

Effects of plant extracts and phytoconstituents on the intestinal transport of indinavir

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Abstract

There is a global rise in the use of herbal products in combination with allopathic medicines, while most patients do not inform their health care providers of the use of these natural products. Both pharmacodynamic and pharmacokinetic interactions between herbal products and conventional drugs must be avoided for the wellbeing of the patient. Increasing evidence from *in vitro* and *in vivo* studies indicate that changed drug pharmacokinetics by co-administered herbs may be attributed to modulation of efflux drug transporters such as P-glycoprotein (P-gp). Garlic (*Allium sativum*), lemon (*Citrus limonum*) and beetroot (*Beta vulgaris*) are widely used by human immunodeficiency virus (HIV) patients, especially following the pronouncement by a former President of South Africa and the Ministers of Health at that time who promoted the use of these botanicals in HIV patients.

The aim of this study was to measure the bi-directional *in vitro* transport of indinavir, a protease inhibitor, in the presence of crude extracts and pure phytoconstituents of *A. sativum* (L-alliin and diallyl disulphide), *C. limonum* (hesperidin and eriocitrin) and *B. vulgaris* (betaine monohydrate and β -carotene) across excised porcine intestinal tissue in Sweetana-Grass diffusion chambers. In the negative control group, the transport of indinavir alone (200 μ M) was determined with no modulator added. In the positive control group, the transport of indinavir was determined in the presence of verapamil (100 μ M), a known P-gp related efflux inhibitor. The control experiments were used to indicate that the effects of the test compounds were caused by their action and not by chance interferences or external factors. Samples collected at pre-determined time intervals were analysed by means of a validated high performance liquid chromatography (HPLC) method and the transport was expressed as the apparent permeability coefficient (P_{app}) and the transepithelial flux (J) from which the efflux ratio (ER) and the net flux (J_{net}) values were calculated. Statistical analysis was used to compare the results of the test compounds with the control groups in order to indicate significant differences.

The mean ER value for indinavir in the negative control group was 1.41 ± 0.170 and in the positive control group it was 0.56 ± 0.0426 . Statistically significant ($p < 0.05$) inhibition of indinavir efflux as indicated by reduced ER values was obtained for L-alliin (ER = 0.280 ± 0.030), diallyl disulphide (ER = 0.505 ± 0.034) and β -carotene (ER = 0.664 ± 0.075). Inhibition of indinavir efflux will lead to increased transport and therefore a potentially higher bioavailability. Statistically significant ($p < 0.05$) promotion of indinavir efflux as indicated by increased ER values was obtained for *C. limonum* crude extract (ER = 5.551 ± 0.575) and hesperidin (ER = 3.385 ± 0.477), which potentially may lead to lower bioavailability.

B. vulgaris crude extract ($p = 0.8452$), betaine monohydrate ($p = 0.9982$), *A. sativum* crude extract ($p = 0.7161$) and eriocitrin ($p = 0.4431$) displayed no statistically significant effect compared to the negative control group on indinavir transport across excised porcine intestinal tissue.

The results from this study demonstrate that L-alliin, diallyl disulphide and β -carotene have an inhibitory effect on indinavir efflux, which may significantly increase indinavir plasma levels after oral administration. *C. limonum* crude extract and hesperidin promote indinavir efflux, which may significantly reduce indinavir plasma levels. These pharmacokinetic interactions between certain drugs and plant extracts may negatively affect the anti-retroviral treatment of HIV patients, but deliberate and controlled inclusion of L-alliin, diallyl disulphide and β -carotene in dosage forms may possibly cause more effective delivery of protease inhibitors after oral administration resulting in less frequent dosing intervals.

Keywords: herbal medicine, *in vitro* models, efflux, P-glycoprotein (P-gp), garlic, lemon, beetroot, Sweetana-Grass diffusion chambers, human immunodeficiency virus (HIV), indinavir.

Uittreksel

Daar is 'n wêreldwye verhoging in die gebruik van kruiemedisyne in kombinasie met allopatiese medikasie, terwyl meeste pasiënte nie hul gesondheidsverskaffers inlig van die gebruik van dié natuurlike medisinale produkte nie. Beide farmakodinamiese en farmakokinetiese interaksies tussen kruiemiddels en konvensionele geneesmiddels moet geïdentifiseer word om die beste behandeling van die pasiënt te verseker. Verskeie *in vitro* en *in vivo* studies vermeld dat die verandering in geneesmiddel farmakokinetika deur gelyktydige toediening van kruiemiddels toegesryf kan word aan die modifisering van efflukstransporters soos P-glikoproteïen (P-gp). Knoffel (*Allium sativum*), suurlemoen (*Citrus limonum*) en beet (*Beta vulgaris*) word gereeld deur menslike immuuniteitsgebrek virus (MIV) pasiënte ingeneem, veral na die uitspraak deur een van die vorige Presidentes van Suid-Afrika en die Minister van Gesondheid van dieselfde era, wat die gebruik van die kruiemiddels aangemoedig het vir die behandeling van MIV pasiënte.

Die doel van hierdie studie was om die twee-rigting *in vitro* transport van die protease inhibeerder, indinavir, te meet in die teenwoordigheid van ru-plant ekstrakte asook suiwer bestandele van *A. sativum* (L-alleïen en dialliedisulfied), *C. limonum* (hesperidien en eriokitrien) en *B. vulgaris* (betaïenmonohidraat en β -karoteen) deur gedissekteerde varkjejumweefsel deur gebruik te maak van die Sweetana-Grass diffusiesisteen. In die negatiewe kontrole groep was die transport van indinavir (200 μ M) alleen gemeet sonder die byvoeging van 'n modulator. In die positiewe kontrole groep was die transport van indinavir in die teenwoordigheid van verapamil (100 μ M), 'n bekende inhibeerder van P-gp verwante effluks, gemeet. Die kontrole eksperimente was gebruik om aan te dui dat die effekte van die toetsverbindinge deur hul eie werking veroorsaak word en nie plaasvind as gevolg van die moontlike inmenging van eksterne faktore nie. Monsters was onttrek volgens voorafbepaalde tidsintervalle en geanaliseer deur 'n gevalideerde hoë-druk vloeistof chromatografie (HDVC) metode en die transport is uitgedruk as die deurlaatbaarheidskoëffisiënt (P_{app}) en transepiteel fluks (J) waarvan die effluksverhouding (EV) en die netto fluks (J_{net}) bereken is. Resultate van die eksperimentele groepe is statisties met die kontrole groepe vergelyk om beduidende verskille aan te dui.

Die gemiddelde EV waarde van indinavir vir die negatiewe kontrolegroep was 1.41 ± 0.170 en vir die positiewe kontrolegroep was dit 0.56 ± 0.0426 . Statisties betekenisvolle ($p < 0.05$) inhibisie van indinavir effluks soos aangedui deur verlaagde EV waardes was verkry vir L-alleïen (EV = 0.280 ± 0.030), dialliedisulfied (EV = 0.505 ± 0.034) en β -karoteen (EV = 0.664 ± 0.075). Inhibisie van indinavir effluks lei tot verhoogde transport van die

geneesmiddel en gevolglik potensiele hoër biobeskikbaarheid. Statisties betekenisvolle ($p < 0.05$) bevordering van indinavir effluks soos aangedui deur verhoogde EV waardes was verkry vir *C. limonum* ru-ekstrak ($EV = 5.551 \pm 0.575$) en hesperidien ($EV = 3.385 \pm 0.477$), wat moontlik 'n laer biobeskikbaarheid van indinavir kan veroorsaak. *B. vulgaris* ru-ekstrak ($p = 0.8452$), betaïen monohidraat ($p = 0.9982$), *A. sativum* ru-ekstrak ($p = 0.7161$) en eriokitrien ($p = 0.4431$) het geen statisties betekenisvolle effek getoon op indinavir transport in vergelyking met die negatiewe kontrolegroep nie.

Die resultate in die studie het dus getoon dat L-alleïen, diallieldisulfied en β -karoteen 'n inhiberende effek op indinavir effluks het, wat 'n betekenisvolle verhoging in indinavir plasmavlakke kan veroorsaak na orale toediening. *C. limonum* ru-ekstrak en hesperidien bevorder indinavir effluks, wat 'n potensiele verlaging in indinavir plasmavlakke mag veroorsaak. Hierdie farmakokinetiese interaksies tussen sekere geneesmiddels en plant ekstrakte kan 'n negatiewe effek hê op MIV-pasiënte se anti-retrovirale behandeling, maar doelgerigte en gekontroleerde byvoeging van L-alleïen, diallieldisulfied en β -karoteen in doseervorme kan moontlik 'n meer effektiewe aflewering van protease inhibeerders veroorsaak na orale toediening, 'n resultaat wat heel moontlik in die toekoms tot minder doseringsintervalle kan lei.

Sleutelwoorde: kruiemiddels, *in vitro* modelle, effluks, P-glikoproteïen (P-gp), knoffel, suurlemoen, beet, Sweetana-Grass diffusiesisteen, menslike immuuniteitsgebrek virus (MIV), indinavir.

Congress proceedings

Congress proceedings

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List of abbreviations

ABC	Adenosine triphosphate-binding cassette
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
ANOVA	One-way analyses of variance
AP-BL	Apical to basolateral
ART	Anti-retroviral treatment
ARV	Anti-retroviral
<i>A. sativum</i>	<i>Allium sativum</i>
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AUC	Area under the plasma concentration-time curve
BCRP	Breast cancer resistance protein
BL-AP	Basolateral to apical
<i>B. vulgaris</i>	<i>Beta vulgaris</i>
<i>C. limonum</i>	<i>Citrus limonum</i>
C _{max}	Maximum plasma concentration
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
DTG	Differential thermal gravimetric
ER	Efflux ratio
FDA	Food and Drug Administration

HAART	Highly active anti-retroviral treatment
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
J	Transepithelial flux
J_{net}	Net flux
KRB	Krebs-Ringer bicarbonate
LRP	Lung cancer-associated resistance protein
MDCK	Madin-Darby canine kidney
MDR	Multi-drug resistance
MRP	Multidrug resistance-associated protein
MRP-2	Multidrug resistance protein
NBD	Nucleotide binding domain
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
OATP	Organic anion transporting polypeptide
PAMPA	Parallel artificial membrane permeability assay
P_{app}	Apparent permeability coefficient
PEPT-1	Oligopeptide transporter 1
P-gp	P-glycoprotein
PI	Protease inhibitors
RSD	Relative standard deviation
R squared	Regression coefficient
Stdev	Standard deviation

TEER	Transepithelial electrical resistance
TGA	Thermogravimetric analyser
USA	United States of America
WHO	World Health Organization

CHAPTER 1

1 INTRODUCTION

1.1 Background

Medicinal plants have been used since the earliest of days and are becoming more popular in the treatment of a number of diseases (Brown *et al.*, 2008:588) regardless of insufficient information concerning the working mechanisms, efficacy and toxicology of these medicines (Zhou *et al.* 2007:664). Herbal medicines are believed to have advantages such as improved patient compliance, reduced side-effects, reasonably low costs and increased accessibility as well as acceptability due to a long history of conventional use (Vermani & Garg, 2002:50). Due to the natural origin of herbal medicines, a general misconception exists that these medicines are safe and are not subjected to side effects and drug interactions (McFadden & Peterson, 2011:65; Pal & Mitra, 2006:2132). Developing countries do not always have sufficient primary health care systems in place and therefore traditional herbal medicines are frequently used in these countries (Brown *et al.*, 2008:588). According to Babb *et al.* (2007:314) up to 80% of the rural population of South Africa consults traditional healers because of their accessibility and affordability. About 70% of patients infected with human immunodeficiency virus (HIV) consume herbal medicines in combination with allopathic medicines such as anti-retroviral treatment (ART) (Gouws *et al.*, 2012:979; Minocha *et al.*, 2011:44; Pal & Mitra, 2006:2131) as it is believed to relieve HIV-related symptoms and reduce viral activity (Gore-Felton *et al.*, 2003:18). The co-administration of herbal medicines with anti-retroviral (ARV) drugs could potentially result in herb-drug pharmacodynamic and/or pharmacokinetic interactions (Brown *et al.*, 2008:588). These herb-drug interactions may cause changed absorption of the co-administered drug, thereby influencing the bioavailability and subsequently the curative effectiveness of the drug (Brown *et al.*, 2008:588; Katragadda *et al.*, 2005:688). One pharmacokinetic type of interaction that may influence the absorption process is the interaction of the co-administered herbal medicine with the drug efflux process (Brown *et al.*, 2008:588).

Some drug molecules are affected by transporter proteins that are responsible for active efflux of drug molecules from the epithelial cells back into the intestinal lumen (Brown *et al.*, 2008:588). One of the key counter transporter efflux proteins is P-glycoprotein (P-gp) (Van Asperen *et al.*, 1998:431; Varma *et al.*, 2003:347), but many other active efflux transporter systems also exist in the gastro-intestinal epithelium (Brown *et al.*, 2008:588). P-gp is located on the apical exterior surface of epithelial cells of the small intestine, which exports toxic substances and xenobiotics out of cells (Balayssac *et al.*, 2005:321; Deferme *et al.*, 2008:187). With respect to the gastro-intestinal tract, efflux therefore limits the

absorption of compounds from the intestinal lumen to the blood stream (Balayssac *et al.*, 2005:321; Chan *et al.*, 2004:34). On the other hand, the bioavailability of some orally administered drugs can be extensively improved by the inhibition of efflux (Evans, 2000:134).

Several studies have shown that ARV drugs, especially protease inhibitors (PIs) (e.g. indinavir), are substrates for efflux transporters such as P-gp, which limits their intestinal absorption. When PIs are administered concurrently with other compounds that act as inhibitors of P-gp, it creates the potential for pharmacokinetic drug interactions (Brown *et al.*, 2008:589). P-gp may limit the distribution of PIs (e.g. indinavir) into several therapeutically relevant compartments, and thus reduce the chance of achieving an optimal treatment regimen. Although these pharmacokinetic interactions may negatively affect the efficacy of ART, the intentional and controlled inclusion of herbal medicines acting as P-gp inhibitors in dosage forms may result in increased permeability and higher ARV plasma levels after oral administration while requiring lower doses to be administered (Huisman *et al.*, 2000:237; Panchagnula *et al.*, 2004:277).

