CRIXIVAN and HMG-CoA Reductase Inhibitors
(See **INTERACTIONS**)
Concomitant use of CRIXIVAN with lovastatin or simvastatin is not recommended. Caution should be exercised if CRIXIVAN is also used concurrently with other HMG-CoA reductase inhibitors that are metabolised by the CYP3A4 pathway. The risk of myopathy, including rhabdomyolysis, may be increased when CRIXIVAN is used in combination with these medicines.

Fat redistribution
Redistribution/accumulation of body fat including central obesity, dorsocervical fat enlargement (buffalo hump), peripheral wasting, breast enlargement and 'cushingoid appearance' have been frequently observed in patients receiving protease inhibitors. The mechanism and long term consequences of these events are currently unknown. A causal relationship has not been established.

Resistance/Cross-resistance
The potential for HIV cross resistance between protease inhibitors has not been fully explored. Therefore, it is unknown what effect initial protease inhibitor therapy will have on the activity of subsequent protease inhibitors.

Concomitant use of CRIXIVAN and St. John's wort *(Hypericum perforatum)*.
Concomitant use of CRIXIVAN and St. John's wort (*Hypericum perforatum*) or products containing St. John's wort is not recommended. Co-administration of CRIXIVAN and St. John's wort has been shown to substantially decrease CRIXIVAN concentrations and may lead to loss of virologic response and possible resistance to CRIXIVAN or to the class of protease inhibitors.

Patients with Coexisting Conditions
There have been reports of spontaneous bleeding in patients with haemophilia A and B treated with protease inhibitors. In some patients, additional factor VII was required. In many of the reported cases, treatment with protease inhibitors was continued or restarted. A causal relationship between protease inhibitor therapy and these episodes has not been established (see **SIDE EFFECTS, Post-Marketed Experience**).

Patients with hepatic insufficiency due to cirrhosis will require a dosage reduction of CRIXIVAN due to decreased metabolism of CRIXIVAN (see **DOSAGE AND DIRECTIONS FOR USE**).

Ingestion of CRIXIVAN with a meal high in calories, fat, and protein reduces the absorption of CRIXIVAN. For optimal absorption, CRIXIVAN should be administered without food but with water 1 hour before or 2 hours after a meal (see **DOSAGE AND DIRECTIONS FOR USE** and **PHARMACOLOGICAL ACTION - PHARMACOKINETICS, Absorption**).

Effects on ability to drive and use machines
There are no data to suggest that CRIXIVAN affects the ability to drive and use machines.

KNOWN SYMPTOMS OF OVERDOSAGE AND PARTICULARS OF ITS TREATMENT
There have been reports of human overdosage with CRIXIVAN. The most commonly reported symptoms were gastrointestinal (e.g. nausea, vomiting, diarrhoea) and renal (e.g. nephrolithiasis, flank pain, haematuria). It is not known whether CRIXIVAN is dialysable by peritoneal or haemodialysis.

IDENTIFICATION
CRIXIVAN 200 mg is a white semi-translucent capsule with "CRIXIVAN™ 200 mg" printed in blue ink.
CRIXIVAN 400 mg is a white semi-translucent capsule with "CRIXIVAN™ 400 mg" printed in green or gold ink.

PRESENTATION
CRIXIVAN 200 mg is available in plastic bottles containing 360 capsules.
CRIXIVAN 400 mg is available in plastic bottles containing 18, 120 or 180 capsules.

STORAGE INSTRUCTIONS
Store in well-closed containers at room temperature, below 30 °C. Protect from moisture.
KEEP OUT OF REACH OF CHILDREN.

REGISTRATION NUMBERS
CRIXIVAN 200 mg - 31/20.2.8/0186
CRIXIVAN 400 mg - 31/20.2.8/0187

NAME AND BUSINESS ADDRESS OF THE HOLDER OF THE CERTIFICATE OF REGISTRATION
MSD (Pty) Ltd
16th Road
HALFWAY HOUSE
1685

DATE OF PUBLICATION OF THIS PACKAGE INSERT
10 August 2007

Namibian Only: Crixivan 200 mg	
Registration Number:	04/20.2.8/1443
Scheduling Status:	NS2

Namibian Only: Crixivan 400 mg	
Registration Number:	04/20.2.8/1442
Scheduling Status:	NS2

Botswana Only: Crixivan 200 mg	
License Number:	BOT9800146
Scheduling Status:	S2

Botswana Only: Crixivan 400 mg	
License Number:	BOT9800326
Scheduling Status:	S2

(WPC-CRX-C-042004, 022005, 072006)

The risk of myopathy, including rhabdomyolysis, may be increased when protease inhibitors, including CRIXIVAN, are used in combination with these drugs (see **SPECIAL PRECAUTIONS**).

St. John's Wort *(Hypericum perforatum)*
Concomitant use of CRIXIVAN and St. John's wort (*Hypericum perforatum*) or products containing St. John's wort is not recommended. Co-administration of CRIXIVAN and St. John's wort has been shown to substantially decrease CRIXIVAN concentrations and may lead to loss of virologic response and possible resistance to CRIXIVAN or to the class of protease inhibitors.

Venlafaxine
Venlafaxine decreases CRIXIVAN plasma concentrations. CRIXIVAN did not affect the plasma concentrations of venlafaxine and active metabolite O-desmethyl-venlafaxine. The clinical significance of this finding is unknown.

Ritonavir
Ritonavir increases CRIXIVAN plasma concentration and CRIXIVAN may affect ritonavir plasma concentration. Currently there are no safety or efficacy data available on the use of this combination in patients.

Sildenafil
Co-administration of CRIXIVAN with sildenafil is expected to substantially increase sildenafil plasma concentrations and may result in an increase in sildenafil-associated adverse events, including hypotension, visual change and priapism (see the manufacturer's complete prescribing information for sildenafil).

Other
Medicines Metabolized by CYP3A4
Co-administration of CRIXIVAN, a CYP3A4 inhibitor, with calcium channel blockers, trazodone and other drugs metabolized by CYP3A4, may result in increased plasma concentrations of these drugs which could increase or prolong their therapeutic and adverse effects.

A formal drug interaction study between CRIXIVAN and didanosine has not been performed. However, a normal (acidic) gastric pH may be necessary for optimum absorption of CRIXIVAN, whereas acid rapidly degrades didanosine which is formulated with buffering agents to increase pH. CRIXIVAN and didanosine should be administered at least one hour apart on an empty stomach (consult the manufacturer's prescribing information for didanosine). Antiretroviral activity was unaltered when didanosine was administered three hours after treatment with CRIXIVAN in one clinical study.

If CRIXIVAN and didanosine are administered concomitantly, they should be administered at least one hour apart on an empty stomach.

Other drugs that induce CYP3A4 less potently than rifampin, such as phenobarbital, phenytoin, carbamazepine, and dexamethasone should be used cautiously together with indinavir since they could also diminish plasma concentrations of indinavir.

PREGNANCY AND LACTATION

CRIXIVAN is contra-indicated in pregnancy and lactation as safety has not been established.

DOSAGE AND DIRECTIONS FOR USE

The recommended dosage of CRIXIVAN is 800 mg (usually given as two 400 mg capsules) orally every 8 hours. **Therapy with CRIXIVAN must be initiated at the recommended dose of 2,4 g/day.**

Since CRIXIVAN must be taken at intervals of 8 hours, a schedule convenient for the patient should be developed.

For optimal absorption, CRIXIVAN should be administered without food but with water 1 hour before or 2 hours after a meal. Alternatively, CRIXIVAN may be administered with other liquids such as skim milk, juice, coffee, or tea, or a light meal, e.g. dry toast with jelly, apple juice, and coffee with skim milk and sugar or corn flakes, skim milk and sugar.

To ensure adequate hydration, it is recommended that the patient drink at least 1,5 litres of liquids during the course of 24 hours.

Concomitant Therapy
Rifabutin
Dose reduction of rifabutin to half the standard dose is recommended (consult the manufacturer's prescribing information for rifabutin) and a dose increase of CRIXIVAN to 1 000 mg every 8 hours are recommended when rifabutin and CRIXIVAN are co-administered.

Ketoconazole
Due to an increase in plasma concentrations of CRIXIVAN, a dosage reduction of CRIXIVAN to 600 mg every 8 hours should be considered when administering ketoconazole concurrently.

Didanosine
If co-prescribed with didanosine (ddI), the two agents should be administered at least one hour apart on an empty stomach.

Itraconazole
Dose reduction of CRIXIVAN to 600 mg every 8 hours is recommended when administering itraconazole 200 mg twice daily concurrently.

Delavirdine
Dose reduction of CRIXIVAN to 600 mg every 8 hours should be considered when administering delavirdine 400 mg three times a day.

Efavirenz
Dose increase of CRIXIVAN to 1 000 mg every 8 hours is recommended when administering efavirenz concurrently.

Patients with Co-existing Conditions
Hepatic Insufficiency due to Cirrhosis
In patients with mild-to-moderate hepatic insufficiency due to cirrhosis, the dosage of CRIXIVAN should be reduced to 600 mg every 8 hours.

Nephrolithiasis
In addition to adequate hydration, medical management in patients who experience nephrolithiasis may include temporary interruption of therapy (e.g. 1 to 3 days) during the acute episode of nephrolithiasis or discontinuation of therapy.

SIDE EFFECTS AND SPECIAL PRECAUTIONS

SIDE EFFECTS
In clinical trials with CRIXIVAN, nephrolithiasis, including flank pain with or without haematuria (including microscopic haematuria), has been reported in approximately 9,8 % (252/2 577) of patients receiving CRIXIVAN at the recommended dose compared to 2,2 % in the control arms. In general these events were not associated with renal dysfunction and resolved with hydration and temporary interruption of therapy (e.g. 1 to 3 days) (see **WARNINGS** and **DOSAGE AND DIRECTIONS FOR USE (Nephrolithiasis)**.

Asymptomatic hyperbilirubinemia (total bilirubin greater than or equal to 42,75 umol/litre), reported predominantly as elevated indirect bilirubin, has occurred in approximately 10 % of patients treated with CRIXIVAN. In less than 1 % this was associated with elevations in ALT or AST.

Hyperbilirubinemia and nephrolithiasis occurred more frequently as doses exceeding 2,4 g/day compared to doses less than or equal to 2,4 g/day.

Post-Marketed Experience
The following additional adverse experiences have been reported in post-marketed experience without regard to causality:

Body as a Whole/Site Unspecified
The following side effects have been reported and the frequencies are unknown:
Abdominal distention; Redistribution/accumulation of body fat in areas such as the back of the neck, breasts, abdomen and retroperitoneum (see **SPECIAL PRECAUTIONS**)

Cardiovascular System
The following side effects have been reported and the frequencies are unknown:
Cardiovascular disorders including myocardial infarction and angina pectoris; Cerebrovascular disorder

Digestive System
The following side effects have been reported and the frequencies are unknown:
Liver function abnormalities; Hepatitis including rare reports of hepatic failure (see **SPECIAL PRECAUTIONS**); Pancreatitis

Haematologic
The following side effects have been reported and the frequencies are unknown:
Increased spontaneous bleeding in patients with haemophilia (see **SPECIAL PRECAUTIONS**); Thrombocytopaenia; Anaemia including acute haemolytic anaemia

Endocrine/Metabolic
The following side effects have been reported and the frequencies are unknown:
New onset diabetes mellitus or hyperglycaemia, or exacerbation of pre-existing diabetes mellitus (see **SPECIAL PRECAUTIONS**); Increases in blood glucose and plasma triglycerides have been reported (see **SPECIAL PRECAUTIONS**)

Hypersensitivity
The following side effects have been reported and the frequencies are unknown:
Anaphylactoid reactions; Vasculitis

Nervous system/Psychiatric
The following side effects have been reported and the frequencies are unknown:
Oral paraesthesia

Skin and Skin Appendage
The following side effects have been reported and the frequencies are unknown:
Hyperpigmentation; Alopecia; Urticaria; Rash, including erythema multiforme and Stevens-Johnson syndrome; Ingrown toenails and/or paronychia

Urogenital System
The following side effects have been reported and the frequencies are unknown:
Nephrolithiasis, generally without renal dysfunction (however there have been reports of nephrolithiasis with renal dysfunction.); Acute renal failure (see **SPECIAL PRECAUTIONS**); Pyelonephritis; Renal insufficiency; Leukocyturia; Crystalluria; Interstitial nephritis sometimes with indinavir crystal deposits; in some patients the interstitial nephritis did not resolve following discontinuation of CRIXIVAN

Clinical Trials
Drug-related clinical adverse experiences of moderate or severe intensity in greater than or equal to 2 % of patients treated with CRIXIVAN alone, CRIXIVAN in combination with zidovudine, or zidovudine alone are presented below:

ADVERSE EXPERIENCE	CRIXIVAN Percent (n=196)	CRIXIVAN Plus Zidovudine Percent (n=195)	Zidovudine Percent (n=196)
Body as a Whole			
Asthenia/fatigue	3,6	9,2	7,7
Abdominal pain	8,7	8,2	5,1
Flank pain	2,6	1,0	0
Malaise	0,5	2,0	1,5
Digestive			
Acid regurgitation	1,0	2,0	0,5
Diarrhoea	4,6	4,1	2,1
Dry mouth	0,5	0	2,1
Nausea	11,7	32,1	14,4
Vomiting	4,1	12,5	4,6
Anorexia	0,5	2,0	3,1
Musculoskeletal System			
Back pain	2,0	1,0	1,5
Nervous System/ Psychiatric			
Dizziness	1,0	3,6	0,5
Headache	5,6	11,7	5,1
Somnolence	1,0	1,5	3,6
Insomnia	3,1	1,5	0
Special Senses			
Taste perversion	2,6	3,6	2,1

In Phase I and II controlled trials, the following side effects were reported significantly more frequently by those randomized to CRIXIVAN-containing arms than by those randomized to nucleoside analogues: rash, upper respiratory infection, dry skin, pharyngitis, taste perversion.

PATIENT INFORMATION LEAFLET
INFORMATION FOR THE PATIENT ABOUT

CRIXIVAN® (INDINAVIR, MSD)

Please read all of this leaflet carefully before you start taking this medicine, even if you have just refilled your prescription. Some of the information in the previous leaflet may have changed. Keep this leaflet. You may need to read it again. If you have further questions, please ask your doctor or your pharmacist. This medicine has been prescribed for you personally and you should not share your medicine with other people. It may harm them, even if their symptoms are the same as yours.

SCHEDULING STATUS S4

PROPRIETARY NAME AND DOSAGE FORM

CRIXIVAN® 200 mg Capsules (200 mg of indinavir)
CRIXIVAN® 400 mg Capsules (400 mg of indinavir)

WHAT CRIXIVAN CONTAINS

CRIXIVAN 200 mg contains 200 mg of indinavir as the active ingredient.
CRIXIVAN 400 mg contains 400 mg of indinavir as the active ingredient.