High profile persons such as one of South Africa's former Presidents and the Ministers of Health at the time have referred to ART as 'toxic' and promoted the use of alternative medicines of natural origin for the treatment of acquired immunodeficiency syndrome (AIDS), including garlic, olive oil, beetroot and lemon (Amon, 2008:4).

Garlic (*Allium sativum*) juice and oil, organic or aqueous extracts and various constituents are believed to have medicinal properties (Amagase *et al.*, 2001:956S). In many parts of the world, *A. sativum* is still used as both a prophylactic and curative agent for several diseases (Dausch & Nixon, 1990:346). *A. sativum* contains high-level sulphur-type compounds (e.g. alliin, allicin, ajoene, allylpropyl disulfide, S-allylcysteine, vinylthiins and S-allylmercaptocysteine), numerous flavonoids/isoflavonoids (e.g. nobiletin, quercetin, rutin and tangeretin), polysaccharides, prostaglandins, saponins, and terpenes (e.g. citral, geraniol, linalool, α - and β -phellandrene) (Zhou *et al.*, 2004:63; Omar & Al-Wabel, 2010:52). According to Dausch and Nixon (1990:347), *A. sativum* is believed to be the richest source of sulphur-containing compounds amongst all vegetables. These compounds are believed to mediate its biological effects, while compounds such as S-allylcysteine, S-allylmercaptocysteine and *N*- α -fructosylarginine may also play a role (Zhou *et al.*, 2004:63). A number of herb-drug interactions were documented for *A. sativum* that involve efflux inhibition. In an *in vitro* study using recombinant human P-gp membranes and applying a colorimetric adenosine triphosphatase (ATPase) assay, garlic and garlic containing products were found to inhibit the activities of P-gp, although the inhibition was low or moderate compared to the known P-gp inhibitor verapamil (Zhou *et al.*, 2004:63).

Several conflicting reports make it difficult to establish the effectiveness and safety of garlic products (Foster *et al.*, 2001:176).

Lemon (*Citrus limonum*) juice has a high content of flavonoids of which the most abundant compounds include eriocitrin and hesperidin (Gil-Izquierdo *et al.*, 2004:324). These flavonoids can interact with transporters such as P-gp to inhibit oxidative and conjugative metabolism *in vitro*. Furthermore, isoflavones, a class of molecules structurally related to the flavonoids, may also alter drug absorption in humans because of their effect on P-gp (Evans, 2000:131).

Beetroot (*Beta vulgaris*) is rich in active compounds such as carotenoids (e.g. β -carotene, β -cryptoxanthin, lutein and zeaxanthin), glycine betaine, saponins, betacyanins, folates, betanin, polyphenols and flavonoids such as quercetin (Figiel, 2010:461). The addition of sugar beet fibre to the diet was shown to drastically decrease gastro-intestinal transit time. Changes in the transit time may potentially influence the absorption and bioavailability of orally administered agents (Ulbricht *et al.*, 2008:1064).

1.2 Research problem

There is a global rise in the use of natural and herbal products in combination with allopathic medicines and in addition most patients do not inform their health care providers of the use of these natural products (Ingersoll, 2005:434). Many herbs have possible interactions with conventional drugs (Ulbricht *et al.*, 2008:1063). Efflux transporters such as P-gp are susceptible to inhibition, activation or induction by herbal medicines and/or their phytoconstituents. The modulation of efflux transporters by herbal products may result in changed absorption and bioavailability of drugs that are substrates for the efflux active transporters (e.g. PIs). Increasing evidence from *in vitro* and *in vivo* studies indicate that changes in drug plasma concentrations following their concomitant administration with herbs may in some instances be attributable to changes in the activity of active drug transporters. Identifying potential modulators of active efflux transporters in the form of herbal medicines is therefore important in terms of patient wellbeing (Zhou *et al.*, 2004:57). Herbs and botanical supplements are widely used in HIV patients (Gouws *et al.*, 2012:979). In addition, many HIV-related conditions such as fatigue, insomnia, depression, nausea and dermatological disorders are also believed to be treated with herbal products (Mills *et al.*, 2005:465). The problem is that the concomitant intake of herbs and ART may result in herb-drug interactions (Tarirai *et al.*, 2010:1515), which should be scientifically investigated in order for healthcare practitioners to provide their patients with the proper advice.

1.3 Research aim and objectives

The purpose of this study was to determine whether crude extracts and pure phytoconstituents of selected plants have an effect on the bi-directional *in vitro* transport of indinavir across excised porcine intestinal tissue.

The objectives of this study were to:

- Develop a validated high performance liquid chromatography (HPLC) method for indinavir analysis.
- Measure the bi-directional *in vitro* transport of indinavir, a protease inhibitor, in the presence of the crude extracts and pure phytoconstituents of *A. sativum* (L-alliin and diallyl disulphide), *C. limonum* (hesperidin and eriocitrin) and *B. vulgaris* (betaine monohydrate and β -carotene) across excised porcine intestinal tissue using Sweetana-Grass diffusion chambers.
- Calculate the apparent permeability coefficient (P_{app}) and transepithelial flux (J) values for indinavir transport in both AP-BL (apical to basolateral) and BL-AP (basolateral to apical) directions from which the efflux ratio (ER) and net flux (J_{net}) values were determined.
- Statistically analyse the transport results in order to indicate significant differences between the experimental groups and the control groups.
- Identify potential interactions of the selected crude extracts and phytoconstituents with indinavir, with specific reference to gastro-intestinal efflux.

1.4 Ethical aspects of research

The excised porcine intestinal tissue was obtained from a local abattoir (Potch Abattoir, Potchefstroom, South Africa) directly after the pigs were slaughtered. Following the completion of all experimental studies, the tissue was disposed of by the Animal Research Centre at the North-West University. Although the pigs are slaughtered for meat production and not for research purposes, ethical approval was obtained from the ethics committee of the North-West University (Ethical Approval Number: NWU-00018-09-A5; Annexure C).

1.5 Structure of dissertation

The structure of this dissertation includes the introductory chapter (Chapter 1), which is followed by two literature review chapters (Chapter 2 and 3). Chapter 4 is a description of

the methods used during this study, while the results and discussion are included in Chapter 5. Chapter 6 includes the conclusion and recommendations for future studies.

CHAPTER 2

2 HERB-DRUG INTERACTIONS

2.1 Introduction

Medicinal plants have been used since the earliest of days and are becoming more popular for health maintenance, disease prevention and treatment because of believed advantages such as fewer side-effects, better patient compliance, relatively low cost and high accessibility as well as high acceptability due to a long history of use (Brown *et al.*, 2008:588; Vermani & Garg, 2002:50). However, herbal preparations are not regulated by authorities such as the American Food and Drug Administration (FDA) or the South African Medicines Control Council (MCC), a fact that complicates the monitoring of its safety and efficacy (Ulbricht *et al.*, 2008:1063). The World Health Organization (WHO) defines herbal medicines as 'finished labelled medicinal products that contain active ingredients from the aerial or underground parts of plants, or other plant material, or combinations thereof, whether in the crude state or as plant preparations. Plant material includes juices, gums, fatty oils, essential oils, and any other substances of this nature. Medicines containing plant material combined with chemically defined active substances, including isolated constituents of plants, are not considered to be herbal medicines' (Tarirai *et al.*, 2010:1515).

Herbal medicines are usually self-administered, sometimes together with therapeutic drugs to control side effects of drugs or to improve general health (Pal & Mitra, 2006:2131). They are typically intricate mixtures of several compounds and both the active ingredient(s) as well as other phytoconstituents could possibly interact with co-administered drugs (Pal & Mitra, 2006:2131). Approximately 70% of patients infected with HIV and 55% of the total population use some form of complimentary therapy which may include herbal medicines (Pal & Mitra, 2006:2131). Most people using herbal products have a misunderstanding of the effects of these products due to the fact that herbal products are generally considered a traditional health aid and no preclinical and clinical research is required (Pal & Mitra, 2006:2132). Furthermore, no accurate process of quality control and the monitoring of adverse effects exist for herbal medicines and herb-drug combinations (Pal & Mitra, 2006:2132).

Traditionally, studies have focused on drug-drug interactions but interactions of herbal products with prescribed medicine are currently receiving more attention due to increasing physician attentiveness of the extensive adverse effects of unidentified herbal use by patients (Pal & Mitra, 2006:2132). One of the main problems that occur with the increased

use of herbal medicines by patients in addition to their allopathic medication is the increased possibility of herb-drug interactions (Brown *et al.*, 2008:588; Tarirai *et al.*, 2010:1516). Identifying these interactions is a difficult process because approximately 70% of patients do not disclose the use of herbal products to their medical practitioner (Hu *et al.*, 2005:1242).

Herb-drug interactions can be pharmacokinetic and/or pharmacodynamic in nature. Pharmacodynamic interactions are herb-drug interactions that cause changes in pharmacological reactions, which may result in promotion or inhibition of the pharmacological activity of a co-administered drug (Tarirai *et al.*, 2010:1517). Pharmacokinetic interactions, on the other hand, interfere with drug absorption processes such as metabolism, distribution and/or elimination (Tarirai *et al.*, 2010:1517). Pharmacokinetic interactions of herbal medicines with drugs become clinically considerable when significant changes occur to the pharmacokinetic parameters of the co-administered drug (Tarirai *et al.*, 2010:1517).

Developing countries do not always provide the required primary health care to all citizens and herbal medicines are therefore frequently used with application of traditional knowledge (Brown *et al.*, 2008:588). Many HIV patients of whom some may have developed AIDS, live in rural areas and are therefore treated by traditional healers because of their convenient access and affordable cost (Brown *et al.*, 2008:588). In a study on the use of traditional medicine amongst people suffering from HIV, it was found that the motives for using these products include an association with certain cultural or religious views, supposed synergism with ARV drugs and to alleviate or prevent the adverse effects of commonly prescribed ARV drugs. Even more controversial, they also believe it to be safe substitutes for ARVs (Müller & Kanfer, 2011:459).

The orally administered ARV agents for treatment of HIV that are currently available comprise four main classes namely the nucleoside reverse transcriptase inhibitors (NRTIs, e.g. abacavir, tenofivir, lamivudine, didanosine, stavudine, zalcitabine and zidovudine), the non-nucleoside reverse transcriptase inhibitors (NNRTIs, e.g. efavirenz, delavirdine, etravirine, rilpivirine and nevirapine), the protease inhibitors (PIs, e.g. saquinavir, ritonavir, indinavir, nelfinavir, amprenavir and lopinavir) and the fusion inhibitors (FIs, e.g. enfuvirtide and maraviroc) (Brown *et al.*, 2008:589). PIs especially indinavir, has been described as substrates for P-gp (Varma *et al.*, 2006:367). Patients infected with HIV who use traditional or complementary medicines in combination with ART may possibly be at risk of the occurrence of adverse pharmacokinetic interactions, especially with reference to the PIs and NNRTIs (Müller & Kanfer, 2011:458).

2.2 Mechanisms of herb-drug interactions

All the potential mechanisms underlying pharmacokinetic and pharmacodynamic herb-drug interactions and their possible effects are summarised in Figure 2.1.

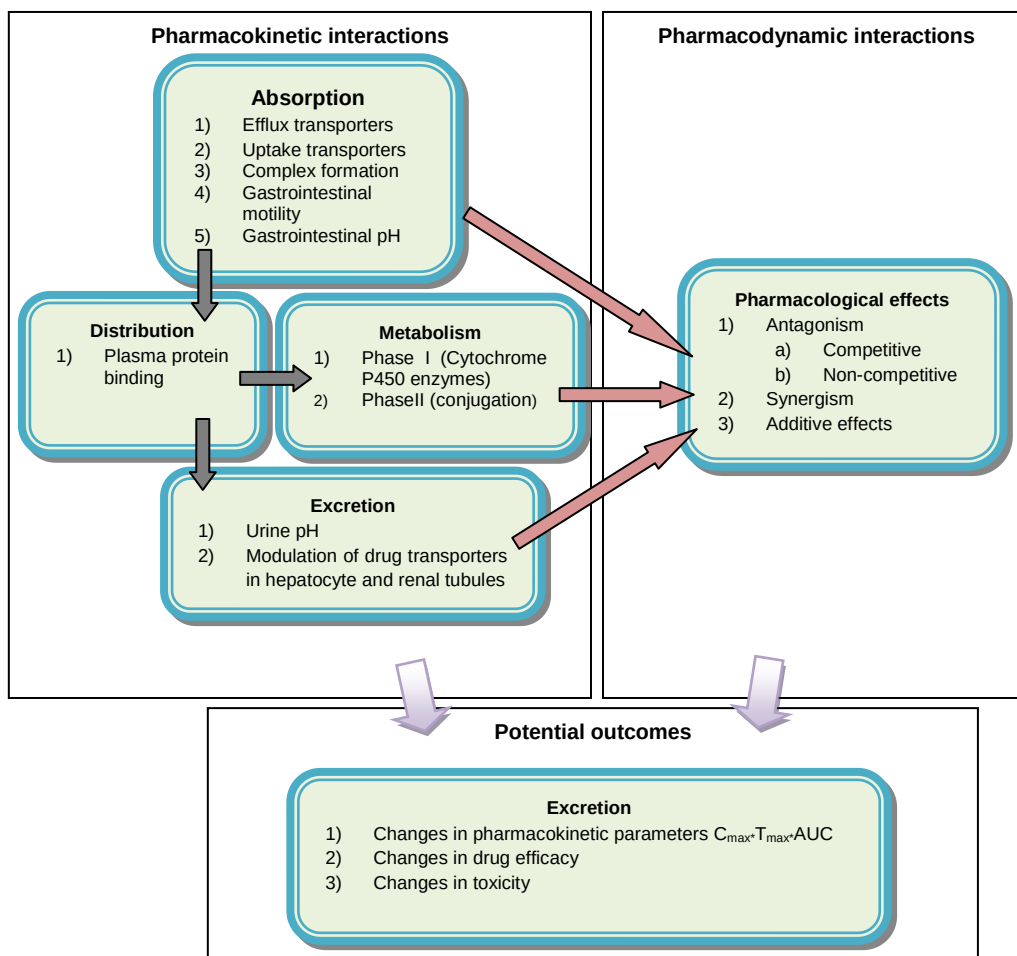


Figure 2.1: Potential mechanisms of herb-drug pharmacokinetic and pharmacodynamic interactions and their possible effects (adapted from Tarirai *et al.*, 2010:1518)

A single herb usually contains more than one alleged phytochemical, each potentially having some degree of pharmacological property. Natural products are therefore typically complex mixtures of many molecules and both the active ingredient(s) as well as other constituents present in the prepared mixture have the potential to interact with different classes of drugs (Pal & Mitra, 2006:2132). Phytoconstituents may therefore be chemically active and able to modulate physiological processes through synergistic and/or antagonistic effects (Bansal *et al.*, 2009:53-54).

Pharmacokinetic interactions can be caused by co-administered herbs due to the promotion or inhibition of efflux transporters such as P-gp and/or metabolism by cytochrome P450 (CYP) enzymes (Borrelli *et al.*, 2007:1393). Herbal components have the potential to interact with efflux transporter proteins and CYP metabolic enzymes in numerous ways (see Figure 2.2):

- A herbal constituent can act as a substrate for a single or several isoforms of CYP enzymes and/or efflux transporters such as P-gp. One substrate competes with another substrate for either metabolism by the same CYP isozyme and/or efflux by a transporter. When a compound acts as an inhibitor of an efflux system, drug efflux will decrease and the result of this interaction is higher plasma concentrations (Pal & Mitra, 2006:2136).
- A compound can also reduce the activity of one of the numerous isoforms of CYP by acting as an inhibitor for CYP enzymes, resulting in increased plasma levels of the co-administered drug (Pal & Mitra, 2006:2136).
- A herbal compound can be a promoter of a number of CYP isoforms and/or efflux transporters, resulting in reduced plasma concentrations (Pal & Mitra, 2006:2136).

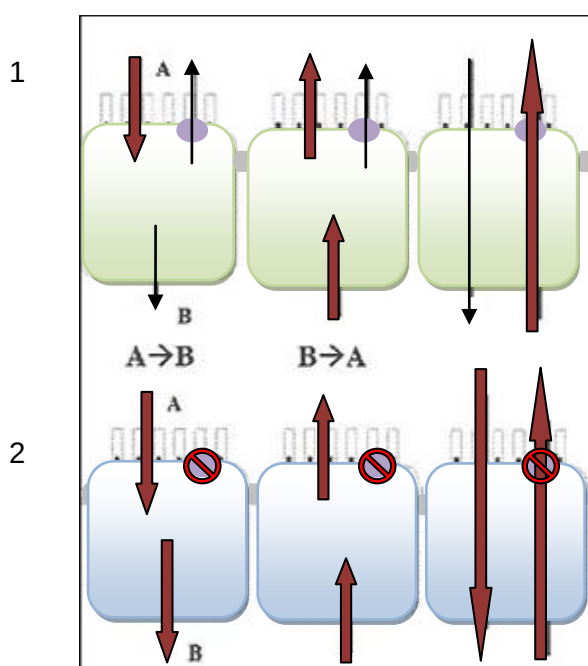


Figure 2.2: Model demonstrating bi-directional transport (absorptive; A→B and secretory; B→A) of P-gp substrates in the (1) absence and (2) presence of a P-gp inhibitor. Key: A, apical; B, basolateral; dark arrows vs. light arrows indicate relative magnitudes of flux (adapted from Varma *et al.*, 2003:354)

2.3 Herb-drug interactions during absorption

Passive diffusion is the main mechanism for the penetration of drugs through biological membranes in the body (Kerns & Di, 2008:103). For a compound to be absorbed or transported by means of passive diffusion, it should have specific physio-chemical properties and membrane permeability (Kerns & Di, 2008:103). A number of trans-membrane transporters are able to influence the permeability of certain drug molecules and are responsible for two essential permeability mechanisms namely active uptake and efflux (Kerns & Di, 2008:103).

The most important function of the intestinal epithelium is to absorb water, electrolytes and nutrients while at the same time acting as a barrier against potentially harmful pathogens and compounds in the lumen (Artursson *et al.*, 1993:1123). The oral route of administration is considered the most popular route of drug intake because of the efficient patient compliance and cost-effectiveness (Norris *et al.*, 1998:136).

Drug absorption across the intestinal epithelium is an intricate process that can occur through several pathways, as illustrated in Figure 2.3. Passive absorption generally occurs through the cell membrane of enterocytes (transcellular route) or via tight junctions between the enterocytes (paracellular route). Carrier-mediated absorption occurs via an active process or by facilitated diffusion (Balimane *et al.*, 2006:E1).

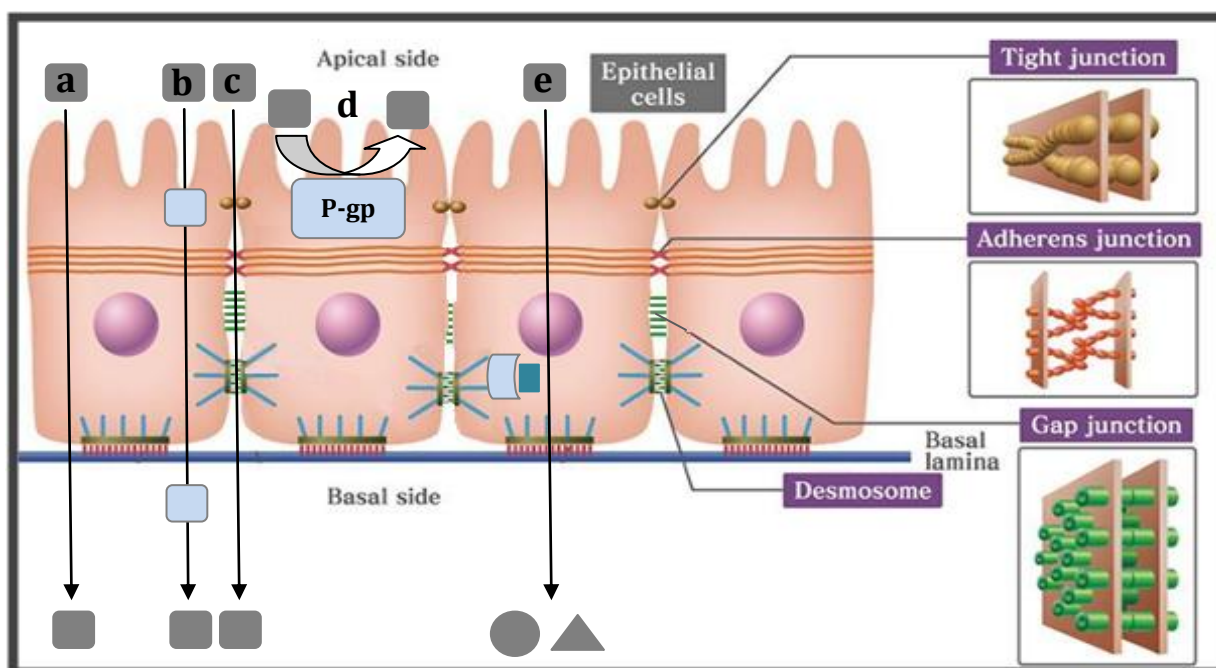


Figure 2.3: Different pathways for transport across the intestinal epithelium (Balimane & Chong, 2005:336; University of Tokyo, 2010)

As indicated in Figure 2.3, molecules cross the intestinal epithelium into the bloodstream via three main pathways, viz a) passive diffusion across cell membranes (transcellular), b) carrier mediated pathways and c) passive diffusion between neighboring cells via tight junctions (paracellular). Drug absorption can be negatively influenced by d) P-gp mediated efflux and e) metabolic enzymes in the cells which may metabolise the compound to pharmacologically inactive entities (Balimane & Chong, 2005:336).

In the intestinal cells, transporters are involved in the absorption as well as the efflux of drugs (Figure 2.4). Enhancement of drug absorption is facilitated by influx transporters such as organic anion transporting polypeptide (OATP) and oligopeptide transporter 1 (PEPT-1) (Kamath *et al.*, 2005: 233). Although there are many suggestions for the contribution of uptake transporters in the intestinal absorption of drugs, information on the transporter molecules responsible for the intestinal absorptive processes is limited (Endres *et al.*, 2006:508). The most investigated absorptive transporter is the PEPT-1, albeit the fact that PEPT-1 predominantly plays a role in the absorption of peptides and has affinity for only a few classes of drugs (Endres *et al.*, 2006:508).

PEPT-1 is present in the intestinal brush border membrane of the human intestine and acts as a transporter for dipeptides and tripeptides, but not for free amino acids or peptides with more than three amino acid residues (Endres *et al.*, 2006:508). Since drugs including oral β -lactam antibiotics, the anti-cancer agent, bestatin and the angiotensin-converting enzyme inhibitors (enalapril and temocapril) are absorbed by PEPT-1, clinically relevant drug interactions may occur at the level of intestinal absorption (Endres *et al.*, 2006:508).

The OATP family such as OATP1A2 and OATP2B1 has been suggested to mediate intestinal absorption of several drugs (Tamai, 2012:508). The OATPs are Na⁺-independent proteins that transport a wide range of endogenous compounds (such as bile acids, steroid conjugates and thyroid hormone) and drugs (such as digoxin, pravastatin and methotexate) (Endres *et al.*, 2006:507). The OATPs are mainly expressed in the organs important in drug absorption and disposition. These organs include the liver, kidney, intestine, brain and placenta (Endres *et al.*, 2006:507). Modulation of uptake transporters such as OATPs can occur and lead to a change in the bioavailability of the co-administered drugs. Because of the extensive tissue expression and substrate selectivity, clinically related inhibitory OATP drug interactions may occur at all levels of drug absorption and disposition (Endres *et al.*, 2006:507).

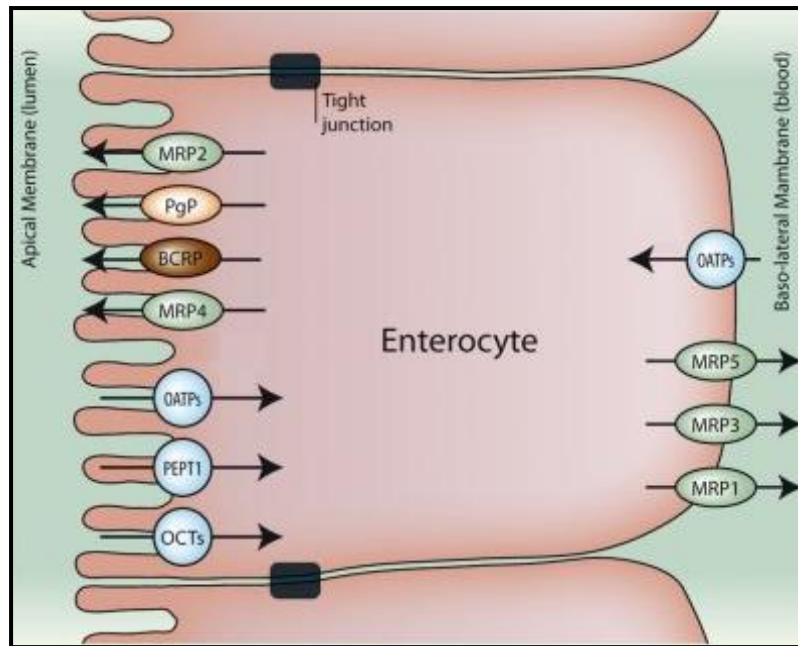


Figure 2.4: Different transporters present in intestinal epithelial cells (Solvo[®] Biotechnology)

Efflux, on the other hand, is the movement of substances from the epithelial cell membrane back into the gastric lumen and absorption can thus be limited by a range of efflux transporters, including P-gp, breast cancer resistance protein (BCRP) and multidrug resistant protein 2 (MRP-2), also depicted in Figure 2.4 (Balimane *et al.*, 2006:E2). The most significant mechanisms for herb-drug absorption interactions include the promotion or inhibition of intestinal drug efflux transporters such as P-gp and MRPs (Pal & Mitra, 2006:2132).

In Figure 2.5 the relationship between P-gp and CYP3A in the intestine is described. The drug is absorbed by passive diffusion processes into the enterocyte where it may be metabolised by CYP3A (Benet & Cummins, 2001:S6).

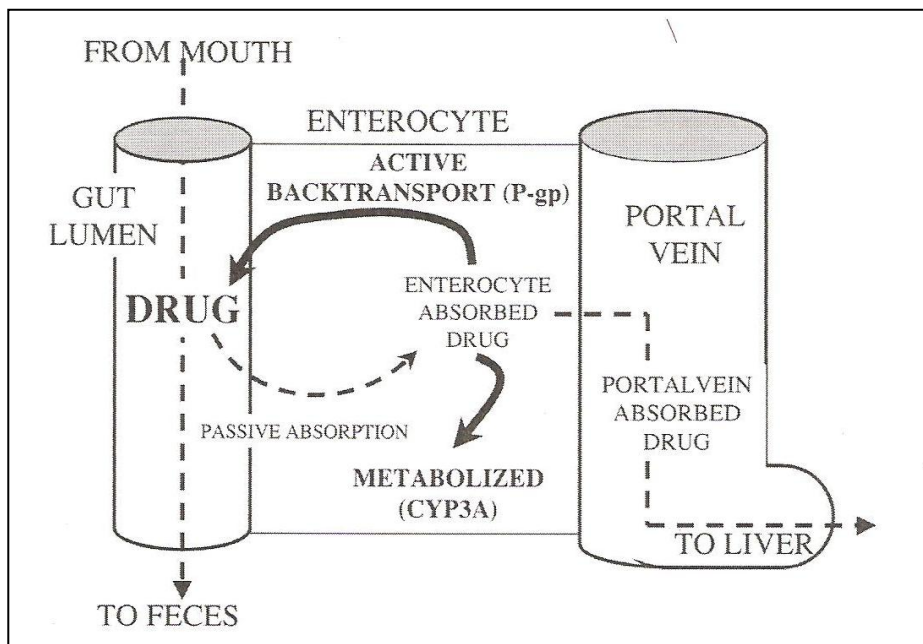


Figure 2.5: Schematic illustration of the enteral cycling of a drug that is a substrate for cytochrome P450 3A enzymes and P-glycoprotein (Benet & Cummins, 2001:S6)

When a drug is subjected to active efflux transport into the intestinal lumen, it allows further access to the enzyme upon subsequent absorption. This effect is illustrated in Figure 2.6, in which drug molecules not subjected to P-gp efflux will pass through the enterocyte only once, while molecules subjected to P-gp efflux may be continually cycled between the enterocyte and the gut lumen, thus allowing the enzyme repeated access to the drug or leading to non-absorption due to efflux and loss through fecal excretion. If P-gp efflux is inhibited, the drug molecules pass through the intestine in a single pass (Benet & Cummins, 2001:S6).