In addition, CRIXIVAN contains the following inactive ingredients: Anhydrous lactose, Magnesium Stearate.

CRIXIVAN contains sugar.

WHAT CRIXIVAN IS USED FOR

CRIXIVAN is a member of a class of medicines called protease inhibitors. It is active against the Human Immunodeficiency Virus (HIV) helping to reduce the amount of virus in the body.

Your doctor has prescribed CRIXIVAN for you because you have HIV infection. CRIXIVAN can help reduce your chances of getting illnesses associated with HIV infection and it can help extend your life. CRIXIVAN can also help lower the amount of HIV in your body (called "viral load") and raise your CD4 (T) cell count. CRIXIVAN may not have these effects in all patients.

HIV is a blood borne disease spread by contact with blood or sexual contact with an infected individual.

BEFORE YOU TAKE CRIXIVAN

Do not take CRIXIVAN:

1. If you are allergic to indinavir or any of the other ingredients of CRIXIVAN.
2. If pregnant or breastfeeding as safety has not been established
3. Children under the age of 18 years, as safety and efficacy have not been established
4. Antituberculosis regimens containing rifampicin must be avoided
5. Safety and efficacy in patients with concomitant liver disease (e.g. Hepatitis B) has not been established
6. Safety and efficacy have not been established in the elderly.

Take special care with CRIXIVAN

What should I tell my doctor before I take CRIXIVAN?

Tell your doctor about any past or present medical problems, including liver disease due to cirrhosis, kidney problems, diabetes, haemophilia, allergies, or high cholesterol and if you are taking cholesterol-lowering medicines called "statins".

You should always tell your doctor about all medications you are taking or plan to take, including those obtained without a prescription, herbal products, or dietary supplements.

Pregnancy and Breastfeeding

If you are pregnant or breastfeeding your baby while taking this medicine, please consult your doctor, pharmacist or other healthcare professional for advice.

It is not known whether CRIXIVAN is harmful to an unborn baby when taken by a pregnant woman. If you are pregnant, you should take CRIXIVAN only if your doctor decides it is clearly needed.

Tell your doctor if you are breastfeeding. Your doctor will tell you to stop breastfeeding while you are taking CRIXIVAN.

Driving and using machinery

There is no specific information to suggest that CRIXIVAN affects your ability to drive and use machinery. However, dizziness and blurred vision have been reported during treatment with CRIXIVAN. If you experience these you should avoid driving or operating machinery.

Taking other medicines with CRIXIVAN

CRIXIVAN may be taken with a number of medications that are commonly used by people with HIV infection. These include zidovudine, didanosine, lamivudine, stavudine, fluconazole, lamivudi, clarithromycin, cimetidine, trimethoprim/sulfamethoxazole and methadone.

Other medications may be taken with CRIXIVAN but require dosage adjustment of that medicine or CRIXIVAN. These include rifabutin, ketoconazole, itraconazole, delavirdine and efavirenz.

Tell your doctor if you are taking calcium channel blockers (medicines to treat hypertension or chest pain).

Tell your doctor if you are taking venlafaxine.

Tell your doctor if you are taking tramadone.

Medications that may not be taken with CRIXIVAN are terfenadine, midazolam, pimozide, cisapride, astemizole, triazolam, rifampin and ergot derivatives such as ergotamine tartrate or ergotamine tartrate and caffeine. Consult your doctor before taking CRIXIVAN with any other medication.

Taking CRIXIVAN with St. John's wort (Hypericum perforatum), an herbal product sold as a dietary supplement, or products containing St. John's wort, is not recommended as it may decrease the effect of CRIXIVAN or other HIV-related drugs.

If you are taking medicines on a regular basis including complementary or traditional medicines, the use of CRIXIVAN with these medicines, may cause undesirable interactions. Please consult your doctor, pharmacist or other healthcare professional for advice.

HOW TO TAKE CRIXIVAN

Always take CRIXIVAN exactly as your doctor has instructed you. You should check with your doctor or pharmacist if you are unsure.

If you have the impression that the effect of CRIXIVAN is too strong or too weak, talk to your doctor or pharmacist.

CRIXIVAN is in capsule form and must be taken orally. Take 800 mg (usually given as two 400 mg capsules) at regular 8-hour intervals. CRIXIVAN must be taken at intervals of 8 hours for full effectiveness.

CRIXIVAN should be taken with water 1 hour before or 2 hours after a meal. If water is not preferred, CRIXIVAN can be taken with skimmed or fat-free milk, juice, coffee, or tea; or a light meal such as dry toast with jam or fruit conserve, juice and coffee with skimmed or fat-free milk and sugar; or corn flakes, skimmed or fat-free milk and sugar. At any other time you can follow your regular diet.

Taking CRIXIVAN with a meal that is high in calories, fat, and protein reduces your body's ability to absorb the medicine and in turn reduces its effectiveness.

It is important to drink at least 1,5 litres of liquids during each day.

It is very important to take CRIXIVAN exactly as your doctor prescribes and that you do not stop taking it without first telling your doctor. Taking CRIXIVAN as described will ensure the full effectiveness of the product.

Do not share medicines prescribed for you with any other person.

If you take more CRIXIVAN than you should

In the event of over dosage, consult your doctor or pharmacist. If neither is available, contact the nearest hospital or poison control centre. The most commonly reported symptoms were gastrointestinal (e.g. nausea, vomiting, diarrhoea) and renal (e.g. nephrolithiasis, flank pain, haematuria).

If you forget to take CRIXIVAN

Take CRIXIVAN 3 times a day at regular 8-hour intervals. However, if you miss a dose, do not take it later in the day. Simply continue to follow your usual schedule. Do not take a double dose to make up for forgotten individual doses.

POSSIBLE SIDE EFFECTS

Any drug may have unintended or undesirable effects, so-called side effects. CRIXIVAN has been shown to be generally well tolerated.

Side effects occurring frequently in patients receiving CRIXIVAN:

Digestive system
Diarrhoea
Nausea

Nervous system/Psychiatric
Headache

Side effects occurring less frequently in patients receiving CRIXIVAN:

Body as a Whole/Site Unspecified
Weakness/fatigue

Digestive system
Dyspepsia

Musculoskeletal system
Severe muscle pain and weakness have occurred in patients taking CRIXIVAN, together with cholesterol-lowering medicines called "statins"

Nervous system and Psychiatric
Dizziness

Skin and Skin Appendage
Dry skin

The following side effects have been reported with an unknown frequency:

Body as a Whole/Site Unspecified
Abdominal pain/swelling
Increased fat appearing in areas such as the neck, breasts, abdomen, and back

Cardiovascular system
Heart problems including heart attack
Stroke

Digestive system
Some patients treated with CRIXIVAN have had liver problems and in rare cases liver failure
Inflammation of the pancreas

Haemic and Lymphatic system
Some patients treated with CRIXIVAN have had rapid breakdown of red blood cells (haemolytic anaemia) which in some cases was severe
In some patients with haemophilia increased bleeding has been reported
Low red blood cell count

Endocrine/Metabolic
Diabetes and high blood sugar (hyperglycaemia) has occurred in patients taking protease inhibitors. In some of these patients, this led to ketoacidosis, a serious condition caused by poorly controlled blood sugar. Some patients had diabetes before starting protease inhibitors, others did not. Some patients required adjustments to their diabetes medication. Others needed new diabetes medication
Increased plasma triglycerides

Hypersensitivity
Allergic reactions

Nervous system/Psychiatric
Numbness of the mouth

Skin and Skin Appendage
Change in skin colour
Hair loss
Ringworm toenails with or without infection
Rash
Severe skin reactions

Special Senses
Taste perversion

Urogenital system
There have been kidney stones and in some of these patients this led to more severe kidney problems including kidney failure
Crystals in the urine
Inflammation of the kidneys; infection of the kidneys; decreased kidney function

Skin and Skin Appendage
Dry skin

Your doctor has a more complete list of side effects.

Tell your doctor promptly about these or any other unusual symptoms. If the condition persists or worsens, seek medical attention.

In addition, tell your doctor if you experience any symptoms that suggest an allergic reaction after taking CRIXIVAN.

Not all side effects reported for this medicine are included in this leaflet. Should your general health worsen while taking this medicine, please consult your doctor, pharmacist or other healthcare professional for advice.

OTHER CONSIDERATIONS

You should know that CRIXIVAN is not a cure for HIV infection and that you may continue to develop infections or other illnesses associated with HIV disease. You should, therefore, remain under the care of your doctor while taking CRIXIVAN.

In some patients with advanced HIV infection (AIDS) and a history of opportunistic infection, signs and symptoms of inflammation from previous infections may occur when combination antiretroviral treatment is started.

The long-term effects of CRIXIVAN are unknown at this time. Treatment with CRIXIVAN has not been shown to reduce the risk of transmission of HIV to others through sexual contact or blood contamination.

CRIXIVAN capsules are sensitive to moisture. At all times, store CRIXIVAN in the original container with the desiccant.

See your doctor for more details.

HOW CAN I LEARN MORE ABOUT CRIXIVAN?

Not all information about the drug is printed here. If you have any additional questions, ask your doctor or pharmacist who have more detailed information about CRIXIVAN and HIV infection.

STORING AND DISPOSING OF CRIXIVAN

How long should I keep my medicine?

Do not use this medicine after the month and year following EXPIRES/VERVAL on the carton and bottle.
Return all unused medicine to your pharmacist.
Do not dispose of unused medicine in drains or sewerage systems (e.g. toilets).

How should I store CRIXIVAN?

Store CRIXIVAN in the well-closed original container at room temperature below 30 °C. Protect from moisture.

Keep all medicines out of reach and sight of children.

PRESENTATION OF CRIXIVAN

CRIXIVAN 200 mg is available in plastic bottles containing 360 capsules.
CRIXIVAN 400 mg is available in plastic bottles containing 18, 120 or 180 capsules.

IDENTIFICATION OF CRIXIVAN

CRIXIVAN 200 mg is a white semi-translucent capsule with "CRIXIVAN™ 200 mg" printed in blue ink.
CRIXIVAN 400 mg is a white semi-translucent capsule with "CRIXIVAN™ 400 mg" printed in green or gold ink.

REGISTRATION NUMBERS

CRIXIVAN 200 mg - 31/20.2.8/0185
CRIXIVAN 400 mg - 31/20.2.8/0187

NAME, BUSINESS ADDRESS AND TELEPHONE NUMBER OF THE HOLDER OF THE CERTIFICATE OF REGISTRATION

MSD (Pty) Ltd
16th Road
Halfway House
1685
011 655 3000

DATE OF PUBLICATION OF THE PATIENT INFORMATION LEAFLET

10 August 2007

MSD

(MPPI-CRX-C-042004, 022005, 072006)

360215

APPENDIX C

CONCENTRATION CALCULATIONS OF

TRANSPORT EXPERIMENTAL GROUPS

Indinavir

Indinavir was provided in the form of Crixivan® capsules (400 mg/capsule). Each capsule contains 658 mg powder in total, of which 400 mg is pure indinavir.

1 M indinavir: 613.79 g in 1000 ml

1 mM indinavir: 0.61379 g in 1000 ml

1 uM indinavir: 0.00061379 g in 1000 ml

200 uM indinavir: 0.12276 g in 1000 ml

$$\text{indinavir concentration} = \frac{10\ x\ 0.12276}{1000}$$

$$\text{indinavir concentration} = 0.0012276 \text{ g in } 10 \text{ ml}$$

$$\text{indinavir concentration} = 1.2276 \text{ mg in } 10 \text{ ml}$$

400 mg indinavir powder in every 658 mg powder

$$\frac{1.23 \text{ mg indinavir}}{10\ ml} = \frac{x}{10\ ml}$$

$$x = 2.02 \text{ mg powder}$$

2.02 mg powder was needed in 10 ml. The transport study required a double concentration so therefore 4.04 mg powder in 10 ml (2.02 mg powder in 5 ml).

Hypoxis hemerocallidea commercial product

Each capsule contained 403.74 mg powder, of which 300 mg *Hypoxis* tuber.

A concentration of 500 ug was required for the transport studies:

$$\frac{0.5\ mg}{300\ mg\ hypoxis\ tuber} = \frac{x}{403.74\ mg\ capsule\ powder}$$

$$x = 0.6729\ mg$$

A double concentration was needed, therefore 1.3458 mg per 1 ml (13.458 mg in 10 ml and 6.729 mg in 5 ml)

Hypoxis hemerocallidea aqueous extract and reference material

For the transport study, a final concentration of 500 ug/ml was required, 0.5 mg *H. hemerocallidea* material in 1 ml. A double concentration needed to be prepared which entailed 1 mg in 1 ml, so 5 mg in 5 ml.

Verapamil

Verapamil has a molar mass of 454.602 g/mol.

1 M verapamil: 454.602 g in 1000 ml

1 mM verapamil: 0.454602 g in 1000 ml

1 uM verapamil: 0.0004545602 g in 1000 ml

100 uM verapamil: 0.0454602 g in 1000 ml

$$x = \frac{10\ x\ 0.0454602}{1000}$$

$$x\ = 0.00045602\ g$$

$x\ = 0.454602\ mg$ verapamil in 10 ml

Double concentration: 0.454602 x 2 = 0.909204 mg in 10 ml. (0.45 mg in 5 ml)

APPENDIX D

RAW DATA

IN VITRO TRANSPORT STUDY

1. Transepithelial electrical resistance (TEER) values

Table D 1: TEER Negative control Indinavir AP-BL

TEER value (Ω)	
0 min	**120 min**
223	253
268	258
267	244

Table D 2: TEER Negative control Indinavir BL-AP

TEER value (Ω)	
0 min	**120 min**
274	281
262	275
205	201

Table D 3: TEER Positive Control Indinavir & Verapamil AP-BL

TEER value (Ω)	
0 min	**120 min**
272	244
281	280
284	236

Table D 4: TEER Positive Control Indinavir & Verapamil BL-AP

TEER value (Ω)	
0 min	**120 min**
287	268
294	238
255	247

Table D 5: TEER Indinavir & *Hypoxis hemerocallidea* aqueous extract AP-BL

TEER value (Ω)	
0 min	**120 min**
235	239
277	268
273	258

Table D 6: TEER Indinavir & *Hypoxis hemerocallidea* aqueous extract BL-AP

TEER value (Ω)	
0 min	**120 min**
231	269
280	239
285	261

Table D 7: TEER Indinavir & *Hypoxis hemerocallidea* commercial product AP-BL

TEER value (Ω)	
0 min	**120 min**
260	284
285	233
229	325

Table D 8: TEER Indinavir & *Hypoxis hemerocallidea* commercial product BL-AP

TEER value (Ω)	
0 min	**120 min**
354	380
317	399
350	380

Table D 9: TEER Indinavir & *Hypoxis hemerocallidea* reference plant material AP-BL

TEER value (Ω)	
0 min	**120 min**
280	241
274	278
298	241

Table D 10: TEER Indinavir & *Hypoxis hemerocallidea* reference plant material BL-AP

TEER value (Ω)	
0 min	**120 min**
292	252
276	258
275	282

2. Transport Calculations

2.1 Indinavir alone AP-BL

Table D 11: Concentration and percentage transport for each sample of indinavir alone (AP-BL) over the pre-determined time intervals. n = 3.