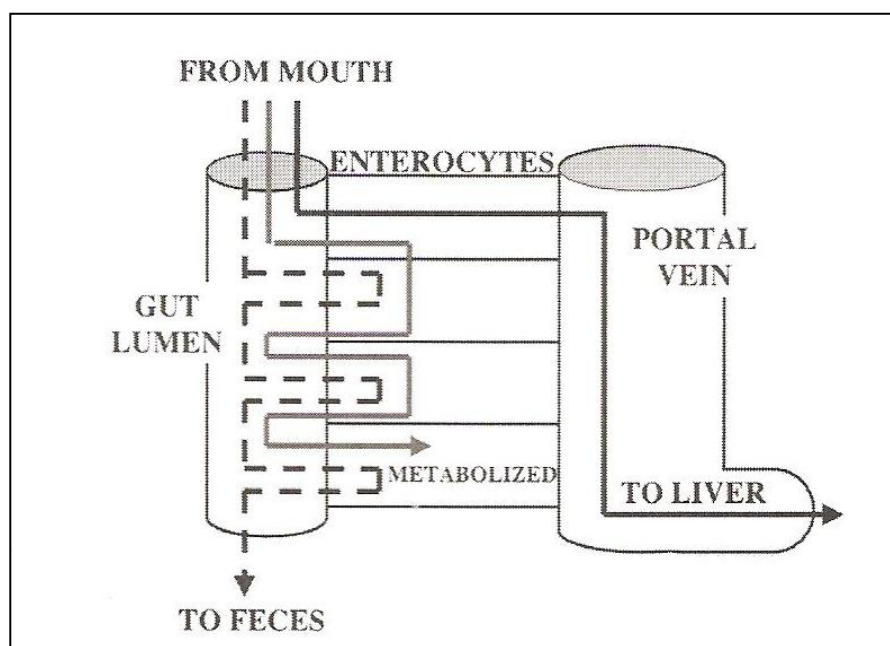


Figure 2.6: Illustration of the possible effects of P-glycoprotein efflux on access of drugs to intestinal cytochrome P450 3A enzymes (Benet & Cummins, 2001:S6)

2.3.1 The ABC efflux proteins

Efflux transporters belong to the adenosine triphosphate-binding cassette (ABC) family and facilitate the export of compounds out of the cell back into the intestinal lumen (Bansal *et al.*, 2009:46). These highly preserved multi-span transmembrane proteins facilitate the movement of a broad range of molecules across the cell membrane by utilising the energy of adenosine triphosphate (ATP) hydrolysis. ABC molecules transport a broad range of substrates which include peptides, lipids, xenobiotics, amino acids, ions, vitamins, carbohydrates, glucuronide and glutathione conjugates (Bansal *et al.*, 2009:46; Sharom, 2008:105). Functional ABC transporters are composed of two randomly linked functional units, each consisting of an ATP-binding cassette and trans-membrane domain complex (Bansal *et al.*, 2009:46).

Multi-drug resistance (MDR) is defined as 'the ability of tumours to show resistance to a broad range of structurally and functionally unrelated drugs when exposed to a single dose' (Bansal *et al.*, 2009:46). MDR causes many malignancies to fail to respond to chemotherapy, which occurs in more than 90% of patients with metastatic cancer. The most important form of resistance to chemotherapy has been associated with the occurrence of at least three molecular pumps (pore model, flippase model and vacuum cleaner model) that actively transport drugs out of the cell. P-gp, a member of the ABC super family, is the most common of these MDR transporters (Aller *et al.*, 2009:1718).

2.3.2 P-glycoprotein

In 1976 Juliano and Ling (Bansal *et al.*, 2009:47) discovered a relationship between the degree of drug resistance displayed by Chinese hamster ovary cells and the occurrence of a 170 kDa membrane glycoprotein. They named it P-glycoprotein (P; permeability), as it was discovered that these molecules caused distorted drug permeability (Berggren, 2006:14). Recent advances in biotechnology confirmed the presence of P-gp not only in tumour cells but also in a great variety of healthy tissue (Bansal *et al.*, 2009:47).

P-gp is an ATP-ase energy dependent membrane bound protein and has been extensively investigated for its role in the absorption, distribution, metabolism, elimination and toxicity of drug molecules (Balimane *et al.*, 2006:2; Berggren, 2006:14).

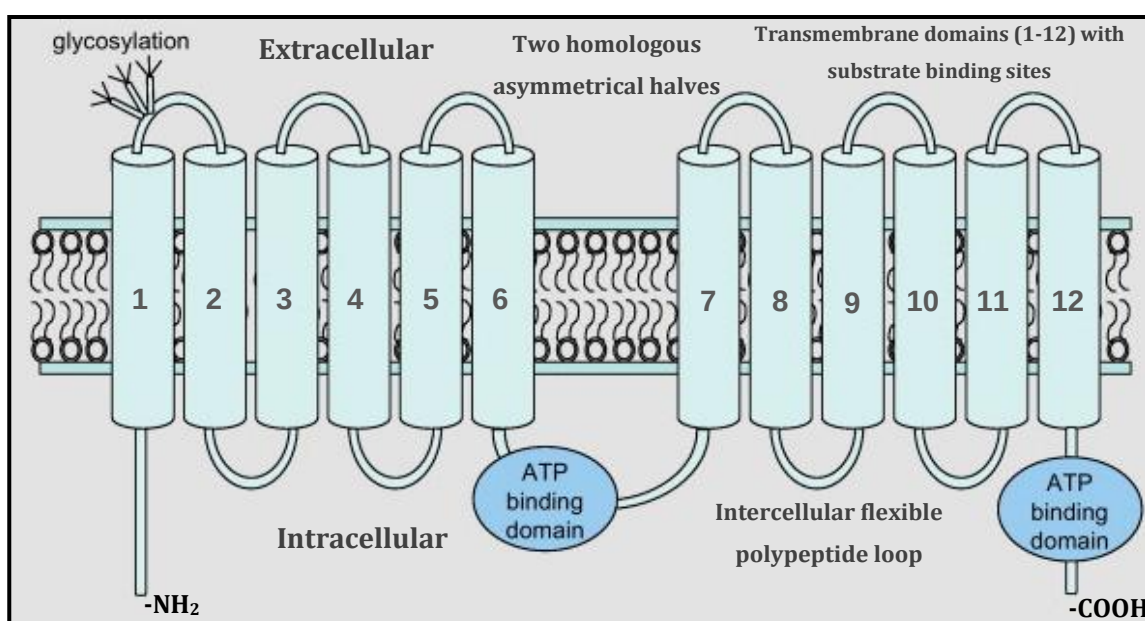


Figure 2.7: A schematic illustration of the structure of P-glycoprotein (Bansal *et al.*, 2009:50)

P-gp has the ability to transport a broad range of relatively hydrophobic, amphipathic drugs out of cells (Huisman *et al.*, 2000:237). Comprising 1 280 amino acids, it is expressed as a single chain divided into two homologous halves, each containing six transmembrane domains and two ATP-binding regions divided by a flexible polypeptide linker as seen in Figure 2.7. The functional unit of P-gp plays an important role in the transport of drug substrates and consists of two transmembrane domains and two nucleotide binding domains (NBD's) (Ambudkar *et al.*, 2006:392).

Located on the apical exterior surface of epithelial cells in the intestine, it acts as biological barrier by exporting toxic substances and xenobiotics from the cell back to the intestinal lumen (Deferme *et al.*, 2008:187). Consequently, P-gp limits or avoids the absorption of a wide range of compounds from the gastro-intestinal lumen (Chan *et al.*, 2004:34). This might limit the net passage of orally administered drugs to the bloodstream, thereby reducing the oral bioavailability of the drug (Huisman *et al.*, 2000:237). Conversely, the bioavailability of some orally administered drugs can be enhanced by inhibiting intestinal P-gp efflux (Evans, 2000:134).

P-gp is present amongst other locations, in the biliary canalicular surfaces of hepatocytes, luminal surfaces of the endothelium in the small and large intestine, endothelial cells of the blood brain barrier, apical surfaces of proximal tubular cells of the kidney and the apical membranes of the foetal-membrane barrier in the placenta (Bansal *et al.*, 2009:47). The high concentration of P-gp on the luminal surfaces of the gastro-intestinal tract (especially the jejunum, ileum and colon) can potentially prevent the absorption of drugs and increase the excretion thereof across the gastro-intestinal tract (Ambudkar *et al.*, 1999:386).

2.3.2.1 Mechanism of P-glycoprotein efflux

P-gp mediated efflux is an osmotically sensitive transport mechanism that generates a concentration gradient utilising ATP energy (Kerns & Di, 2008:111). The mechanism underlying P-gp efflux is illustrated in Figure 2.8. The first step in the drug efflux process is the stereo-specific recognition of the drug molecule by P-gp followed by ATP-binding and hydrolysis. The energy released following ATP binding is spent to facilitate the efflux of the substrate to the outside of the cell through the central pore of P-gp. Two ATP-molecules are expended during the transport process of one substrate molecule (Kerns & Di, 2008:111). One ATP-molecule is hydrolysed for the actual transport of the substrate while the other is hydrolysed to facilitate in resetting the pump for the next transport cycle (Suana & Ambudkar, 2000:2519). The discharge of adenosine diphosphate (ADP) from the nucleotide binding site ends the first transport cycle (Bansal *et al.*, 2009: 51).

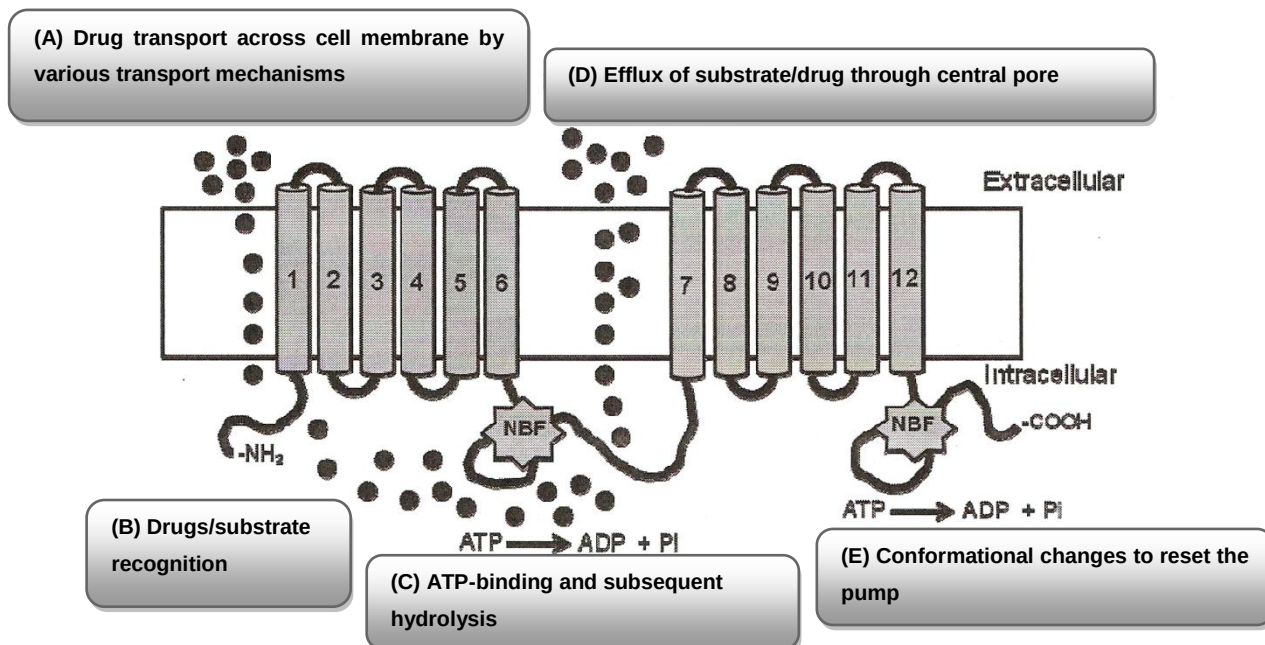


Figure 2.8: Mechanism of P-glycoprotein mediated efflux (adapted from Bansal *et al.*, 2009:51)

The three models (Figure 2.9) hypothesised for substrate transport by P-gp propose two potential sites for the primary substrate interactions. In the 'pore model' (classical pump) drugs associate with P-gp in the cytosolic compartment and are transported through a protein channel out of the cell. In the 'flippase model' drugs are transported from the inner (cytosolic) side of the plasma membrane where it binds to P-gp within the plane of the membrane, to the outer (extracellular) side of the membrane from where the drug molecules passively diffuse into the extracellular fluid (Hochman *et al.*, 2002:259).