Time	Inj vol	Peak area	Correction 1	Correction 2	Concentration (µg/ml)	% Transport	
M 1	25	3146.90	3146.90	3146.90	52.19	100	
0	25	0	0.00	0.00	0.00	0	
20	25	14.63	14.63	14.63	0.24	0.46	
40	25	72.45	73.62	80.02	1.33	2.54	
60	25	78.80	84.60	91.95	1.52	2.92	
80	25	99.27	105.57	114.75	1.90	3.65	
100	25	132.25	140.19	152.38	2.53	4.84	
120	25	135.97	146.55	159.29	2.64	5.06	
M 2	25	2970.85	2970.85	2970.85	49.27	100.00	
0	25	0.00	0.00	0.00	0.00	0.00	
20	25	14.35	14.35	14.35	0.24	0.48	
40	25	63.34	64.49	70.10	1.16	2.36	
60	25	89.59	94.65	102.89	1.71	3.46	
80	25	113.09	120.26	130.71	2.17	4.40	
100	25	136.34	145.39	158.03	2.62	5.32	
120	25	147.37	158.28	172.05	2.85	5.79	
M 3	25	2378.57	2378.57	2378.57	39.45	100.00	
0	25	0.00	0.00	0.00	0.00	0.00	
20	25	43.47	43.47	43.47	0.72	1.83	
40	25	86.14	89.62	97.41	1.62	4.10	
60	25	116.46	123.35	134.07	2.22	5.64	
80	25	136.76	146.08	158.78	2.63	6.68	
100	25	160.85	171.79	186.73	3.10	7.85	
120	25	180.32	193.19	209.99	3.48	8.83	

Table D 12: P_{app} values for each sample of indinavir alone (AP-BL)

	Slope	**1/A.60.C0**	**P_{app}**
M1	0.044721483	3.56888E-05	1.59606E-06
M2	0.051940303	3.56888E-05	1.85369E-06
M3	0.073413403	3.56888E-05	2.62004E-06
Average			2.02326E-06
STDEV.P			4.34895E-07

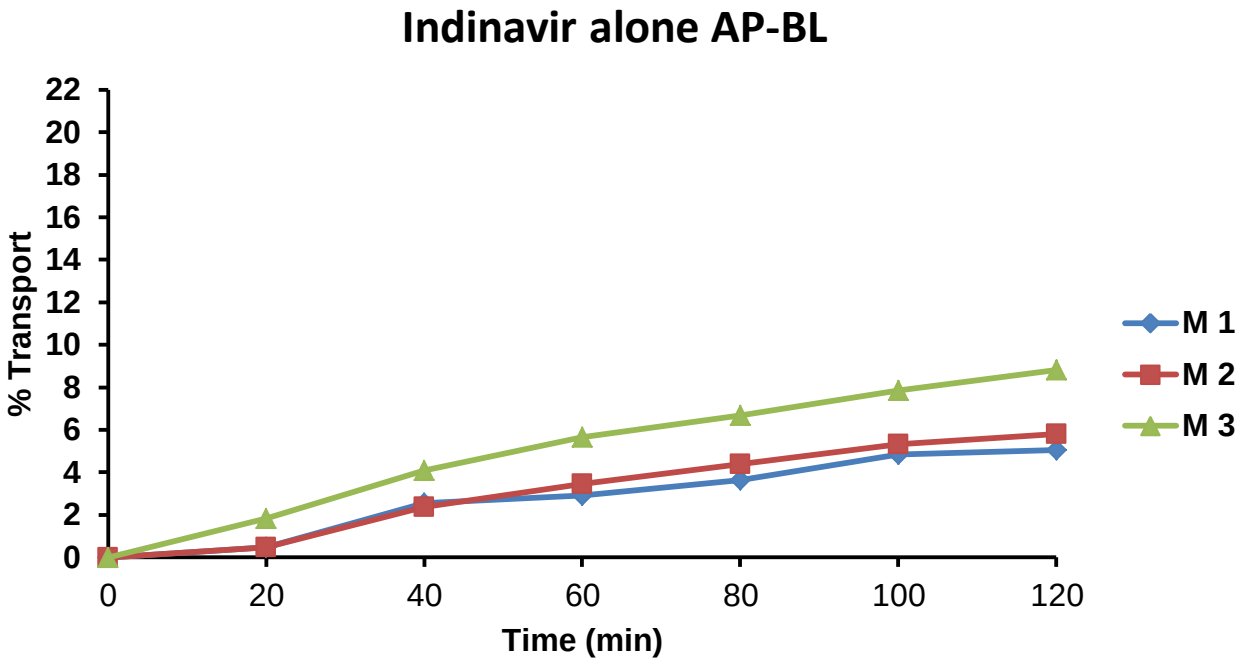

Figure D 1: Percentage of indinavir transport for each of the samples (Indinavir alone AP-BL). n = 3.

Table D 13: Average transport and standard deviation for each sample (indinavir alone AP-BL) over the pre-determined time intervals. n = 3.

Time (min)	M1	M2	M3	Average transport	STDEV.P
0	0	0	0	0	0
20	0.464870825	0.48295673	1.827465242	0.925097599	0.638112998
40	2.542869712	2.359528696	4.095235665	2.999211358	0.778612203
60	2.922007842	3.463155048	5.636782861	4.00731525	1.173196089
80	3.646597314	4.399915015	6.675510459	4.907340929	1.287553107
100	4.842142371	5.319389942	7.850636892	6.004056401	1.320185831
120	5.061919971	5.791105678	8.828295847	6.560440499	1.631012744

2.2 Indinavir alone BL-AP

Table D 14: Concentration and percentage transport for each sample of indinavir alone (BL-AP) over the pre-determined time intervals. n = 3.

Time	Inj vol	Peak area	Correction 1	Correction 2	Concentration (µg/ml)	% Transport	
M 1	25	2555.28	2555.28	2555.28	42.38	100	
0	25	0	0.00	0.00	0.00	0	
20	25	254.36	254.36	254.36	4.22	9.95	
40	25	428.92	449.27	488.34	8.10	19.11	
60	25	605.03	639.35	694.94	11.52	27.20	
80	25	741.86	790.26	858.98	14.25	33.62	
100	25	815.79	875.13	951.23	15.78	37.23	
120	25	960.00	1025.26	1114.41	18.48	43.61	
M 2	25	2765.99	2765.99	2765.99	45.87	100.00	
0	25	0.00	0.00	0.00	0.00	0.00	
20	25	265.67	265.67	265.67	4.41	9.61	
40	25	514.33	535.58	582.15	9.65	21.05	
60	25	674.61	715.76	778.00	12.90	28.13	
80	25	915.80	969.77	1054.09	17.48	38.11	
100	25	1097.76	1171.03	1272.86	21.11	46.02	
120	25	1169.59	1257.41	1366.75	22.67	49.41	
M 3	25	3566.75	3566.75	3566.75	59.15	100.00	
0	25	0.00	0.00	0.00	0.00	0.00	
20	25	200.69	200.69	200.69	3.33	5.63	
40	25	335.47	351.53	382.10	6.34	10.71	
60	25	530.12	556.96	605.39	10.04	16.97	
80	25	754.28	796.69	865.97	14.36	24.28	
100	25	847.67	908.01	986.97	16.37	27.67	
120	25	1037.23	1105.04	1201.14	19.92	33.68	

Table D 15: P_{app} values for each sample of indinavir alone (BL-AP)

	Slope	**1/A.60.C0**	**Papp**
M1	0.356938082	3.56888E-05	1.27387E-05
M2	0.42522683	3.56888E-05	1.51758E-05
M3	0.283362783	3.56888E-05	1.01129E-05
Average			1.26758E-05
STDEV.P			2.06742E-06

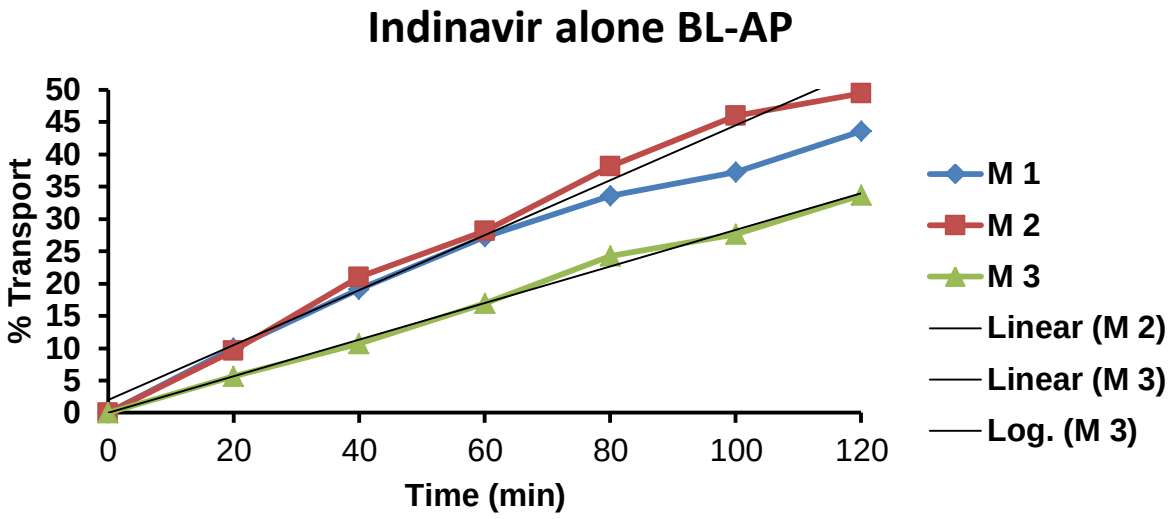

Figure D 2: Percentage of indinavir transport for each of the samples (Indinavir alone BL-AP). n = 3.

Table D 16: Average transport and standard deviation for each sample (indinavir alone BL-AP) over the pre-determined time intervals. n = 3.

Time (min)	M1	M2	M3	Average Transport	STDEV.P
0	0	0	0	0	0
20	9.9543189	9.605022072	5.626736	8.395358932	1.962899
40	19.11084864	21.04682214	10.7127	16.95679096	4.48542
60	27.19638945	28.12729553	16.9732	24.09896172	5.052986
80	33.61582612	38.10911025	24.27896	32.0012999	5.760397
100	37.22618008	46.01810169	27.67133	36.97187051	7.492197
120	43.61220863	49.41285914	33.6759	42.23365698	6.498115

2.3 Indinavir & Verapamil AP-BL

Table D 17: Concentration and percentage transport for each sample of indinavir with verapamil (AP-BL) over the pre-determined time intervals. n = 3.

Time	Inj vol	Peak area	Correction 1	Correction 2	Concentration (µg/ml)	% Transport	
M 1	25	3077.05	3077.05	3077.05	48.61	100	
0	25	0	0.00	0.00	0.00	0	
20	25	65.95	65.95	65.95	1.04	2.14	
40	25	192.50	197.77	214.97	3.40	6.99	
60	25	295.82	311.22	338.29	5.34	10.99	
80	25	419.56	443.23	481.77	7.61	15.66	
100	25	541.76	575.32	625.35	9.88	20.32	
120	25	595.71	639.05	694.62	10.97	22.57	
M 2	25	3361.50	3361.50	3361.50	53.11	100.00	
0	25	0.00	0.00	0.00	0.00	0.00	
20	25	75.42	75.42	75.42	1.19	2.24	
40	25	193.54	199.57	216.93	3.43	6.45	
60	25	268.39	283.88	308.56	4.87	9.18	
80	25	389.38	410.85	446.58	7.06	13.29	
100	25	485.75	516.90	561.85	8.88	16.71	
120	25	566.89	605.75	658.43	10.40	19.59	
M 3	25	2650.26	2650.26	2650.26	41.87	100.00	
0	25	0.00	0.00	0.00	0.00	0.00	
20	25	100.23	100.23	100.23	1.58	3.78	
40	25	250.21	258.23	280.68	4.43	10.59	
60	25	256.37	276.39	300.42	4.75	11.34	
80	25	429.32	449.83	488.95	7.72	18.45	
100	25	656.51	690.86	750.93	11.86	28.33	
120	25	633.84	686.37	746.05	11.79	28.15	

Table D 18: P_{app} values for each sample of indinavir with verapamil (AP-BL)

	Slope	**1/A.60.C0**	**Papp**
M1	0.20134436	3.56888E-05	7.18574E-06
M2	0.168812683	3.56888E-05	6.02472E-06
M3	0.252523498	3.56888E-05	9.01226E-06
Average			7.40757E-06
STDEV.P			1.2297E-06

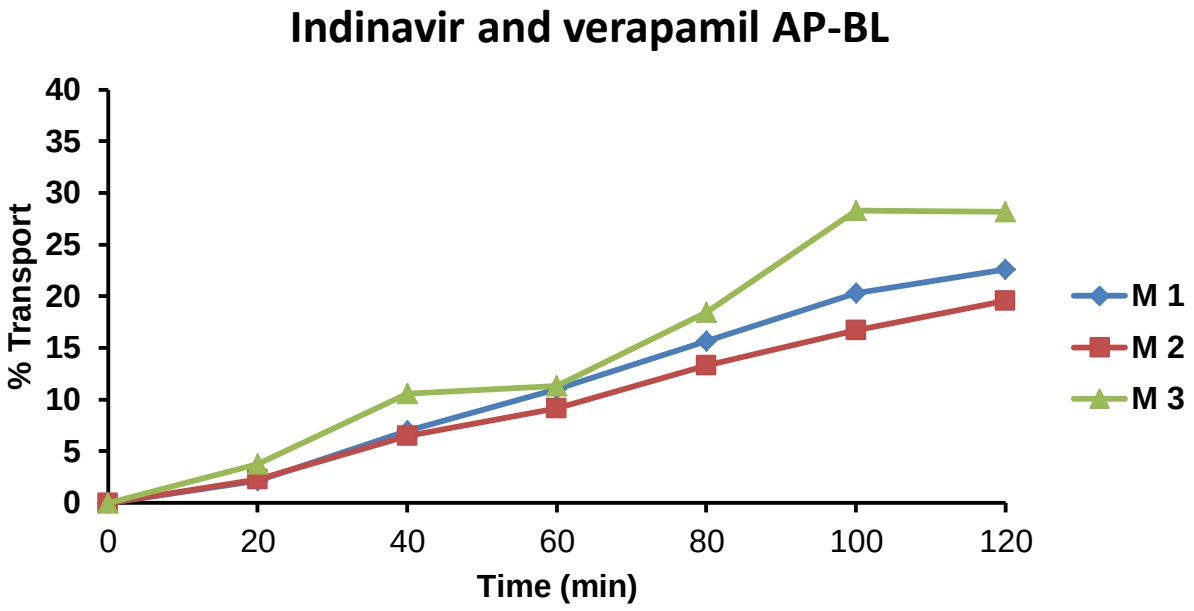

Figure D 3: Percentage of indinavir transport for each of the samples (Indinavir with verapamil AP-BL). n = 3.