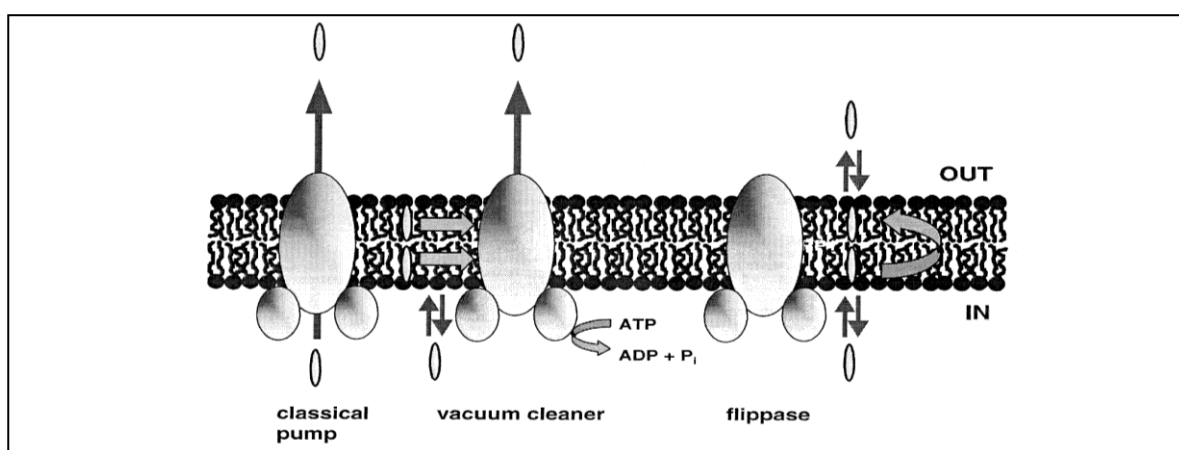


Figure 2.9: Models illustrating the mechanism of drug efflux by P-glycoprotein (Sharom, 2006:979)

The third model combines features of both the 'classical pump' and 'flippase model' and is known as the 'hydrophobic vacuum cleaner channel'. This model suggests that P-gp identifies substrates contained in the inner side of the plasma membrane. Substrates are absorbed from the inner side and transported through a protein channel after which it is released into the extracellular media (Hochman *et al.*, 2002:259).

2.3.2.2 Inhibition of P-glycoprotein

Inhibition of P-gp could be a useful intervention to improve the oral absorption of drugs and reduce drug excretion. This effect could possibly be exploited in future to reduce the applicable drug doses and to ensure better drug penetration in target tissues (Ambudkar *et al.*, 1999:389). Given the role P-gp plays in the alteration of various pharmacokinetic parameters, methods to overcome P-gp mediated efflux have been investigated. Consequently, a number of different P-gp inhibitors have been identified (see Table 2.1) (Zhou *et al.*, 2004:47). Several non-cytotoxic substances are able to reverse or inhibit the P-gp mediated efflux of drugs, including the calcium channel antagonists (e.g. verapamil), PIs (e.g. indinavir), cyclosporine and diterpene (Zhou *et al.*, 2004:59).

First-generation inhibitors possess pharmacological activity and are used for indications that are independent of their action on P-gp. The second-generation inhibitors consist of agents with low pharmacological activity compared to the first-generation compounds but exhibit a higher affinity for P-gp. Applying the knowledge obtained from the structure-activity relationships evident in the first two generations of P-gp inhibitors, the third-generation P-gp inhibitors were developed. These molecules selectively inhibit the actions of P-gp and are used to optimise the treatment of patients diagnosed with MDR tumours (Bansal *et al.*, 2009:52).

Table 2.1: Different classes of P-glycoprotein modulators (Bansal *et al.*, 2009:55)

P-glycoprotein Inhibitors						P-glycoprotein Promoters	
1 st Genertion	2 nd Generation	3 rd Generation					
		Synthetic compounds	Pharmaceutical Excipients	Herbs and Fruits	Herbal constituents		
Verapamil	Dexverapamil	LY 335979	C8/C10 Glycerol	Grapefruit	Flavonoids	Adriamycin	
Felodipine	Gallopamil	XR 9576 (tariquidar)	Cremophor	Orange	Quercetin	Daunomycin	
Nifedipine	PSC 833 (Valspodar)	OC 144-093	Solutol HS-15	Apricot	Iso-quercetin	Mitoxantrone	
Diltiazem	Reversin 125	R 101,933	Labrasol	Mint	Spiraeoside	Dexamethasone	
Chloorpromazine	VX-710 (Biricodar)	Mitotane (NSC-38721)	Softigen 767	Strawberry	Rutin	Cyclosporin A	
Quinidine	Reversin 121	Annamycin	Aconnon E	African potato	Kaempferol	St. John's Wort	
Cyclosporin A	MS-209		Sucrose esters	Green tea	Naringenin		
Erythromycin	GF 120918		Sucrose monolaurate	Rosemary extract	Naringin		
Fluphenazine			Polysorbates	Garlic	Biochanin A		
Reserpine			Tween 80	Milk Thistle	Morin		
Tamoxifen			Span 20	Ginseng	Silymarin		
			Tocopherol esters	Black pepper	Genistein		
			A-tocopheryl-PEG-1000-succinate	Ginko	Bergamottin		
			PEG esters	Onion	Catechin		
							Bergamottin
							Hesperidin
							Phloretin
	Tangeretin						
Monoterpenoids							
(R)-(+)-citronellal							
				(S)-(-)-beta-citronellol			

The fact that P-gp can be inhibited raises the question whether a significant positive improvement can be attained in the normal pharmacokinetic parameters of the various drugs influenced by P-gp mediated efflux. These drugs include the PIs used in the treatment of HIV and AIDS and although this strategy will not cure the condition, every possible mechanism to optimise the current treatment regimens must be exploited. In theory, the concomitant administration of P-gp inhibitors with PIs has the potential to improve the oral bioavailability and reduce the pre-absorption excretion of the latter. As stated earlier, these changes in pharmacokinetic parameters can possibly be exploited to lower the total daily dose of PI therapy, thereby improving patient compliance and minimising the experience of PI associated adverse effects. Increased bioavailability of PIs could also result in higher plasma levels and theoretically delay the development of PI-resistant HIV variants. A number of highly active anti-retroviral treatment (HAART) drug combinations that are currently prescribed already exploit P-gp inhibition, including the combination of saquinavir and ritonavir that results in the increased oral bioavailability of the first due to the inhibition of P-gp caused by the latter (Huisman *et al.*, 2000:240). However, this synergistic interaction can be hazardous if drug levels become toxic due to the inconsiderate combination (whether intentional or unintentional) of drugs prescribed at normal doses with P-gp inhibitors (Brown *et al.*, 2008:589).

In the past few years, governments all over the world have promised to make more ARV drugs available and accessible to provide basic treatment to everybody (Amon, 2008:1). Attention has been focused on how to finance this ideal and how to improve health care delivery systems (Amon, 2008:1). Employing strategies such as the optimisation of the pharmacokinetic parameters of the various ARV drug therapies, can aid in reaching this ideal.

2.4 Models to study drug absorption and pharmacokinetic interactions

Despite remarkable improvements in drug delivery methods in the last few decades, the oral route still remains the favoured route of administration for most new chemical entities. The oral route is preferred due to its apparent advantages such as convenience, low cost and high patient compliance compared to alternative routes (Balimane *et al.*, 2000:301). However, compounds that are developed for oral administration must have sufficient aqueous solubility and intestinal permeability in order to achieve therapeutic concentrations in the blood plasma (Balimane *et al.*, 2000:301). The assessment of permeability properties is an essential step in the development of new drug candidates. Appropriate cost-effective screening models have been developed to assess these properties (Balimane & Chong, 2005:335) and will be reviewed shortly in the following sections.

Drug discovery scientists use various techniques when evaluating the intestinal epithelial permeability of drug candidates during the initial drug development process. Figure 2.10 illustrates some of the most validated preclinical methods for the determination of drug permeability currently used throughout the industry which can be divided into *in vitro*, *in vivo*, *in situ* and *in silico* models. The *in vitro* models can be based on the use of either excised animal tissue (e.g. the Ussing chamber, membrane vesicles and everted gut), cells (e.g. Caco-2 cells and Mardin-Darby canine kidney [MDCK] cells), membranes (e.g. parallel artificial membrane permeability assay [PAMPA]) and immobilised artificial membranes. *In vivo* screens are done on live animals, albeit in any tissue expressing P-gp, while *in situ* methods are performed in the actual cells within the intact organ under investigation, whether *in vivo* or *ex vivo* (in this case the small intestine). *In silico* is a term used to describe computer-generated models for the investigation of certain phenomena viz the study of drug permeability (Balimane & Chong, 2005:336).

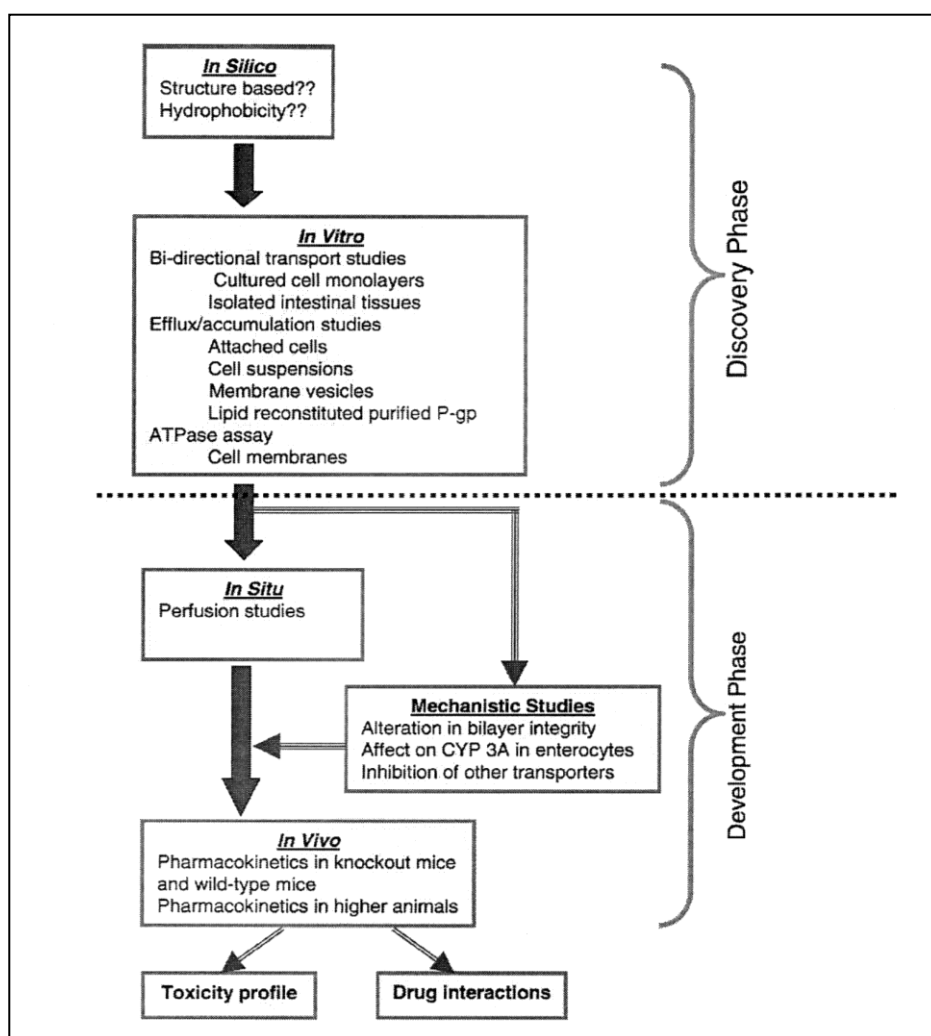


Figure 2.10: Illustration of different models for screening of the drug discovery and development phases (Varma *et al.*, 2003:353)

2.4.1 *In vivo* models to study intestinal absorption

As the anatomy of mammals demonstrate functional similarity with that of humans (Hidalgo, 2001:389), the characteristics of drug absorption in animals can in most cases be sufficient as a reliable predictor of the biological factors that can influence the intestinal absorption of drugs in humans. Using *in vivo* models, drugs are administered orally to measure their permeation from the gut to the blood and tissue compartments of the body (Hidalgo, 2001:389).

One of the main advantages of *in vivo* models is the occurrence of the dynamic interplay between the mesenteric blood circulation, the mucous layer and all the diverse biological factors that influence drug absorption. Another important advantage of studies with live animals is that the species used for the absorption studies, could also be used to study the pharmacological and toxicological properties of the drug and P-gp modulator under investigation (Hidalgo, 2001:389).

However, one of the most important disadvantages of *in vivo* models is that the variables inherent to the absorption process cannot be separated. For example, it is not possible to identify the various individual rate-limiting factors that play a role in absorption (Le Ferrec *et al.*, 2001:653). Furthermore, large amounts of drug is required, the analytical methods necessary for plasma analysis are invasive and complex, while these models also only provide limited insight into the mechanisms of drug absorption. They are therefore ideal for quantitative, but not qualitative analysis (Hidalgo, 2001:389).

In conclusion, *in vivo* methods provide insight into the actual physio-chemical permeability characteristics of drugs in living mammals and probably represent the most accurate predictor of human drug absorption (Nejdfors *et al.*, 2000:501).

2.4.2 *In situ* models to study intestinal absorption

A powerful research technique for the analysis of intestinal transport and metabolism is the use of vascular perfused intact sections of the small intestines of mammals. In this model, the abdominal cavity of an anaesthetised animal is exposed by laparotomy. *In situ* methods allow the researcher to analyse drug factors such as formulation-independent breakdown in the stomach under acidic conditions. Although the experimental animal is anaesthetised and surgically manipulated, mesenteric blood flow stays intact. However, recent studies demonstrated that anaesthesia can influence intestinal drug absorption, which complicates the choice of anaesthetic (Le Ferrec *et al.*, 2001:653).

In situ methods have several advantages over *in vivo* models. Certainly one of the most pronounced advantages is the fact that *in situ* models do not require the drug to pass through the stomach and therefore prevent the precipitation of acidic compounds. Furthermore, drug secretion into the intestinal lumen after intravenous administration can also be investigated, enabling the researcher to study the efflux of xenobiotics into the intestinal lumen by means of P-gp, multidrug resistance-associated protein (MRP) and lung cancer-associated resistance protein (LRP) (Le Ferrec *et al.*, 2001:653).