Table D 19: Average transport and standard deviation for each sample (indinavir with verapamil AP-BL) over the pre-determined time intervals. n = 3.

Time (min)	M1	M2	M3	Average transport	STDEV.P
0	0	0	0	0	0
20	2.143351587	2.243691804	3.781967	2.723003355	0.74992
40	6.986269316	6.453222099	10.59076	8.010082768	1.83774
60	10.99386492	9.179230468	11.33552	10.50287292	0.94629
80	15.65683467	13.28513845	18.44906	15.79701091	2.11049
100	20.32302953	16.71428948	28.33431	21.79054385	4.85602
120	22.57430684	19.58733019	28.15005	23.43723061	3.54857

2.4 Indinavir & Verapamil BL-AP

Table D 20: Concentration and percentage transport for each sample of indinavir with verapamil (BL-AP) over the pre-determined time intervals. n = 3.

Time	Inj vol	Peak area	Correction 1	Correction 2	Concentration (µg/ml)	% Transport	
M 1	25	2321.72	2321.72	2321.72	36.68	100	
0	25	0	0.00	0.00	0.00	0	
20	25	104.86	104.86	104.86	1.66	4.52	
40	25	277.98	286.37	311.27	4.92	13.41	
60	25	320.21	342.45	372.23	5.88	16.03	
80	25	438.50	464.12	504.48	7.97	21.73	
100	25	496.69	531.77	578.01	9.13	24.90	
120	25	599.44	639.17	694.76	10.98	29.92	
M 2	25	2239.37	2239.37	2239.37	35.38	100.00	
0	25	0.00	0.00	0.00	0.00	0.00	
20	25	57.76	57.76	57.76	0.91	2.58	
40	25	196.09	200.72	218.17	3.45	9.74	
60	25	249.59	265.28	288.35	4.56	12.88	
80	25	327.83	347.79	378.04	5.97	16.88	
100	25	416.93	443.15	481.69	7.61	21.51	
120	25	507.34	540.70	587.71	9.28	26.24	
M 3	25	2540.83	2540.83	2540.83	40.14	100.00	
0	25	0.00	0.00	0.00	0.00	0.00	
20	25	85.93	85.93	85.93	1.36	3.38	
40	25	212.49	219.37	238.44	3.77	9.38	
60	25	338.22	355.22	386.11	6.10	15.20	
80	25	379.85	406.91	442.29	6.99	17.41	
100	25	488.22	518.61	563.71	8.91	22.19	
120	25	550.09	589.15	640.38	10.12	25.20	

Table D 21: P_{app} values for each sample of indinavir with verapamil (BL-AP)

	Slope	**1/A.60.C0**	**Papp**
M1	0.247950182	3.56888E-05	8.84904E-06
M2	0.220953336	3.56888E-05	7.88556E-06
M3	0.216503006	3.56888E-05	7.72673E-06
Average			8.15378E-06
STDEV.P			4.95884E-07

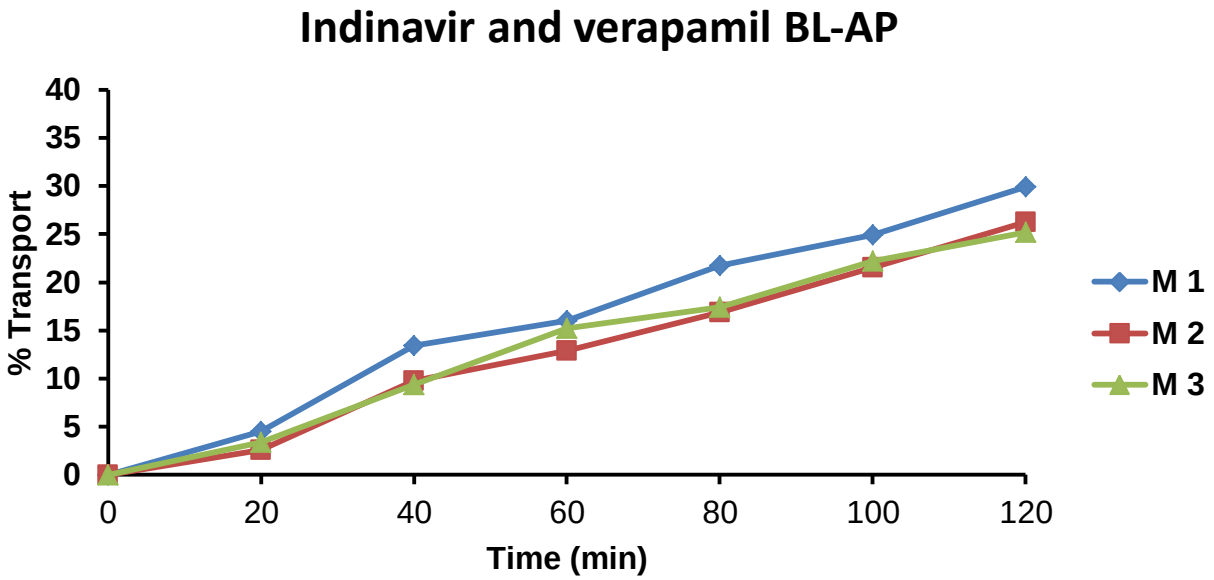

Figure D 4: Percentage of indinavir transport for each of the samples (Indinavir with verapamil BL-AP). n = 3.

Table D 22: Average transport and standard deviation for each sample (indinavir with verapamil BL-AP) over the pre-determined time intervals. n = 3.

Time (min)	M1	M2	M3	Average transport	STDEV.P
0	0	0	0	0	0
20	4.516667815	2.579430822	3.382044	3.492714362	0.79473587
40	13.40705805	9.742406201	9.384358	10.84460747	1.81781267
60	16.03253519	12.87617092	15.19614	14.70161459	1.3351838
80	21.72856656	16.88140397	17.40738	18.67244991	2.17164289
100	24.89571929	21.50992119	22.18592	22.86385186	1.46301193
120	29.92416353	26.24462996	25.20364	27.12414463	2.02500933

2.5 Indinavir & *Hypoxis hemerocallidea* aqueous extract AP-BL

Table D 23: Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* aqueous extract (AP-BL) over the pre-determined time intervals. n = 3.

Time	Inj vol	Peak area	Correction 1	Correction 2	Concentration (µg/ml)	% Transport
M 1	25	7053.87	7053.87	7053.87	116.98	100
0	25	0	0.00	0.00	0.00	0
20	25	26.73	26.73	26.73	0.44	0.38
40	25	128.02	130.15	141.47	2.35	2.01
60	25	218.86	229.10	249.03	4.13	3.53
80	25	259.70	277.21	301.31	5.00	4.27
100	25	386.86	407.64	443.09	7.35	6.28
120	25	582.27	613.22	666.55	11.05	9.45
M 2	25	6849.63	6849.63	6849.63	113.59	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	41.77	41.77	41.77	0.69	0.61
40	25	149.97	153.31	166.64	2.76	2.43
60	25	201.01	213.01	231.53	3.84	3.38
80	25	247.77	263.85	286.79	4.76	4.19
100	25	362.11	381.93	415.14	6.88	6.06
120	25	495.89	524.86	570.49	9.46	8.33
M 3	25	6771.00	6771.00	6771.00	112.29	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	56.66	56.66	56.66	0.94	0.84
40	25	190.25	194.78	211.72	3.51	3.13
60	25	240.65	255.87	278.12	4.61	4.11
80	25	312.77	332.02	360.90	5.98	5.33
100	25	436.74	461.76	501.91	8.32	7.41
120	25	530.01	564.95	614.07	10.18	9.07

Table D 24: P_{app} values for each sample of indinavir with *H. hemerocallidea* aqueous extract (AP-BL)

	Slope	**1/A.60.C0**	**Papp**
M1	0.075748536	3.56888E-05	2.70337E-06
M2	0.067218765	3.56888E-05	2.39896E-06
M3	0.076004596	3.56888E-05	2.71251E-06
Average			2.60495E-06
STDEV.P			1.45705E-07

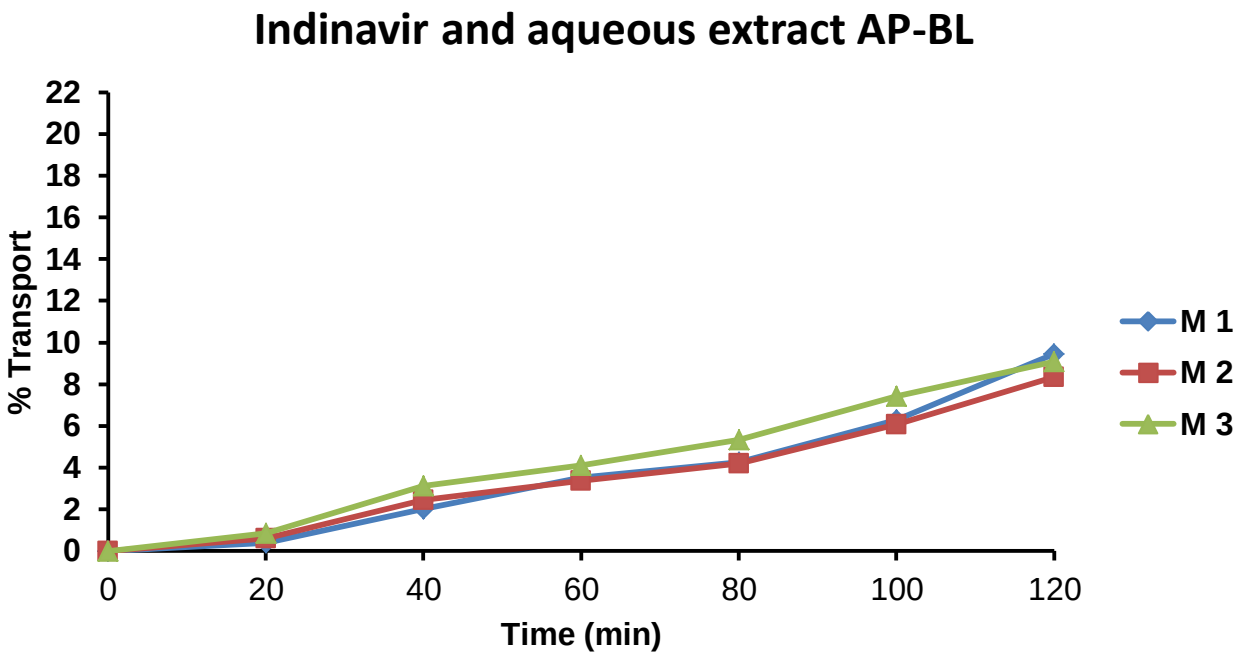

Figure D 5: Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* aqueous extract AP-BL). n = 3.

Table D 25: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* aqueous extract AP-BL) over the pre-determined time intervals. n = 3.

Time (min)	M1	M2	M3	Average transport	STDEV.P
0	0	0	0	0	0
20	0.378903921	0.609853379	0.836756	0.608504587	0.18692
40	2.00558918	2.432873175	3.126826	2.521762865	0.4620384
60	3.530349119	3.380251856	4.10749	3.672696893	0.313492
80	4.271569464	4.186960717	5.330011	4.596180496	0.5200451
100	6.281446336	6.060804027	7.412714	6.584988005	0.5921813
120	9.449371628	8.328839933	9.069158	8.949123247	0.4652627

2.6 Indinavir & *Hypoxis hemerocallidea* aqueous extract BL-AP

Table D 26: Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* aqueous extract (BL-AP) over the pre-determined time intervals. n = 3.

Time	Inj vol	Peak area	Correction 1	Correction 2	Concentration (µg/ml)	% Transport
M 1	25	7310.22	7310.22	7310.22	121.23	100
0	25	0	0.00	0.00	0.00	0
20	25	265.10	265.10	265.10	4.40	3.63
40	25	452.55	473.76	514.96	8.54	7.04
60	25	719.28	755.48	821.17	13.62	11.23
80	25	943.52	1001.06	1088.11	18.04	14.88
100	25	1196.51	1271.99	1382.60	22.93	18.91
120	25	1336.08	1431.80	1556.31	25.81	21.29
M 2	25	8040.00	8040.00	8040.00	133.33	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	182.45	182.45	182.45	3.03	2.27
40	25	478.24	492.83	535.69	8.88	6.66
60	25	641.98	680.24	739.39	12.26	9.20
80	25	863.17	914.52	994.05	16.49	12.36
100	25	1228.16	1297.21	1410.01	23.38	17.54
120	25	1300.14	1398.39	1519.99	25.21	18.91
M 3	25	7279.41	7279.41	7279.41	120.72	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	286.19	286.19	286.19	4.75	3.93
40	25	427.56	450.46	489.63	8.12	6.73
60	25	580.14	614.35	667.77	11.07	9.17
80	25	842.12	888.53	965.80	16.02	13.27
100	25	1153.05	1220.42	1326.55	22.00	18.22
120	25	1264.70	1356.95	1474.94	24.46	20.26

Table D 27: P_{app} values for each sample of indinavir with *H. hemerocallidea* aqueous extract (BL-AP)

	Slope	**1/A.60.C0**	**Papp**
M1	0.182647539	3.56888E-05	6.51847E-06
M2	0.16598791	3.56888E-05	5.92391E-06
M3	0.171268391	3.56888E-05	6.11236E-06
Average			6.18491E-06
STDEV.P			2.48091E-07

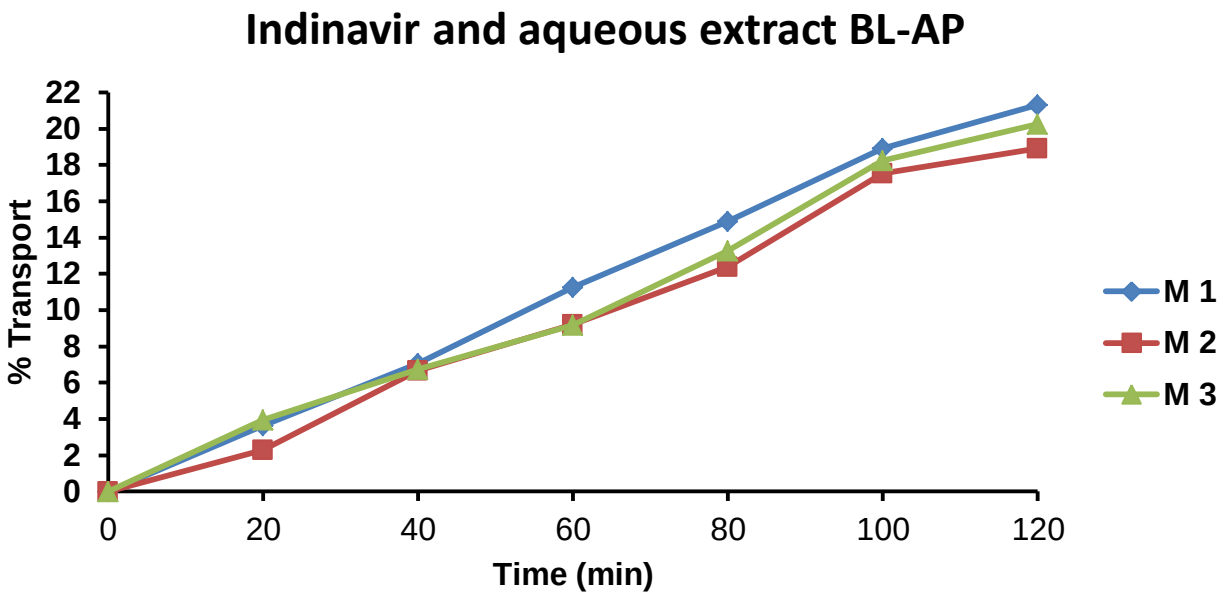

Figure D 6: Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* aqueous extract BL-AP). n = 3.

Table D 28: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* aqueous extract BL-AP) over the pre-determined time intervals. n = 3.

Time (min)	M1	M2	M3	Average transport	STDEV.P
0	0	0	0	0	0
20	3.626366101	2.269271891	3.931539	3.275725787	0.7224933
40	7.044361349	6.662804142	6.72618	6.811115052	0.1669471
60	11.23322601	9.196387319	9.173397	9.867670255	0.9656393
80	14.8847777	12.36377562	13.2675	13.50535216	1.0428461
100	18.91324069	17.53744488	18.22328	18.2246559	0.5616671
120	21.28948538	18.90530404	20.26183	20.15220648	0.9764197

2.7 Indinavir & *Hypoxis hemerocallidea* commercial product AP-BL

Table D 29: Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* commercial product (AP-BL) over the pre-determined time intervals. n = 3.

Time	Inj vol	Peak area	Correction 1	Correction 2	Concentration (µg/ml)	% Transport
M 1	25	3397.05	3397.05	3397.05	58.75	100
0	25	0	0.00	0.00	0.00	0
20	25	61.08	61.08	61.08	1.06	1.80
40	25	129.13	134.01	145.66	2.52	4.29
60	25	268.51	278.84	303.08	5.24	8.92
80	25	364.21	385.69	419.23	7.25	12.34
100	25	404.44	433.57	471.27	8.15	13.87
120	25	511.40	543.76	591.04	10.22	17.40
M 2	25	6277.95	6277.95	6277.95	108.58	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	49.24	49.24	49.24	0.85	0.78
40	25	172.48	176.42	191.76	3.32	3.05
60	25	339.25	353.05	383.75	6.64	6.11
80	25	440.72	467.86	508.54	8.80	8.10
100	25	501.72	536.98	583.68	10.09	9.30
120	25	595.39	635.52	690.79	11.95	11.00
M 3	25	6449.86	6449.86	6449.86	111.55	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	95.90	95.90	95.90	1.66	1.49
40	25	185.41	193.08	209.87	3.63	3.25
60	25	334.13	348.97	379.31	6.56	5.88
80	25	487.86	514.59	559.34	9.67	8.67
100	25	581.56	620.59	674.55	11.67	10.46
120	25	643.14	689.67	749.64	12.97	11.62

Table D 30: P_{app} values for each sample of indinavir with *H. hemerocallidea* commercial product (AP-BL)

	Slope	**1/A.60.C0**	**Papp**
M1	0.15071257	3.56888E-05	5.37875E-06
M2	0.098360386	3.56888E-05	3.51036E-06
M3	0.103980277	3.56888E-05	3.71093E-06
Average			4.20001E-06
STDEV.P			8.37504E-07

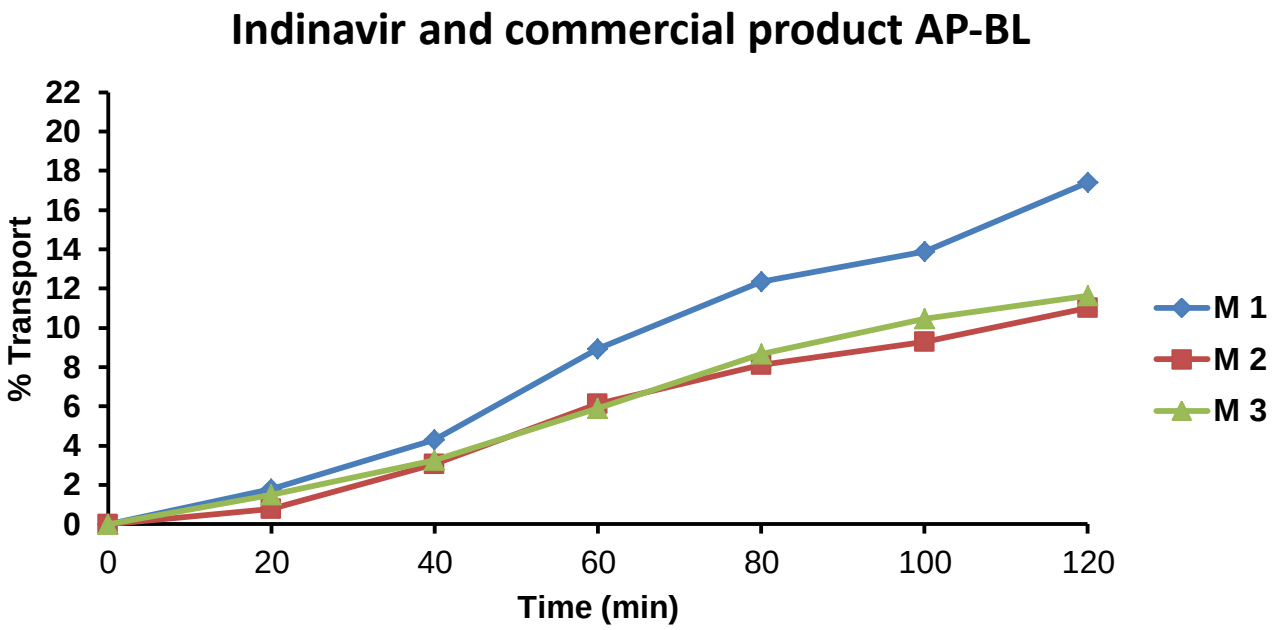

Figure D 7: Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* commercial product AP-BL). n = 3.

Table D 31: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* commercial product AP-BL) over the pre-determined time intervals. n = 3.

Time (min)	M1	M2	M3	Average Transport	STDEV.P
0	0	0	0	0	0
20	1.797912895	0.784364185	1.486907086	1.356394722	0.42394603
40	4.287978124	3.054485196	3.253814943	3.532092754	0.540650908
60	8.921904747	6.112595917	5.880933305	6.971811323	1.382163802
80	12.34099535	8.100455494	8.672101819	9.704517555	1.878821663
100	13.8730465	9.297229016	10.45841922	11.20956491	1.942110758
120	17.39858496	11.00337206	11.62254791	13.34150164	2.87990607

2.8 Indinavir & *Hypoxis hemerocallidea* commercial product BL-AP

Table D 32: Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* commercial product (BL-AP) over the pre-determined time intervals. n = 3.

Time	Inj vol	Peak area	Correction 1	Correction 2	Concentration (µg/ml)	% Transport
M 1	25	7144.77	7144.77	7144.77	123.57	100
0	25	0	0.00	0.00	0.00	0
20	25	186.48	186.48	186.48	3.23	2.61
40	25	439.95	454.86	494.42	8.55	6.92
60	25	713.97	749.16	814.31	14.08	11.40
80	25	837.26	894.37	972.14	16.81	13.61
100	25	1073.38	1140.36	1239.52	21.44	17.35
120	25	1281.61	1367.48	1486.39	25.71	20.80
M 2	25	7154.33	7154.33	7154.33	123.73	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	212.81	212.81	212.81	3.68	2.97
40	25	387.92	404.94	440.15	7.61	6.15
60	25	734.06	765.09	831.62	14.38	11.62
80	25	798.46	857.18	931.72	16.11	13.02
100	25	969.45	1033.32	1123.18	19.43	15.70
120	25	1144.02	1221.58	1327.80	22.96	18.56
M 3	25	7180.93	7180.93	7180.93	124.19	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	155.27	155.27	155.27	2.69	2.16
40	25	417.50	429.92	467.30	8.08	6.51
60	25	611.83	645.23	701.34	12.13	9.77
80	25	876.05	924.99	1005.43	17.39	14.00
100	25	1032.60	1102.69	1198.57	20.73	16.69
120	25	1180.85	1263.46	1373.33	23.75	19.12

Table D 33: P_{app} values for each sample of indinavir with *H. hemerocallidea* commercial product (BL-AP)

	Slope	**1/A.60.C0**	**Papp**
M1	0.176027405	3.56888E-05	6.28221E-06
M2	0.157140322	3.56888E-05	5.60815E-06
M3	0.167724091	3.56888E-05	5.98587E-06
Average			5.95874E-06
STDEV.P			2.75851E-07

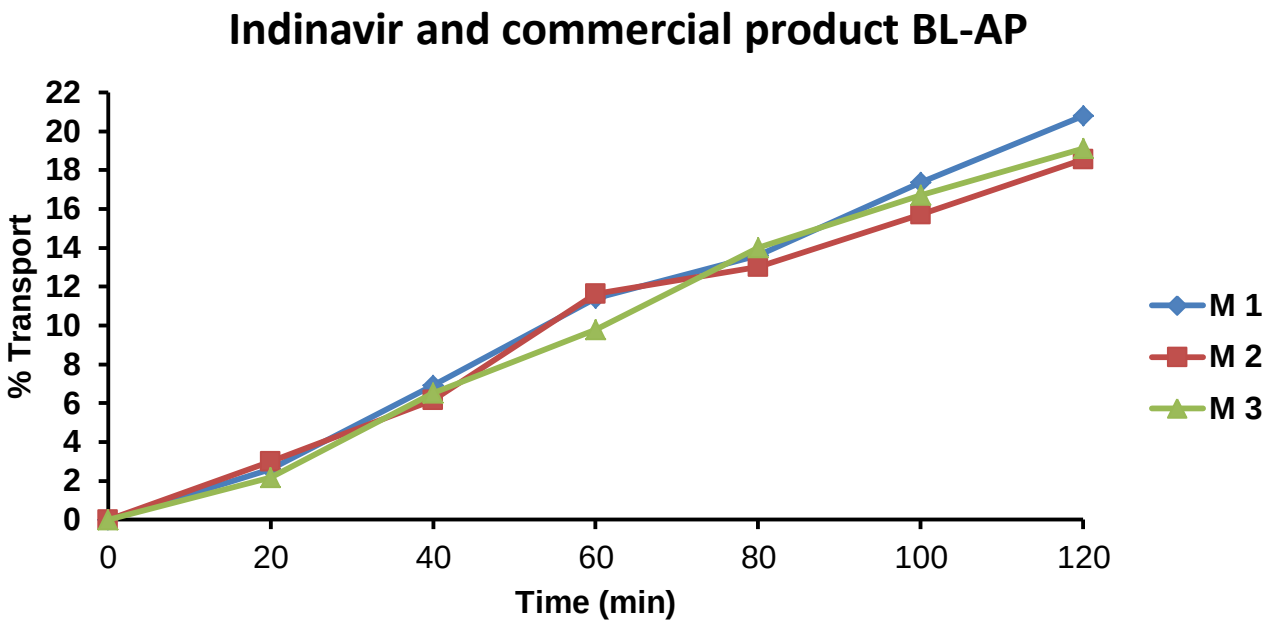

Figure D 8: Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* commercial product BL-AP). n = 3.

Table D 34: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* commercial product BL-AP) over the pre-determined time intervals. n = 3.

Time (min)	M1	M2	M3	Average Transport	STDEV.P
0	0	0	0	0	0
20	2.609993409	2.974560344	2.162214	2.582255851	0.332219
40	6.920004053	6.152245018	6.507567	6.526605358	0.313725
60	11.39722897	11.62399036	9.766667	10.92929543	0.827298
80	13.60637047	13.0231168	14.00138	13.5436224	0.401831
100	17.34869732	15.6992691	16.69105	16.57967248	0.677966
120	20.80385755	18.55943036	19.12467	19.49598526	0.95316

2.9 Indinavir & *Hypoxis hemerocallidea* reference plant material AP-BL

Table D 35: Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* reference plant material (AP-BL) over the pre-determined time intervals. n = 3.