2.4.3 *In vitro* models to study intestinal absorption

Due to both ethical and time constraints, live animal studies cannot be used as a high volume screening tool in the early stages of drug development. Subsequently, *in vitro* models for the assessment of intestinal absorption have been developed for this purpose (Deferme *et al.*, 2008:187). Various *in vitro* methods are available to determine the intestinal absorption potential of drug candidates. Each method has different advantages and disadvantages that must be taken into account upon deciding which method will suit the outcome of the current experiment the best.

In vitro techniques for the assessment of permeability are less labour and cost-intensive compared to *in vivo* animal models (Balimane *et al.*, 2000:305). One of the major concerns with all *in vitro* systems is that the effects of physiological factors such as gastric emptying, gastrointestinal transit rate, and gastrointestinal pH cannot be integrated in the data analysis. The successful application of *in vitro* models to predict drug absorption across the intestinal mucosa depends on how accurately the *in vitro* model mimics the characteristics of the *in vivo* intestinal epithelium (Balimane *et al.*, 2000:305).

2.4.3.1 Animal tissue-based models to study intestinal absorption

To obtain viable human tissues for permeability studies on a regular basis is problematic. As the intestinal tissue of mammals display similar physiological and anatomical characteristics of that in humans, permeability screening for drug discovery is often carried out using tissue obtained from various animal species (Balimane *et al.*, 2000:305). Excised animal tissue models have been employed for the past seven decades to investigate the mechanisms underlying the absorption of nutrients from the intestine (Balimane *et al.*, 2000:305). These models include the Ussing chamber, everted gut technique and isolated membrane vesicles.

2.4.3.1.1 Ussing chambers

Transport of ions across intestinal tissue obtained from animals is frequently utilised to study drug absorption (Balimane *et al.*, 2000:305). Ussing & Zehran (1951) introduced Ussing chambers to study the active transport of sodium in the form of electric current in isolated

frog skin samples. However, these chambers have since been used to study ion transport over a diverse range of different membranes (Le Ferrec *et al.*, 2001:654).

In short, tissues are isolated, dissected into strips of appropriate size and clamped onto a suitable device after which the rate of drug transport across the membrane is measured (Balimane *et al.*, 2000:305). The drug can be introduced to either the mucosal (apical) or the serosal (basolateral) side of the enterocytes. A unique advantage of this model is that the transepithelial electric resistance (TEER) of the membrane can be measured easily. This value reflects the viability of the intestinal tissue during the transport studies. Another important advantage of Ussing chambers is the fact that using excised tissue ensures the examination of suitable membrane characteristics for drug transport. As all factors influencing drug absorption are expressed at near-physiological levels in the excised tissue, it is possible to study the combined effect of both intracellular modulators of absorption (e.g. enzymes) and membrane-bound modulators such as the active transporters in the intestine (Berggren, 2006:18). In addition, the Ussing chamber technique is an ideal method to study the effect of anatomical differences on the absorption of drugs by using intestinal tissues obtained from different intestinal regions. The amount of drug required is relatively small compared to most *in vivo* models and the collected samples are analytically pure which ensures rapid and accurate quantitative analysis. Major shortcomings of this technique however, include the absence of blood supply and nerve innervation, rapid loss of tissue viability over the course of the experiment and changes in the morphology and functionality of the transporter proteins during tissue preparation (Balimane *et al.*, 2000:305-306).

The relative uncomplicated methodology underlying this model makes it an ideal *in vitro* model to study drug transport (Le Ferrec *et al.*, 2001:654), albeit the fact that the drug being investigated must demonstrate ion-like transport.

2.4.3.1.2 Everted gut technique

In 1953 the everted gut technique was described by Wilson & Wiseman (1953:116) with the purpose of studying the transport of sugars and amino acids from the mucosal to the serosal side of intestinal membranes. In short, the mesenteric border of the small intestine of a mammal is stripped manually. To evert the gut, the ileal end is pushed with a rod through the lumen of the gut until it protrudes from the duodenal opening. The everted intestine is then slipped off the rod and immersed in the appropriate nutrient mixture (containing the nutrient(s) being investigated) at room temperature. The everted intestine is tied off on one end using a thread ligature while a second ligature is placed loosely around the other end. After the weight of the tissue is recorded, a blunt needle attached to a syringe is inserted in the cavity (previously the basolateral side) and the loose ligature pulled tight over the needle.

Due to a lack of modern technology in 1953, an appropriate buffer together with a small amount of O₂ was injected into the cavity and as the needle was withdrawn, the ligature was tied off. The total weight of the tissue containing the buffer was then weighed and the initial amount of buffer in the cavity was calculated. After completion of the experiment, the closed sac was weighed again and the increase in weight was calculated (Wilson & Wiseman, 1953:116).

Since the initial development of the method, several changes to the protocol have been made which improved the reliability of the method. The eversion of the intestine exposes the highly active mucosa to the well-oxygenated medium, while the distension increases the surface area of the exposed apical side and reduces the thickness of the wall. The relatively small amount of fluid inside the cavity allows a rapid rise in concentration of transported nutrients. Both active and passive transport can be analysed which makes it an ideal model to study nutrient absorption holistically (Balimane *et al.*, 2000:305).

Recent interest in P-gp activity in the gastro-intestinal tract makes this model ideal to evaluate the role of efflux transporters in the intestinal absorption of drugs by comparing the transport kinetics of drugs in the presence and absence of P-gp inhibitors and substrates. One of the advantages of this model is that the sample volume on the serosal side is relatively small which will cause rapid accumulation of drugs. As is the case with most *in vitro* methods, disadvantages of the method include the lack of blood flow and nerve innervation which may result in rapid loss of tissue viability. Everting the tissue can also lead to morphological damage interfering with the results (Balimane *et al.*, 2000:305). Another disadvantage is the presence of the muscularis mucosa, which is often not removed from the intestinal tissue. Thus, the test-compound passes from the lumen into the lamina propria (where blood and lymph vessels are present) and across the muscularis mucosa, a path that does not mimic the actual intestinal barrier (Le Ferrec *et al.*, 2001:654).

2.4.3.1.3 Isolated membrane vesicles

Brush border membrane vesicles have been isolated from several mammal species and used extensively in transport studies (Balimane *et al.*, 2000:306) which can be prepared from either intestinal scrapings or isolated enterocytes. Membrane vesicles provide the option to examine the interaction of drugs with a specific membrane of interest. In addition, this method allows for total manipulation of the solute environment both inside and outside the vesicle, therefore making it an ideal system for absorption studies. In comparison with other *in vitro* techniques, membrane vesicles are a more convenient option when low quantities of drug are available (Balimane *et al.*, 2000:306).

Vesicles can be cryopreserved and several experiments can be performed with vesicles prepared from a single laboratory animal. However, prepared vesicles are not necessarily pure and may be contaminated by other membranes or organelles. The isolation of vesicles often damages the transporter proteins and enzymes, while sensitive analytical methods are required to analyse membrane vesicles to accommodate the small quantities of analyte being investigated (Balimane *et al.*, 2000:306).

2.4.3.2 Membrane-based models to study intestinal absorption

2.4.3.2.1 Parallel artificial membrane permeability (PAMPA) models

The PAMPA model utilises a hydrophobic filter material coated with a mixture of lecithin/phospholipids dissolved in an inert organic solvent such as dodecane which creates an artificial lipid membrane barrier and mimics the endothelial membrane of the human intestine. In a number of studies significant correlations have been demonstrated between the flux of several well characterised drugs across the PAMPA system and the human intestine, indicating that the PAMPA system may be a reliable method to simulate physiological drug absorption inherent to humans (Balimane & Chong, 2000:304). If sample quantities are high, the PAMPA system has the advantage of screening large numbers of samples in a very short duration of time using 96-well microtiter plates joined with rapid analysis using a spectrophotometer. Although the PAMPA technique is much less labour intensive than cell culture models, they appear to have similar predictability (see paragraph 2.4.3.3). A major disadvantage of this model is that it does not provide for the absorption of compounds that are actively absorbed by drug transporters. Despite this limitation, PAMPA models may serve as an important primary permeability screen in the early stages of the drug discovery process because of its high volume and rapid output (Balimane *et al.*, 2006:E13).

2.4.3.3 Cell-based *in vitro* models

Different types of cell monolayer models that mimic *in vivo* intestinal epithelium in humans have been developed. Unlike enterocytes, human tumour cells grow quickly into confluent monolayers followed by spontaneous differentiation providing a near to ideal system for transport studies. Currently leading cell culture models for estimating transcellular intestinal flux include Caco-2, HT-29, T-84, MDCK and LLC-PK1 cells (Balimane *et al.*, 2000:306).

2.4.3.3.1 Caco-2 cells

The Caco-2 cell model is probably the most accepted and extensively studied cell-based model for examining the permeability of drugs through intestinal epithelium. Caco-2 cells are isolated human colon adenocarcinoma cells that differentiate spontaneously in culture and

possess apical brush borders with well-established tight junctions between adjacent cells. This is similar to the structure and organisation of the intestinal epithelium in humans (Figure 2.11) (Balimane *et al.*, 2005:337).

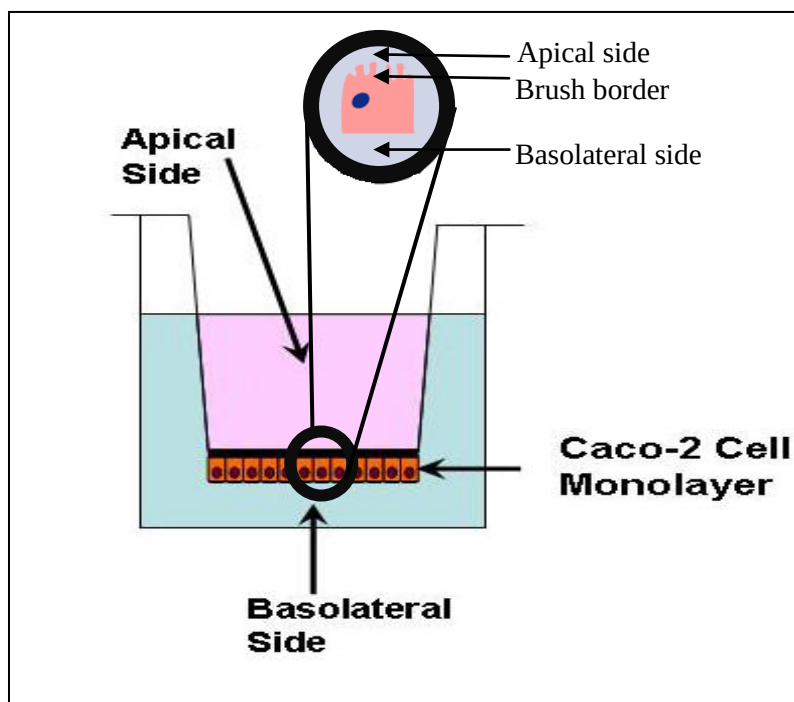


Figure 2.11: Distinctive experimental setup of the Caco-2 method (adapted from Apre dica: Caco-2 permeability assay services; Bohets *et al.*, 2001:374)

Despite the fact that drug permeability has been investigated in Caco-2 cells for the past 10 to 15 years (Balimane *et al.*, 2005:337), it is difficult to compare the absolute drug permeability coefficient values obtained from Caco-2 cells with that of other *in vitro* or *in vivo* models, especially for compounds that are almost exclusively absorbed via the paracellular pathway. This can probably be explained by the differences in culture conditions and the varying nature of different cell sub-populations compared to the relative uniformity of tissue obtained from mammals (Balimane *et al.*, 2005:337). Another limitation of the Caco-2 cell model is that the concentration of the pharmaceutically important transporters expressed by these cells is significantly lower than that found in mammals and humans. Furthermore hydrophilic compounds with a low molecular weight demonstrate poor permeability in Caco-2 cells regardless of its adequate absorption in humans. However, compounds that demonstrate a high degree of permeability in this model are characteristically well absorbed *in vivo*. In addition, the use of certain organic solvents may influence the structural integrity of the tight junctions between the Caco-2 cells. As many novel compounds are procured in organic solvents, this is a major limitation of the Caco-2 cell model (Balimane *et al.*, 2000:306).

Despite these limitations, the Caco-2 cell model provide valuable information on compound permeability and is still the most applied intestinal cell culture model (Balimane *et al.*, 2000:306).

2.5 Conclusion

Following the above discussion, it is evident that drug-transporter interactions may be influenced by a diverse range of variables such as transporter inhibition and promotion, as well as drug influx or efflux. These interactions can be clinically significant if the elimination of the drug in question or the distribution thereof in target tissues is mediated primarily by drug transporters and if the interaction results in drug concentrations that fall outside the normal therapeutic window. However, thoroughly investigated and validated, drug-transporter interactions can be beneficial when they are applied for the optimisation of drug kinetics and ultimately improve the therapeutic outcomes of the current treatment regimens (Endres *et. al.*, 2006:502). As it is, one of the greatest challenges facing the pharmaceutical industry today, is to improve as much as possible on the current treatment regimens to our disposal. As such, it is important to develop high-volume, cost-effective and predictable permeability models that can be used during the developmental phases of drug discovery. All of the discussed models have inherent advantages and disadvantages that need to be considered upon deciding on the appropriate method of permeability analysis. It is possible that not only one, but two or more of these methodologies need to be applied in the process of drug discovery, before validated and reproducible data can be obtained (Balimane & Chong, 2005:342).

CHAPTER 3

3 BRIEF OVERVIEW OF SELECTED MEDICINAL PLANTS

3.1 Introduction

According to clinical and preclinical studies a close relationship exists between dietary lifestyles and the occurrence of certain diseases. It has been demonstrated that sufficient intake of fruits, vegetables and herbs included in a diet may decrease the risk for the development of certain diseases and even prevent a number of acute and chronic conditions (Amagase *et al.*, 2001:955S). For many years, vitamins and herbal supplements have been self-administered either for the improvement of physical and mental health or to manage side effects of other drugs. The popularity of herbal medicines are drastically increasing worldwide, regardless of the fact that their mechanisms of action is still not explained and evidence for their efficacy and toxicological properties is insufficient. Herbal products such as St. John's wort, garlic, ginko biloba, ginseng and milk thistle are being used worldwide in the hope of promoting general health and for the treatment of many conditions such as colds, inflammation, central nervous system diseases, depression, high blood pressure and diabetes (Pal & Mitra, 2006:2131; Zhou *et al.*, 2007:664).