Time	Inj vol	Peak area	Correction 1	Correction 2	Concentration (µg/ml)	% Transport
M 1	25	7423.62	7423.62	7423.62	117.28	100
0	25	0	0.00	0.00	0.00	0
20	25	42.33	42.33	42.33	0.67	0.57
40	25	199.50	202.89	220.53	3.48	2.97
60	25	297.72	313.68	340.96	5.39	4.59
80	25	376.61	400.43	435.25	6.88	5.86
100	25	636.63	666.75	724.73	11.45	9.76
120	25	650.60	701.53	762.53	12.05	10.27
M 2	25	7550.33	7550.33	7550.33	119.28	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	106.36	106.36	106.36	1.68	1.41
40	25	277.74	286.25	311.14	4.92	4.12
60	25	376.57	398.79	433.47	6.85	5.74
80	25	477.82	507.95	552.12	8.72	7.31
100	25	569.91	608.14	661.02	10.44	8.75
120	25	679.47	725.07	788.12	12.45	10.44
M 3	25	7450.70	7450.70	7450.70	117.71	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	90.36	90.36	90.36	1.43	1.21
40	25	212.20	219.42	238.51	3.77	3.20
60	25	312.17	329.14	357.77	5.65	4.80
80	25	411.78	436.76	474.74	7.50	6.37
100	25	429.18	462.12	502.31	7.94	6.74
120	25	513.85	548.19	595.86	9.41	8.00

Table D 36: P_{app} values for each sample of indinavir with *H. hemerocallidea* reference plant material (AP-BL)

	Slope	**1/A.60.C0**	**Papp**
M1	0.093021661	3.56888E-05	3.31983E-06
M2	0.08785424	3.56888E-05	3.13541E-06
M3	0.06825082	3.56888E-05	2.43579E-06
Average			2.96368E-06
STDEV.P			3.8079E-07

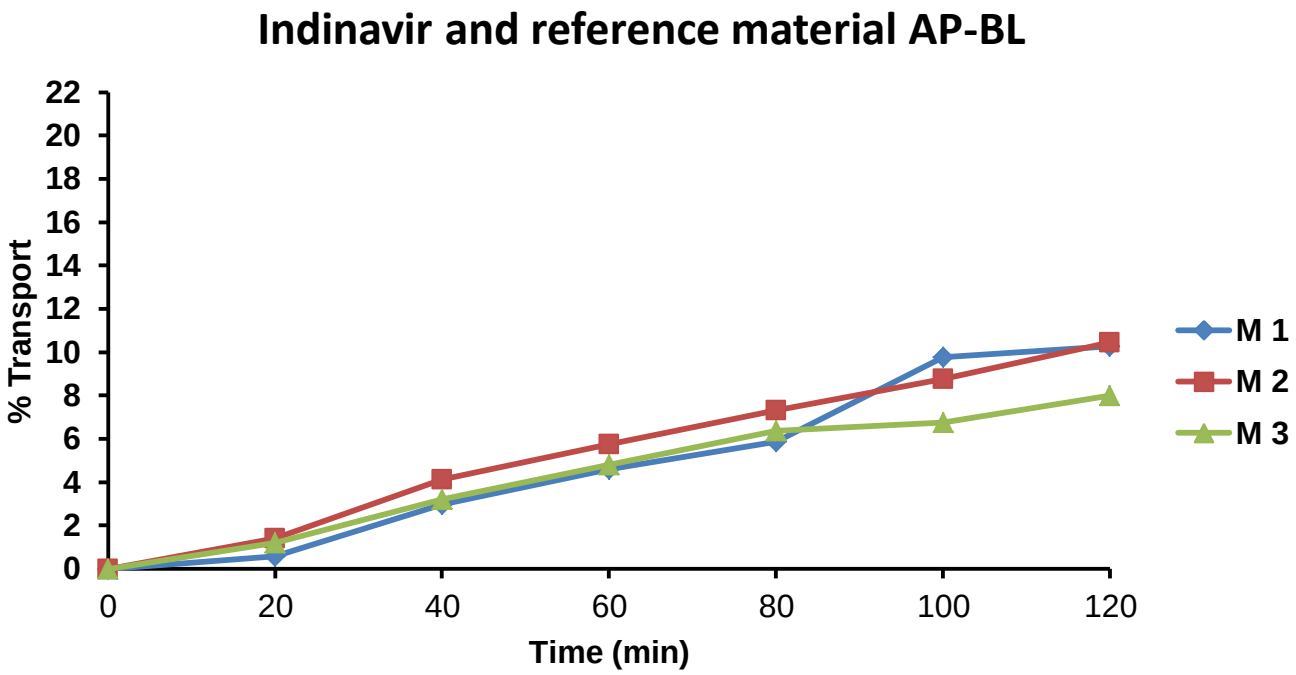

Figure D 9: Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* reference plant material AP-BL). n = 3.

Table D 37: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* reference plant material AP-BL) over the pre-determined time intervals. n = 3.

Time (min)	M1	M2	M3	Average transport	STDEV.P
0	0	0	0	0	0
20	0.570159168	1.408683859	1.212837	1.063893358	0.35816112
40	2.970676353	4.120867858	3.201113	3.430885873	0.49687803
60	4.592852239	5.741032736	4.801779	5.045221437	0.49935135
80	5.863055801	7.312482345	6.371703	6.515747098	0.60042808
100	9.762525759	8.75480417	6.74176	8.41969657	1.25578119
120	10.2716725	10.4381731	7.997341	9.569062289	1.11345135

2.10 Indinavir & *Hypoxis hemerocallidea* reference plant material BL-AP

Table D 38: Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* reference plant material (BL-AP) over the pre-determined time intervals. n = 3.

Time	Inj vol	Peak area	Correction 1	Correction 2	Concentration (µg/ml)	% Transport
M 1	25	7271.59	7271.59	7271.59	114.88	100
0	25	0	0.00	0.00	0.00	0
20	25	251.21	251.21	251.21	3.97	3.45
40	25	599.64	619.74	673.63	10.64	9.26
60	25	928.72	976.69	1061.62	16.77	14.60
80	25	1156.94	1231.23	1338.30	21.14	18.40
100	25	1435.06	1527.62	1660.45	26.23	22.83
120	25	1540.39	1655.20	1799.13	28.42	24.74
M 2	25	7085.37	7085.37	7085.37	111.94	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	285.50	285.50	285.50	4.51	4.03
40	25	524.81	547.65	595.27	9.40	8.40
60	25	817.72	859.71	934.46	14.76	13.19
80	25	1047.33	1112.75	1209.51	19.11	17.07
100	25	1246.49	1330.28	1445.95	22.84	20.41
120	25	1474.81	1574.53	1711.45	27.04	24.15
M 3	25	7184.07	7184.07	7184.07	113.50	100.00
0	25	0.00	0.00	0.00	0.00	0.00
20	25	138.90	138.90	138.90	2.19	1.93
40	25	571.35	582.46	633.11	10.00	8.81
60	25	852.65	898.36	976.47	15.43	13.59
80	25	1152.57	1220.79	1326.94	20.96	18.47
100	25	1391.19	1483.40	1612.39	25.47	22.44
120	25	1609.86	1721.15	1870.82	29.56	26.04

Table D 39: P_{app} values for each sample of indinavir with *H. hemerocallidea* reference plant material (BL-AP)

	Slope	**1/A.60.C0**	**Papp**
M1	0.218083246	3.56888E-05	7.78313E-06
M2	0.203373943	3.56888E-05	7.25817E-06
M3	0.230004179	3.56888E-05	8.20857E-06
Average			7.74996E-06
STDEV.P			3.88708E-07

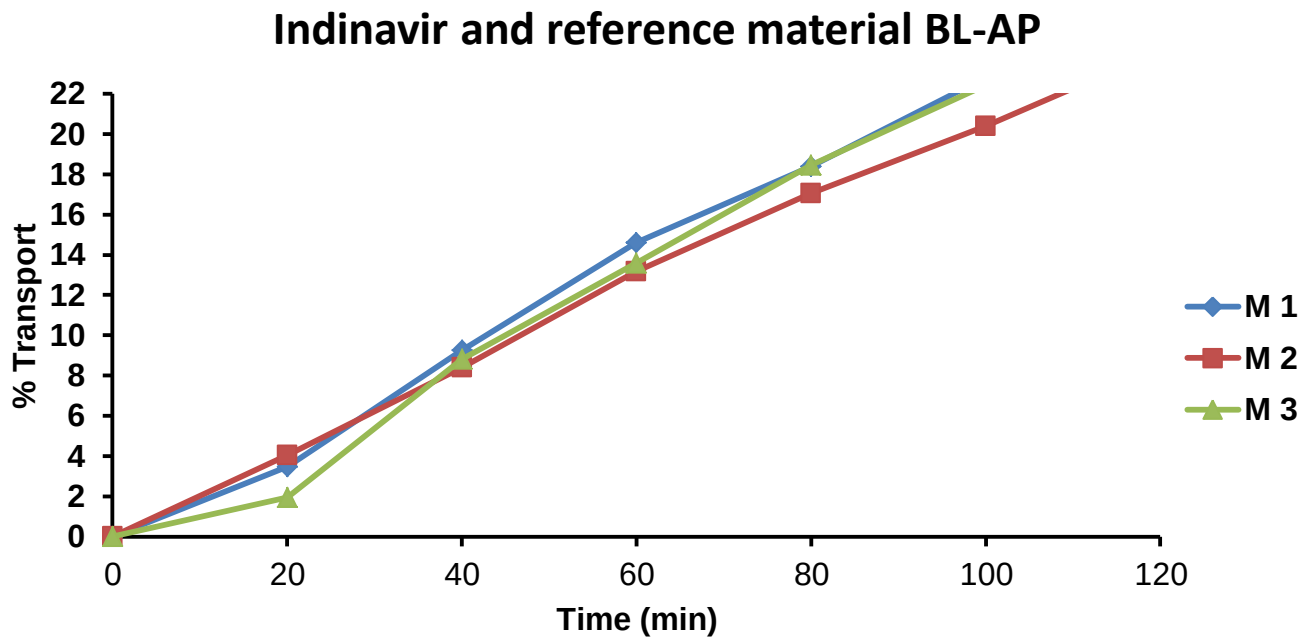

Figure D 10: Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* reference plant material BL-AP). n = 3.

Table D 40: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* reference plant material BL-AP) over the pre-determined time intervals. n = 3.

Time (min)	M1	M2	M3	Average transport	STDEV.P
0	0	0	0	0	0
20	3.4546747	4.029498	1.933510531	3.139227638	0.884278
40	9.263825596	8.40136	8.812697066	8.825960886	0.352225
60	14.59955091	13.18862	13.59222239	13.79346535	0.593326
80	18.4044551	17.07054	18.47060472	17.98186627	0.644971
100	22.83480797	20.40757	22.44397005	21.89545071	1.064118
120	24.74190726	24.15469	26.04117118	24.97925683	0.788227

IN VIVO bioavailability study

Table D 41: Weight of rats used in the *in vivo* study

Rat	Weight (g) of rats used in Indinavir PK-study (a=Acute; C=Chronic)										
	G1	**G2**	**G3**	**G4A**	**G4C**	**G5A**	**G5C**	**G6A**	**G6C**	**G7A**	**G7C**
R 1	258	281	285	288	296	280	293	264	270	270	307
R 2	303	301	318	325	304	291	294	304	275	225	260
R 3	262	293	292	309	297	304	272	255	284	287	260
R 4	294	320	291	256	264	290	269	276	273	220	308
R 5	260	299	338	295	275	311	257	237	263	270	295

Indinavir alone (Negative control)

Table D 42: Indinavir concentration for each rat (indinavir alone). n = 5.

Time (h)	Conc. (ng/ml)							
	R 1	**R 2**	**R 3**	**R 4**	**R 5**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0
0.5	1091	1926	1036	744.5	745.3	1109	484	198
1	684.9	588.5	538.8	489.5	270.0	514.3	155	63.1
2	255.1	261.0	130	204.9	133.8	197.0	63.2	25.8
4	35.15	31.15	43.54	32.62	29.93	34.48	5.43	2.21
6	18.80	13.12	28.02	19.51	12.30	18.35	6.31	2.57
8	8.993	5.561	18.38	13.730	14.01	12.13	4.95	2.02

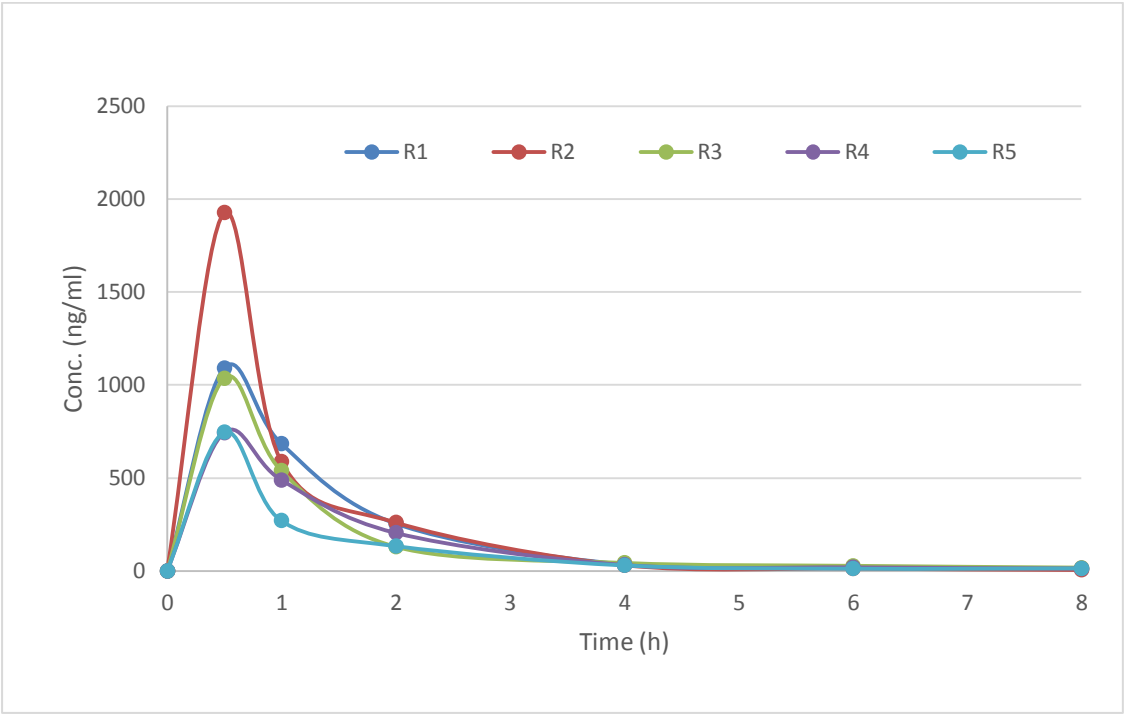

Figure D 11: Indinavir concentration over time for each rat (indinavir alone). n = 5.

Indinavir with ketoconazole (Positive control)

Table D 43: Indinavir concentration for each rat (indinavir with ketoconazole). n = 4.

Time (h)	Conc. (ng/ml)							
	R 1	R 2	R 3	R 4	R 5	Mean	STDEV	SEM
0	0	0		0	0	0	0	0
0.5	257.4	543.2		1006.0	949.5	689	354	144
1	208.4	232.3		421.5	321.8	296	97	39.5
2	147.8	221.8		306.7	276.4	238	70	28.5
4	28.23	59.78		82.52	24.98	49	27	11.17
6	51.39	91.17		50.23	25.77	55	27	11.05
8	57.55	187.50		39.09	82.28	92	66	27.07

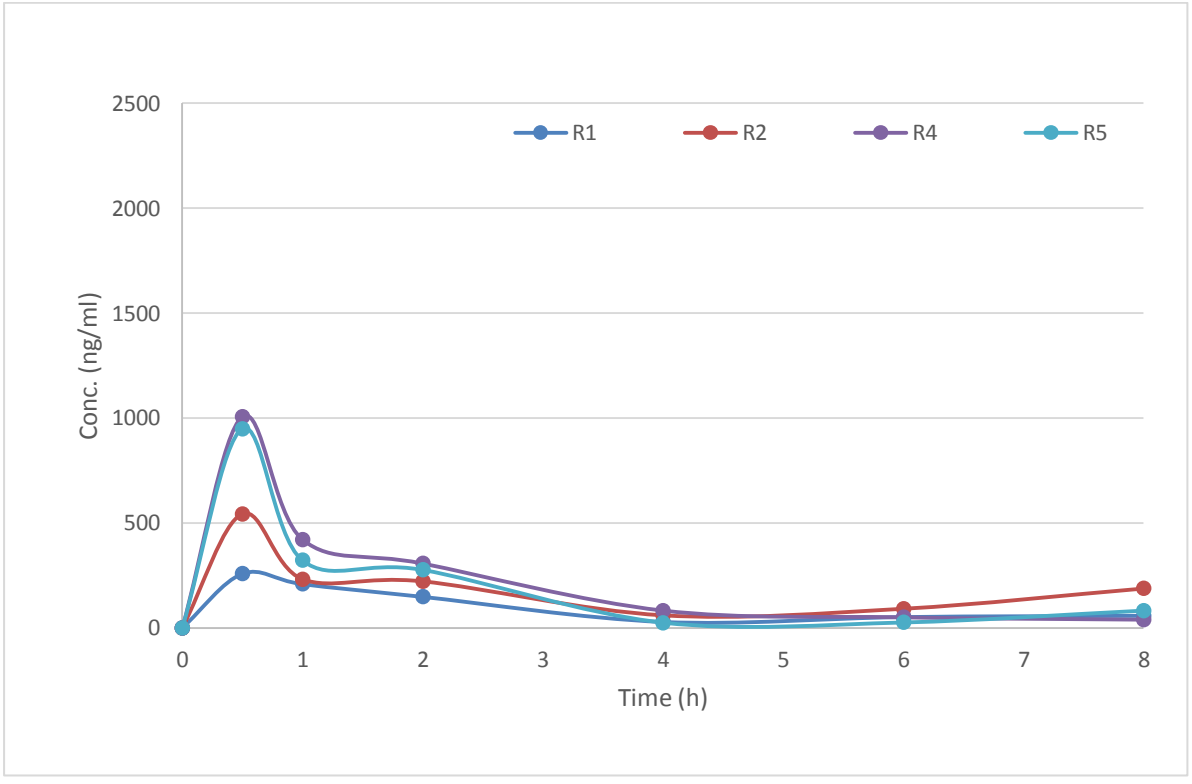

Figure D 12: Indinavir concentration over time for each rat (indinavir with ketoconazole). n = 4.

Indinavir with verapamil (Positive control)

Table D 44: Indinavir concentration for each rat (indinavir with verapamil). n = 5.

Time (h)	Conc. (ng/ml)							
	R 1	R 2	R 3	R 4	R 5	Mean	STDEV	SEM
0	0	0	0	0	0	0	0	0
0.5	203	1191	288	1160.0	119.2	592	536	219
1	156.6	776.5	262.4	1096.0	372.9	532.9	393	160.3
2	206.8	128.4	290	312.8	218.8	231.3	73.1	29.8
4	46.82	40.52	49.99	37.91	46.21	44.29	4.94	2.02
6	86.34	48.59	22.32	28.91	87.73	54.78	31.00	12.65
8	39.440	37.710	24.78	9.591	67.34	35.77	21.33	8.71

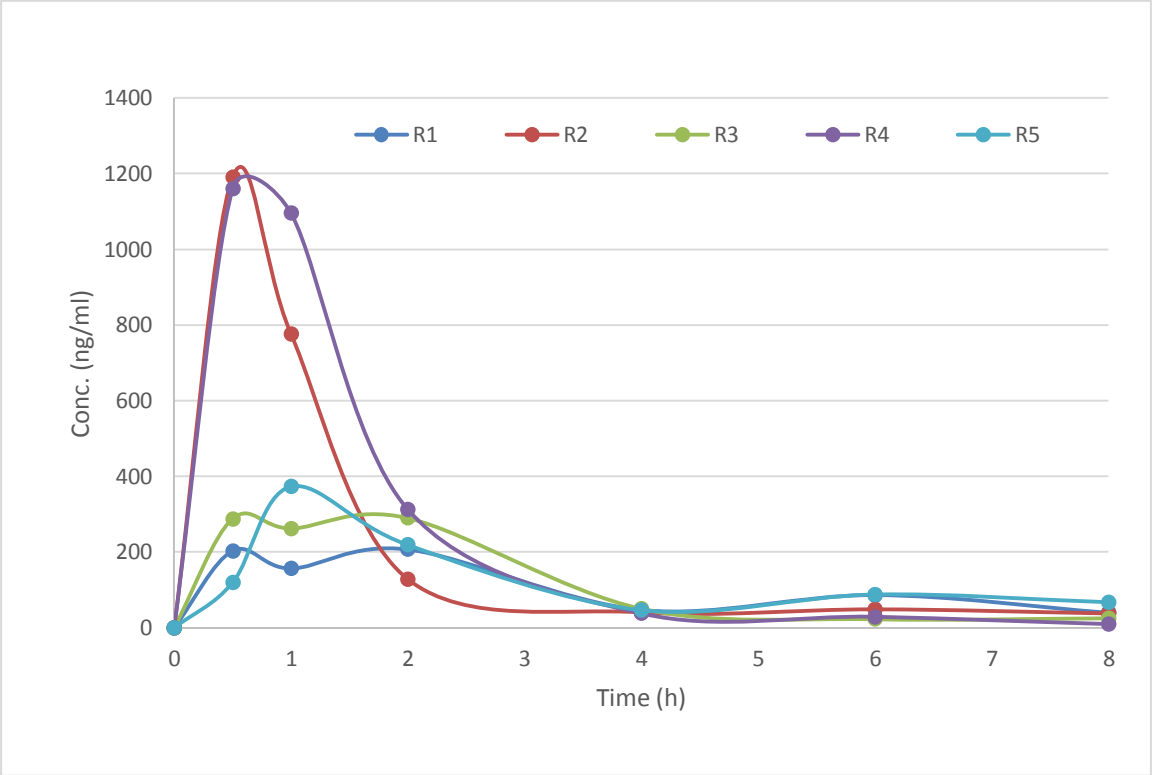

Figure D 13: Indinavir concentration over time for each rat (indinavir with verapamil). n = 5.

Indinavir with *H. hemerocallidea* aqueous extract (acute)

Table D 45: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* aqueous extract, acute). n = 5.

Time (h)	Conc. (ng/ml)							
	R 1	R 2	R 3	R 4	R 5	Mean	STDEV	SEM
0	0	0	0	0	0	0	0	0
0.5	373.2	1091	67.02	1107.0	596.7	647.0	453.5	185.1
1	114.8	787.0	340.2	1667.0	295.5	640.9	624.6	255.0
2	199.0	222.7	311.8	192.7	143.7	214.0	61.77	25.21
4	105.60	47.51	49.67	26.47	45.97	55.04	29.75	12.14
6	238.30	30.55	37.41	23.25	45.24	74.95	91.68	37.42
8	26.66	24.86	30.22	13.89	23.39	23.80	6.101	2.490

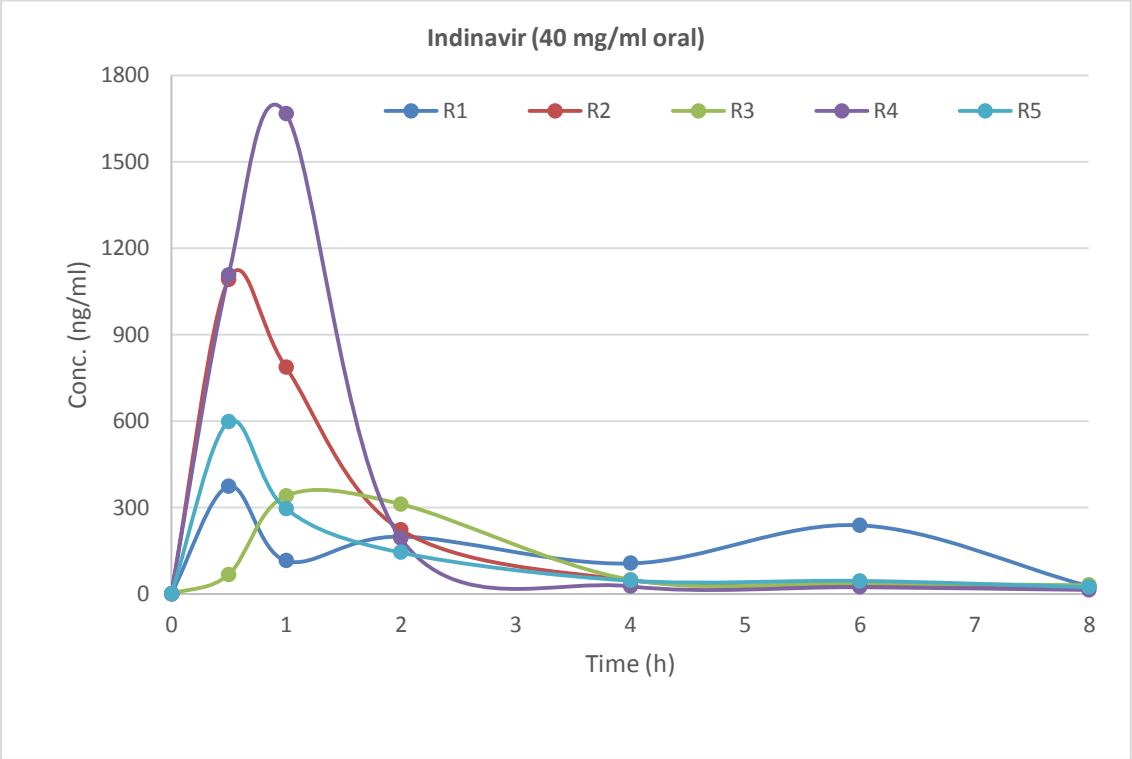

Figure D 14: Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* aqueous extract, acute). n = 5.

Indinavir with *H. hemerocallidea* aqueous extract (chronic)

Table D 46: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* aqueous extract, chronic). n = 5.

Time (h)	Conc. (ng/ml)							
	R 1	R 2	R 3	R 4	R 5	Mean	STDEV	SEM
0	0	0	0	0	0	0	0	0
0.5	1254	1439	873	1466.0	2381.0	1483	555	227
1	564.7	664.4	742.6	590.5	1163.0	745.0	244	99.5
2	249.7	200.6	158	113.7	357.4	215.8	93.9	38.3
4	58.75	38.75	54.92	62.18	33.93	49.71	12.58	5.14
6	47.35	26.15	31.42	45.12	18.67	33.74	12.30	5.02
8	62.980	15.620	19.81	21.640	17.44	27.50	19.97	8.15

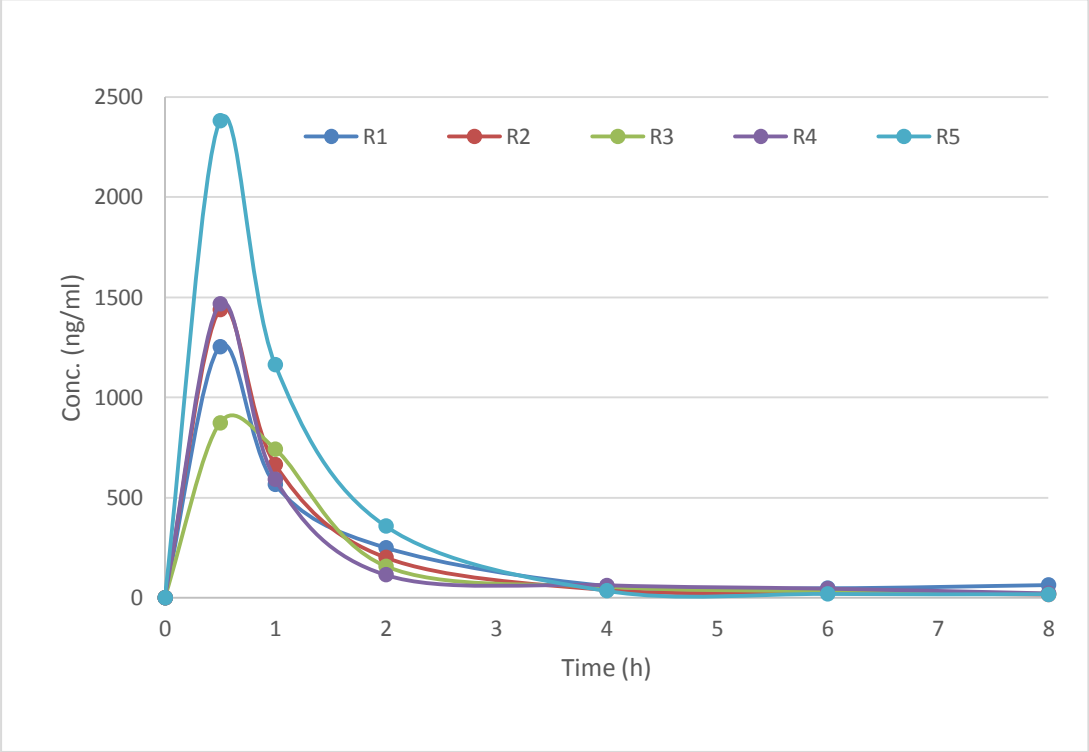

Figure D 15: Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* aqueous extract, chronic). n = 5.

Indinavir with *H. hemerocallidea* commercial product (acute)

Table D 47: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* commercial product, acute). n = 5.

Time (h)	Conc. (ng/ml)							
	R 1	**R 2**	**R 3**	**R 4**	**R 5**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0
0.5	1506	304.8	544.6	263.6	226.4	569.1	538.3	219.7
1	597.2	312.4	176.2	1245	1033	672.8	458.1	187.0
2	243.8	245.0	108.8	227.3	370.7	239.1	92.86	37.90
4	29.11	54.85	59.43	41.40	484.6	133.9	196.4	80.17
6	20.93	101.6	35.30	38.03	38.19	46.81	31.45	12.84
8	6.420	14.13	22.82	25.63	11.30	16.06	8.009	3.269

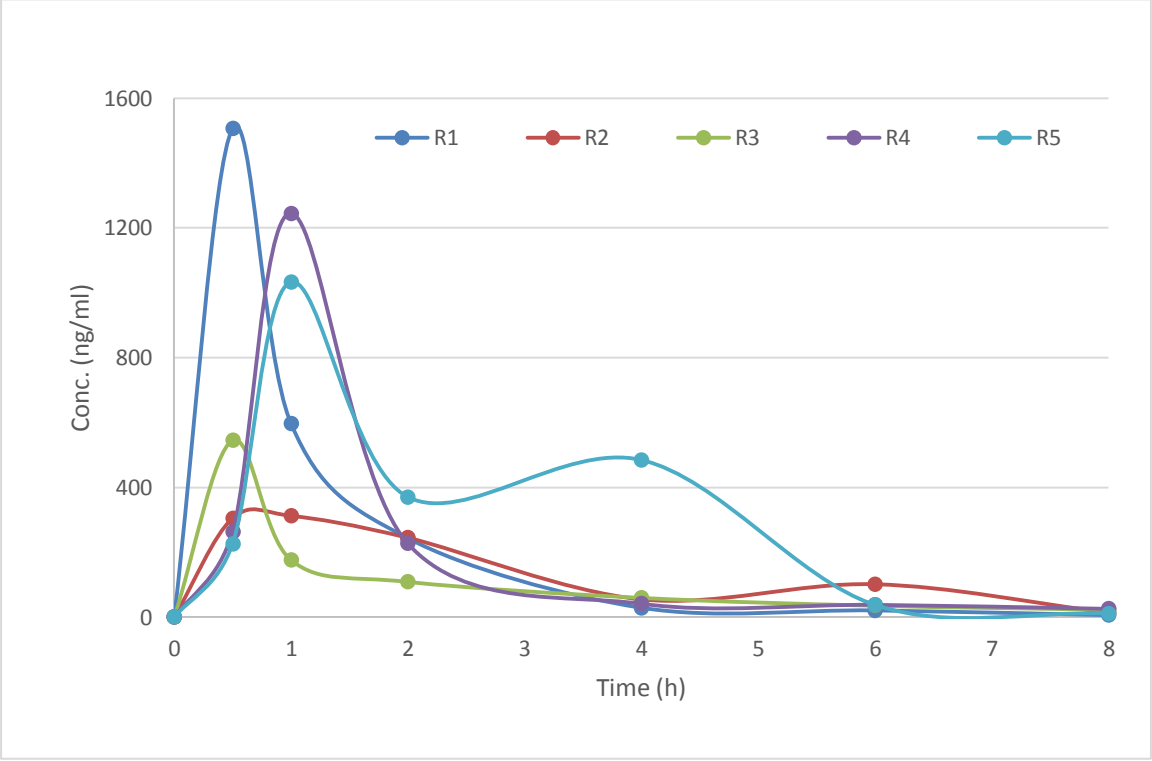

Figure D 16: Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* commercial product, acute). n = 5.