A previous President of South-Africa and the two Health Ministers in his cabinet have referred to ART as 'toxic', while they promoted alternative medicines of natural origin for the treatment of HIV/AIDS, including garlic, olive oil, beetroot and lemon. The insecurity and fear provoked by this incurable, life-threatening and stigmatised disease, can easily cause people suffering from HIV/AIDS to revert to these alternative 'cures' (Amon, 2008:4). In this study, some of the most common natural and herbal substances used in conjunction with ART, viz *A. sativum*, *C. limonum* and *B. vulgaris*, will be investigated. In the remainder of Chapter 3, a short review will be given of their most important biological and physiological properties.

3.2 Garlic (*Allium sativum*)

3.2.1 Botany of *Allium sativum*

Allium sativum is a member of the Liliaceae family and contains large concentrations of chemical compounds that are believed to have health promoting and beneficial properties in humans (Omar & Al-Wabel, 2010:51). A fully grown *A. sativum* plant is about 30 to 80 cm high and features 6 to 12 flat bifacial leaves that enclose the lower part of the stalk. The bulb is divided into approximately 15 cloves (Figure 3.1), which are created by the axillary bud and its collateral buds (Sendl, 1995:324).



Figure 3.1: Picture illustrating the bulbs and cloves of *Allium sativum* (Belsinger, 2004)

3.2.2 Chemical composition of *Allium sativum*

Blackwood and Fulder (as indicated by Omar & Al-Wabel, 2010:52) reported that one clove of *A. sativum* weighs between 3 and 6 grams and contains 1 g carbohydrates, 0.2 g protein, 0.05 g fiber, 0.01 g fat as well as trace amounts of vitamins A, B₁, B₂, B₃ and C. *A. sativum* contains approximately ten different natural sugars such as glucose, fructose, inulin and arabinose. It is believed that *A. sativum* is richer in adenosine (a nucleic acid acting as a building block for deoxyribonucleic acid [DNA]) than any other food source. Adenosine is a major constituent that displays anti-platelet action. *A. sativum* also contains approximately 33 sulphur-containing compounds (e.g. alliin, allicin, ajoene, allylpropyl disulphide, sallylcysteine, vinylthiines and S-allylmercaptocystein), numerous flavonoids/isoflavonoids (e.g. nobiletin, quercetin, rutin and tangeretin), polysaccharides, prostaglandins, saponins, and terpenes (e.g. citral, geraniol, linalole, α - and β -phellandrene) (Omar & Al-Wabel, 2010:52; Zhou *et al.*, 2004:63).

According to Dausch and Nixon (1990:347), *A. sativum* is one of the richest vegetable sources of sulphur-containing compounds, believed to be the reason for its positive biological effects. However, other compounds such as S-allylcysteine, S-allylmercaptocysteine and N- α -fructocyl arganine may also be important in this regard (Zhou *et al.*, 2004:63).

Allicin (diallylthiosulphate) is responsible for the characteristic smell of *A. sativum*. Combined with Vitamin B₁ (thiamin) it is called allithiamine which is easily absorbed from the intestinal lumen. Allicin only exists in *A. sativum* after the cloves have been crushed during which the enzyme allinase is being activated. Alliin is a non-protein amino acid with four isomers of

which one is present in *A. sativum*. Allicin is subsequently metabolised to produce diallyl sulphide, diallyl disulphide, diallyl trisulphide, allyl-methyltrisulphide, and dithiins (Figure 3.2). This process takes place within hours at room temperature and within minutes during cooking. Allicin can easily diffuse into vesicles or into the cytoplasm of red blood cells (Omar & Al-Wabel, 2010:52).

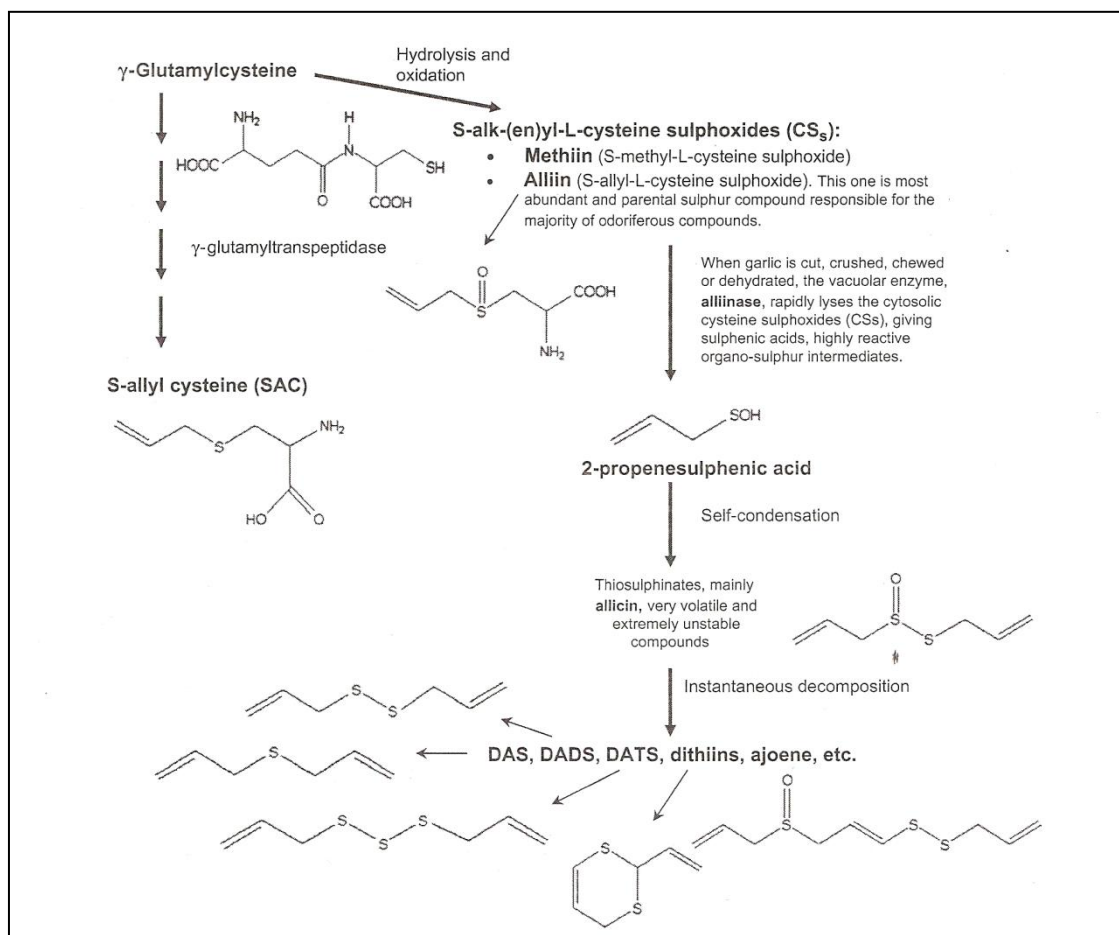


Figure 3.2: Metabolic pathways of processed *Allium sativum* and the formation of organo-sulphur compounds (Corzo-Martínez *et al.*, 2007:610)

Not only does allicin react with high and low molecular weight tiols and oxidants, but it also displays a high grade of membrane permeability (Omar & Al-Wabel, 2010:52).

3.2.3 Medicinal applications of *Allium sativum*

A. sativum is one of the most commonly prescribed herbal additives and remedies and is used for a diverse range of indications which include the flavouring of food, traditional healing and as a supplement to improve mental and physical health (Borrelli *et al.*, 2007:1386; Foster *et al.*, 2001:176).

Several studies have aimed to investigate the potential benefits of alliums such as *A. sativum* with regards to its proposed role in the prevention and cure of disease (Amagase *et al.*, 2001:955S). A wide range of *A. sativum* preparations are available on the market and therefore it is of great importance to elucidate the potential effects of this herbal product on the pharmacokinetics of co-administered allopathic drugs (Amagase *et al.*, 2001:955S).

The pharmacological effects of *A. sativum* are believed to be diverse and include among others anti-bacterial, anti-viral and anti-fungal action (Borrelli *et al.*, 2007:1386; Omar & Al-Wabel, 2010:51). Furthermore, evidence suggests that *A. sativum* has been used since ancient times for the treatment of worm infestations, earaches, flatulence, leprosy, severe diarrhoea, constipation, fever, respiratory infections, wounds and symptoms of aging (Amagase *et al.*, 2001:955S; Omar & Al-Wabel, 2010:51).

An effect of *A. sativum* that may prove clinically significant is its ability to activate additional mechanisms to eliminate exogenous substances such as carcinogens (Dausch & Nixon, 1990:350). Possibly one of the most significant effects of *A. sativum* and its associated sulphur phytoconstituents are the ability to decrease the risk for cancer and modify the biological activity of existing tumours. A recent investigation by Omar & Al-wabel (2010) has demonstrated a decreased incidence of breast, skin, colon, uterine, oesophageal and pulmonary tumours associated with the use of *A. sativum* (Omar & Al-wabel, 2010:52).

Although *A. sativum* is generally regarded as a safe herbal additive, several adverse effects have been documented. During clinical trials, numerous complaints were filed by the participants including halitosis and body odour as well as flatulence, nausea and bloating (Borrelli *et al.*, 2007:1394). Furthermore, the topical application of *A. sativum* may cause contact dermatitis by means of both primary irritation and local allergic reactions. Other adverse effects that were reported, but not yet statistically demonstrated, include a higher risk for bleeding, oesophageal and abdominal pain, Ménière's disease and myocardial infarction (Borrelli *et al.*, 2007:1394).

3.2.4 Interactions of *Allium sativum* with allopathic drugs

Some constituents in *A. sativum* can cause inhibition and/or promotion of both P-gp mediated efflux and CYP-mediated metabolism. A number of herb-drug interactions are documented that involve efflux inhibition amongst other mechanisms. Although to a lesser extent than the known P-gp inhibitor, verapamil, an *in vitro* study using recombinant human P-gp membranes and a colometric ATPase assay, demonstrated that crushed *A. sativum* and pure *A. sativum* constituents inhibit P-gp activity (Zhou *et al.*, 2004:63).

3.2.4.1 Cardiovascular drugs

Recently, several clinical studies indicated that the concomitant administration of *A. sativum* and allopathic drugs can result in clinically significant pharmacokinetic and pharmacodynamic interactions, especially if combined with anti-coagulants (e.g. warfarin) (Fugh-Berman, 2000:135). *A. sativum* has been demonstrated to inhibit platelet aggregation (Vaes & Chyka, 2000:1479) while a number of case reports also indicated that the use of *A. sativum* is associated with an increased risk of excessive post-operative bleeding and spontaneous spinal epidural haematoma (Petry, 1995:483). Therefore, it is advised that *A. sativum* administration, whether for medicinal purposes or as food additive, should be withdrawn 4 to 8 weeks before surgery or in patients who chronically receive warfarin (Hu *et al.*, 2005:1244).

3.2.4.2 Protease inhibitors

Almost all the ARV drugs are eliminated by intestinal and hepatic CYP3A4 enzymes and function as substrates for P-gp. In an investigation by Hu *et al.* (2005:1243), *A. sativum* caplets (each containing 3.6 mg powdered *A. sativum* extract) were co-administered with the PI, saquinavir (at therapeutic dosage) to ten healthy volunteers, while ten different patients only received saquinavir (control group). The co-administration of saquinavir with *A. sativum* resulted in an average decrease of 51% in the saquinavir plasma area under the plasma concentration-time curve (AUC) after 8 hours compared to the saquinavir plasma AUC of the control group. The authors explained that the decreased bioavailability of saquinavir (a substrate for P-gp) in the group receiving both *A. sativum* and saquinavir, could be the result of *A. sativum* induced P-gp promotion resulting in an increase in saquinavir efflux (Hu *et al.*, 2005:1243).

In another study (Chavez *et al.*, 2006:2148), 1200 mg saquinavir was administered to ten healthy patients three times daily on days 1 to 4, 22 to 25 and 36 to 39 after meals (where day 1 is accepted to be the first day of drug administration). On days 5 to 25 patients were given two *A. sativum* capsules containing 4.64 mg allicin and 11.2 mg alliin twice daily. Another ten patients received only saquinavir and sufficed as the control group. Mean saquinavir AUC, mean C_{max} and 8-hour plasma levels declined on average with 51%, 54% and 49% respectively in the patients receiving both the *A. sativum* constituents and saquinavir compared to the control group. However, after a ten day *A. sativum* washout period, the saquinavir AUC, C_{max} and 8-hour plasma levels in this group returned to 65%, 61% and 71% respectively, of the values measured in the control group. In correspondence with the data of Hu *et al.* (2005), this also suggests a possible promotion of CYP3A4 metabolism and/or promotion of P-gp mediated efflux of saquinavir. It is advisable that

patients taking PIs should therefore be cautious with the concomitant administration of *A. sativum* (Chavez *et al.*, 2006:2148).

A recent study established that raw garlic and garlic products inhibited the activities of P-gp *in vitro*, although inhibition was low when compared to the known P-gp inhibitor verapamil (Hu *et al.*, 2005:1243). This creates controversy with previously mentioned studies and stresses the importance for further studies on the effect of *A. sativum* and its compounds on the metabolising enzymes CYP4A3 and efflux transporter P-gp (Foster *et al.*, 2001:176).

3.2.4.3 Analgesic drugs

Recent studies performed in mice indicated that *A. Sativum* may act as a hepatoprotectant (Hu *et al.*, 2005:1245). This has been demonstrated by a reduction in the plasma concentration of oxidative, but not non-oxidative, paracetamol metabolites when administered simultaneously with diallyl sulfone constituents derived from *A. sativum*. The authors assume the mechanism for this interaction to be based on the inhibition of CYP2E1, the predominant enzyme accountable for the bioactivation of paracetamol. In another study using liver microsomes, the oxidation of paracetamol to its toxic metabolite, *N*-acetyl-*p*-benzoquinone imine, was drastically inhibited by diallyl sulfone. These findings could possibly explain the hepatoprotective effect of diallyl sulfone. In fact, it has been demonstrated that administering diallyl sulfone at a dose of 25 mg/kg within 20 minutes following exposure to toxic paracetamol concentrations, provides adequate protection against hepatic damage (Hu *et al.*, 2005:1245).