Indinavir with *H. hemerocallidea* commercial product (chronic)

Table D 48: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* commercial product, chronic). n = 5.

Time (h)	Conc. (ng/ml)							
	R 1	**R 2**	**R 3**	**R 4**	**R 5**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0
0.5	1052	1043	1634	2049	884.8	1333	491.7	200.7
1	525.8	396.4	651.2	513.4	316.9	480.7	128.6	52.47
2	234.8	189.1	354.3	161.0	142.2	216.3	84.67	34.56
4	54.35	62.56	48.71	28.94	49.67	48.85	12.40	5.062
6	20.22	34.76	38.14	12.92	19.78	25.16	10.77	4.395
8	10.14	47.91	13.65	-	13.86	21.39	17.8	7.25

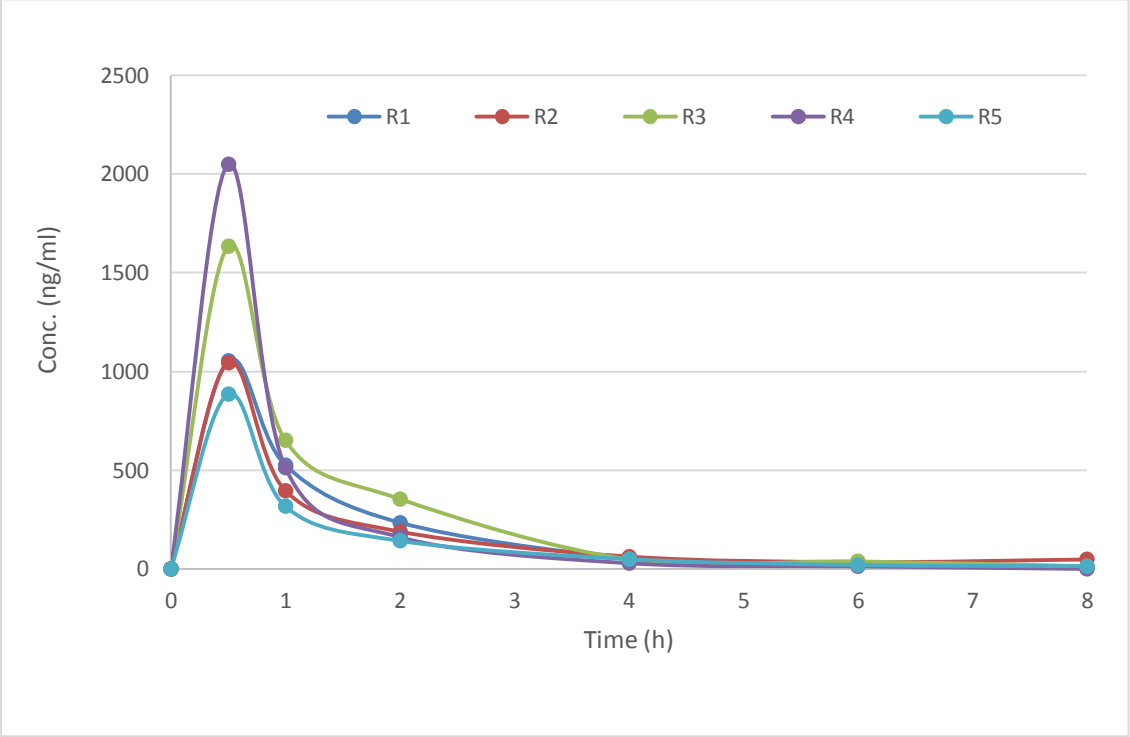

Figure D 17: Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* commercial product, chronic). n = 5.

Indinavir with *H. hemerocallidea* reference plant material (acute)

Table D 49: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* reference plant material, acute). n = 5.

Time (h)	Conc. (ng/ml)							
	R 1	R 2	R 3	R 4	R 5	Mean	STDEV	SEM
0	0	0	0	0	0	0	0	0
0.5	1450	611.1	1388	463.7	1011	984.8	444.7	181.5
1	374.6	794.8	670.6	331.4	429.2	520.1	202.0	82.43
2	83.39	127.5	430.4	152.8	320.2	222.9	146.6	59.85
4	38.62	18.85	39.05	32.76	63.10	38.48	16.00	6.532
6	35.08	22.03	20.69	97.53	18.89	38.84	33.42	13.64
8	28.82	18.32	135.5	44.84	13.89	48.27	50.19	20.49

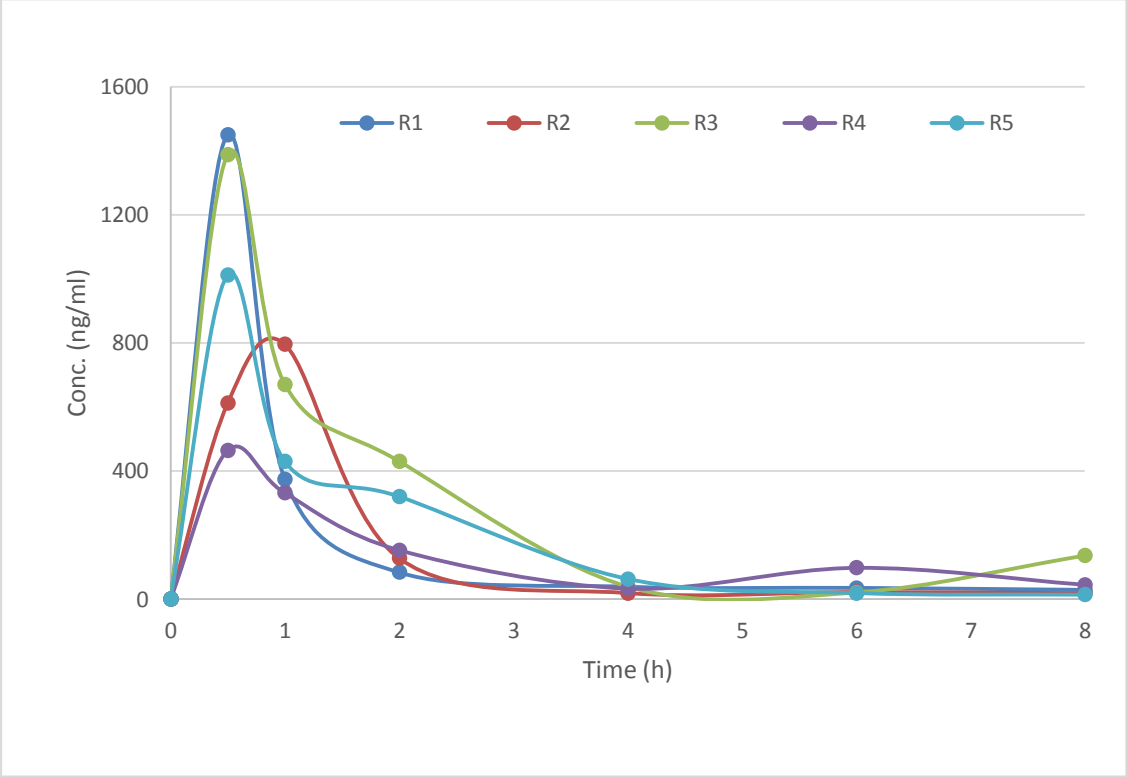

Figure D 18: Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* reference plant material, acute). n = 5.

Indinavir with *H. hemerocallidea* reference plant material (chronic)

Table D 50: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* reference plant material, chronic). n = 5.

Time (h)	Conc. (ng/ml)							
	R 1	**R 2**	**R 3**	**R 4**	**R 5**	**Mean**	**STDEV**	**SEM**
0	0	0	0	0	0	0	0	0
0.5	2497	1247	2031	931.1	3299	2001	954.6	389.6
1	729.7	314.8	734.2	330.2	1380	697.8	432.9	176.7
2	206.0	185.0	369.4	184.2	342.5	257.4	90.87	37.09
4	112.5	45.62	139.00	62.73	78.48	87.67	37.84	15.44
6	44.08	30.51	51.84	26.02	25.66	35.62	11.75	4.795
8	33.48	14.75	14.24	24.50	19.49	21.29	7.978	3.256

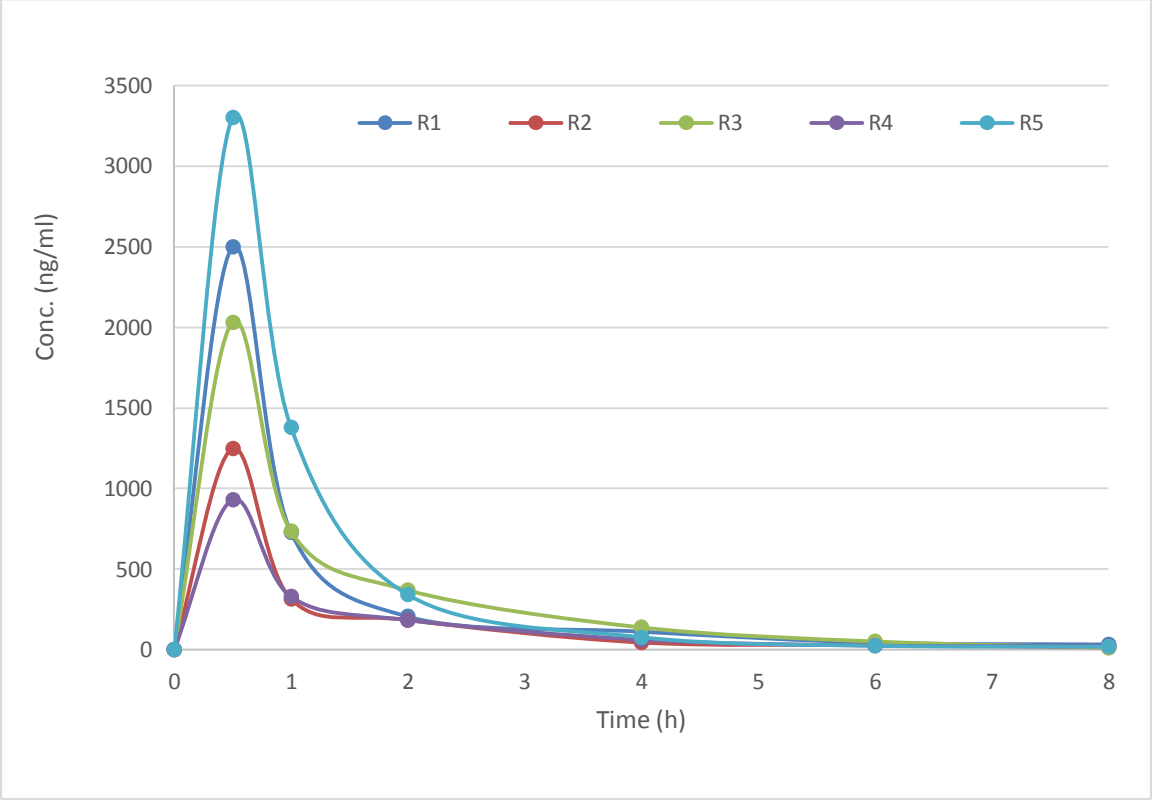

Figure D 19: Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* reference plant material, chronic). n = 5.

APPENDIX E

ETHICS LETTER OF APPROVAL

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Animal Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 404 7682
Email: nosi.tsama@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/animalethics/forms

17 March 2016

Dr L Wiesner
Pharmacology
Old Main Building

Dear Dr Wiesner

PROTOCOL TITLE: PHARMACOKINETIC INTERACTIONS BETWEEN HYPOXIS HEMEROCALLIDEA AND INDINAR IN SPRAGUE DAWLEY RATS

FHS AEC REF NO: 015/041

Thank you for submitting your protocol to the Faculty of Health Sciences (FHS) Animal Ethics Committee (AEC) for review.

I am pleased to inform you that the FHS AEC has **approved** your protocol, which will terminate on **30 March 2019**

Number of animals & species: 66 Rats

Please quote the FHS AEC REF NO (above) in all future correspondence.

Please note that the approval of this protocol imposes the following obligations on the principal investigator (PI):

1. To submit an annual mandatory progress report. The first annual report for this protocol is due on **28 February 2017**. The forms can be accessed from http://www.health.uct.ac.za/fhs/research/animalethics/forms
2. To submit a final mandatory report on the **28 February 2019**, please access the final report form from: http://www.health.uct.ac.za/fhs/research/animalethics/forms
3. Ensuring that all study participants perform within the confines of the procedures and experimental design of the protocol as approved, or as amended.
4. Ensuring that all study participants comply with all applicable national legislation, UCT policies, FHS AEC policies and standard operating procedures (SOPs) and national standards (SANS 10386: 2008).

5. Ensuring that you as the PI immediately alert the FHS AEC to any event involving the welfare of the animals which has occurred during the course of the study, as well as the actions that were taken to respond to these events.
6. Ensuring that you as the PI alert the FHS AEC to any new or unexpected ethical issues that arose during the course of the study, and how these issues were addressed.
7. Ensuring that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of SAVC-registered veterinary professionals or SAVC-registered para-veterinary professionals.
8. If the PI or any study participant is in any way uncertain how to respond to any of these obligations or deal with any of the issues referred to above, they must consult with FHS AEC.
9. All animals found dead must be reported to the RAF on the appropriate form: http://www.health.uct.ac.za/fhs/research/animalethics/forms
10. All animals found in distress must be reported to the RAF on the appropriate form.

My best wishes for a successful research and /or teaching endeavour.

Yours sincerely

PROF PJ COMMERFORD
CHAIR, FHS AEC

AEC REF# 015/041