3.3 Lemon (*Citrus limonum*)

3.3.1 Botany of *Citrus limonum*

The essential oil derived from *C. limonum* is commonly used as flavouring agent in food, a fragrance in the manufacturing of perfume as well as for a number of pharmaceutical applications (Lüker *et al.*, 2002:3160). The oil of *C. limonum* is extracted from the peel or the flavedo of the fruit which contains numerous vesicles filled with oil (Figure 3.3) (Lüker *et al.*, 2002:3160).



Figure 3.3: Illustration of the *Citrus limonum* plant including leaves, flowers and fruit (ABC Local, 2012)

3.3.2 Chemical composition of *Citrus limonum*

Flavonoids, occurring as glycosides in most fruit, are an abundant group of polyphenolic compounds with a low molecular weight and represent one of the most significant classes of biologically active compounds. They are structurally similar to their parent compound flavones (2-phenyl chromone or 2-phenyl benzopyrone) and are appropriately represented by a $C_6-C_3-C_6$ carbon frame configuration (with the C_6 components being aromatic rings) (Garg *et al.*, 2001:655).

C. limonum, or the lemon fruit, is a rich source of nutrients such as flavonoids, citric acid, vitamin C and minerals (Gil-Izquierdo *et al.*, 2004:324). Although aldehydes, such as citral, are minor components present in the essential oil of *C. limonum*, they contribute more to the characteristic lemon flavour than do the main compounds, which are mostly olefinic monoterpenes (Lüker *et al.*, 2002:3160). Monoterpenes are ten-carbon (C_{10}) molecules and consist of two head-to-tail coupled isoprene units (each five-carbons; C_5). Most secondary metabolites do not play a significant role in the viability and survival of the species they are found in, but are beneficial for plants as they provide natural protection against herbivores and pathogens or serve as attraction for pollinators (Lüker *et al.*, 2002:3160). *C. limonum* juice is well known for its high flavonoid content, particularly the flavanone and flavone type glycosides (Gil-Izquierdo *et al.*, 2004:324). Hesperidin and eriocitrin (flavonoids), as well as

small amounts of diosmetin 6,8-di-C-glucoside and diosmin are the predominant bioflavonoids present in *C. limonum* juice (González-Molina *et al.*, 2009:353).

Hesperidin is a flavanone glycoside coupled to the disaccharide rutinose and when in the form of an aglycone, is called hesperitin (Garg *et al.*, 2001:656). Rutinose itself (C₁₂H₂₂O₁₀) is composed of one rhamnose and one glucose molecule. After oral ingestion, hesperidin is transformed in the intestine to hesperitin by alpha-rhamnosidase and beta-glucosidase producing bacteria (Garg *et al.*, 2001:656).

3.3.3 Medicinal applications of *Citrus limonum*

A balanced diet became an important factor in living a healthy life in modern society. It is a well known fact that fruits and vegetables contribute to a well-balanced diet, especially regarding the role they play in disease prevention (González-Molina *et al.*, 2009:353). Ascorbic acid (vitamin C) is present in citrus fruit (abundantly in *C. limonum*), and while it is believed to be the most valuable health-promoting property in fruit, recent studies demonstrated that the flavonoids in fruit may also contribute to the disease-preventing properties (Okwu & Emenike, 2006:1).

Furthermore, bruising caused by capillary fragility were originally believed to be due to a deficiency in ascorbic acid, while early studies also demonstrated that crude, but not purified, supplements of ascorbic acid (or what was believed to be ascorbic acid) relieved this type of tissue damage (Garg *et al.*, 2001:655). However, later investigations suggested that the bioflavonoids, previously termed 'vitamin P', are responsible for curing the bruises and improving the permeability and integrity of the capillary lining (Garg *et al.*, 2001:655). A numerous number of diseases are associated with increased permeability of blood capillaries, including chronic venous insufficiency, diabetes mellitus type 2, and scurvy (Garg *et al.*, 2001:660). Furthermore, conditions such as oedema, excessive bleeding and hypertension most often have an ethiological basis in an increased capillary permeability (Garg *et al.*, 2001:660).

In another clinically relevant investigation, it has been demonstrated that hesperidin (in combination with diosmin), plays a protective role against inflammation, both *in vivo* and *in vitro*, possibly by inhibiting eicosanoid synthesis and/or acting as an anti-oxidant and free radical scavenger (Garg *et al.*, 2001:662). At least one *in vitro* study indicated that hesperidin and other flavonoids inhibit the growth of *Helicobacter pylori* (Garg *et al.*, 2001:662). In addition, hesperidin demonstrates anti-fungal activity against *Botrytis cinerea*, *Trichoderma glaucum* and *Aspergillus fumigates*, while anti-viral action was also reported against herpes simplex type 1, para-influenza 3, polio virus type 1 and respiratory syncytial

virus (Garg *et al.*, 2001:662). Case reports have indicated that hesperidin also may exhibit anti-allergic and anti-anaphylactic activity (Garg *et al.*, 2001:662).

Concerning the endocrinological system it was reported that hesperidin can play a role in the regulation of oestrogen levels and may reduce the pain, inflammation and abdominal swelling associated with the fluctuation in oestrogen levels (Garg *et al.*, 2001:664). In one clinical trial, 94 women experiencing hot flushes and other menopausal symptoms were given a combination of 900 mg hesperidin, 300 mg hesperidin methylchalcone and 1200 mg vitamin C daily. After one month of treatment 53% of the women did not experience hot flushes anymore and in 34% the symptoms decreased. A control group was not included in the study. A small number of adverse effects have been documented including a slight body odour and perspiration that caused minor stains on clothing (Garg *et al.*, 2001:664).

Diosmin also displays pharmacological activity and is therefore used as an adjunctive therapy in the treatment of diseases of the circulatory system. Because of its ability to improve muscular tone and vascular resistance to inflammation, diosmin is used in the treatment of diseases such as chronic venous insufficiency and rheumatic arthritis (Del Río *et al.*, 2004:457).

3.3.4 Interactions of *Citrus limonum* with allopathic drugs

Recent studies aimed to investigate whether the bioflavonoids in grapefruit would influence the permeation of vincristine across the blood-brain barrier (Mitsunaga *et al.*, 2000:193). Indeed, the results demonstrated that the hesperidin in grapefruit increased the central nervous system uptake of vincristine compared to the control. The increase in the uptake of vincristine is claimed to be the result of hesperidin-induced inhibition of P-gp mediated drug efflux (Garg *et al.*, 2001:658).

3.4 Beetroot (*Beta vulgaris*)

3.4.1 Botany of *Beta vulgaris*

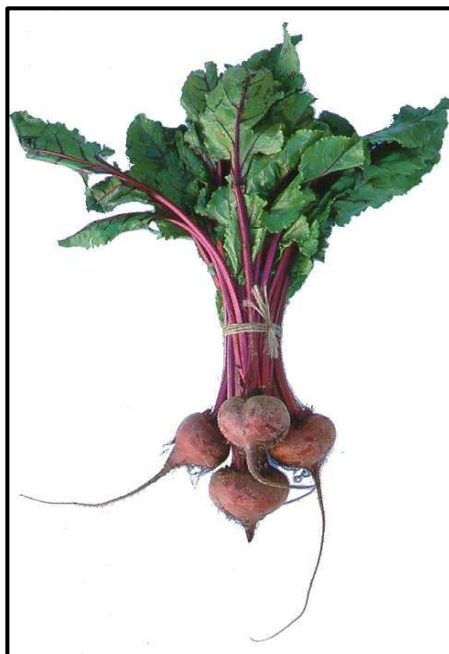


Figure 3.4: Illustration of the *Beta vulgaris* plant (Robertson, 2012)

3.4.2 Chemical composition of *Beta vulgaris*

Beta vulgaris contains high amounts of carotenoids, glycine betaine (*N,N,N*-trimethylglycine), saponins, betacyanines, folates, betanin (the main colouring agent in *B. vulgaris*), polyphenols and flavanoids (Figiel, 2010:461). Carotenoids is a collection of natural pigments responsible for the colour of fruits and vegetables varying from yellow to red. The main carotenoids present in *B. vulgaris* include β -carotene, lutein and zeaxanthin (Dias *et al.*, 2009:813).

Glycine betaine is a small zwitterionic compound produced by a number of organisms such as bacteria, invertebrates, plants and mammals. In humans, glycine betaine can be absorbed after dietary intake or it can be synthesised in the liver from choline (De Zwart *et al.*, 2003:198). Folates are of great nutritional importance as they can only be absorbed from the diet, while they display biological properties similar to that of folic acid and typically occur in the form of poly-glutamate molecules (Jastrebova *et al.*, 2003:579).

3.4.3 Medicinal applications of *Beta vulgaris*

The proposed medicinal effects of *B. vulgaris* are attributed to its bioactive components e.g. betaine, betanin, folic acid, biotin, pectin, and the betaxanthins, flavonoids, polyphenols and vitamins (e.g. thiamine, riboflavin, pyridoxine and ascorbic acid). Primarily, betanin, the

flavonoids (especially quercetin) and other polyphenols demonstrate anti-oxidant effects (Váli *et al.*, 2007:173).

In addition to its anti-oxidant effects, the polyphenols in *B. vulgaris* display anti-inflammatory and anti-viral properties. They may prevent liver injury associated with high concentrations of lipid peroxide and pro-inflammatory nitric intermediaries following excessive oxidative reactions (Váli *et al.*, 2007:176).

The carotenoids on the other hand, have an important role to play in human health as they act as regulators of cell differentiation, proliferation and communication and modulate numerous functions of the immune system and tumour metabolism (Dias *et al.*, 2009:808). Several studies have demonstrated a strong relationship between the use of carotenoids and a viable immune system as well as a decrease in the risk for cancer, cardiovascular disease, cataracts and muscular degeneration (Dias *et al.*, 2009:808). Minor adverse effects resulting from the use of *B. vulgaris* include, allergic skin reactions a pink colouration of urine, which was established in approximately 10% to 14% of the cases (Ulbricht *et al.*, 2008:1065).

According to the American Academy of Paediatrics, infants younger than three months of age should avoid the intake of *B. vulgaris* because of the high nitrate content and the risk of nitrate poisoning (Ulbricht *et al.*, 2008:1065). However, breastfed infants of mothers who ingest *B. vulgaris* are not at risk of nitrate poisoning, as the amount of nitrates secreted in breast milk, is negligible (Ulbricht *et al.*, 2008:1065).

3.4.4 Interactions of *Beta vulgaris* with allopathic drugs

At least one clinical study demonstrates that the administration of *B. vulgaris* fibre decreases gastro-intestinal transit time (Ulbricht *et al.*, 2008:1065). However, fibre in general has the same effect and it has not yet been elucidated whether *B. vulgaris* fibre plays a more dramatic role than other fibre in the regulation of gastro-intestinal motility. A relative increase in the secretion of motilin, a prokinetic gastro-intestinal hormone usually only secreted during fasting periods, was observed after meals in obese patients with type 2 diabetes mellitus who also took a *B. vulgaris* fibre supplement (Ulbricht *et al.*, 2008:1065). Thus the intake of *B. vulgaris* fibre may increase the transit time. Increased or decreased transit time may potentially affect the absorption and bioavailability of various orally administered agents (Ulbricht *et al.*, 2008:1065).

3.5 Conclusion

There is a global rise in the use of natural and herbal products in combination with allopathic medicines, while most patients do not inform their health care providers of the use of these natural products (Ingersoll, 2005:434). Many herbs display pharmacological properties and thus it is of utmost importance to study the possible interactions between herbs and conventional drugs. The modulation and expression of P-gp by herbal constituents may result in altered absorption and bioavailability of drugs that are P-gp substrates. Increasing evidence from *in vitro* and *in vivo* studies indicate that the distorted drug concentrations observed after the concomitant administration of herbs may be attributed to a change in the activity of drug transporters such as P-gp (Ulbricht *et al.*, 2008:1063).

With a South African ART roll-out program that drastically increased in quantity during the past five years (Tarirai *et al.*, 2010:1515), it is a logical assumption that these additives are used concomitantly with allopathic ART. Many HIV-related conditions such as weakness due to an affected immune system, insomnia, depression, nausea and dermatological disorders are also treated with herbal medicines by traditional healers and may result in clinically relevant drug interactions (Tarirai *et al.*, 2010:1515).

CHAPTER 4

4 EXPERIMENTAL METHODS

4.1 Introduction

Absorption of drug molecules from the gastro-intestinal tract is a complex process. Passive diffusion accounts for most of the drug absorption processes and follows one of either two pathways. The transcellular route applies when drug molecules pass through the cell membranes of enterocytes, while transport through tight junctions and intercellular spaces are known as the paracellular route (Balimane *et al.*, 2006:E1). *In vitro* transport studies across excised intestinal sheets from mammals are widely used to study and predict drug absorption. This method includes the isolation of intestinal tissue, dissecting it into strips, clamping it on a suitable diffusion apparatus and measuring the drug transport across the tissue in a controlled environment (Balimane *et al.*, 2000:305).

Various *in vitro* methods have been used for the assessment of the intestinal permeability of drug candidates (Balimane *et al.*, 2000:305). The successful application of *in vitro* models to predict drug absorption across the intestinal mucosa depends on how effective the *in vitro* model mimics the *in vivo* intestinal epithelium (Balimane *et al.*, 2000:305). The main application of *in vitro* experimental techniques is the measurement of the interaction of drug molecules with the isolated tissue (Grass, 1997:204). A number of *in vitro* intestinal tissue models such as Caco-2 cell monolayers and excised intestinal tissue models using intestinal sheets obtained from rats, rabbits, monkeys, dogs and porcines have been employed to investigate the permeability of drugs (Tarirai *et al.*, 2012:255).

The present study made use of the Sweetana-Grass diffusion apparatus to determine the bi-directional *in vitro* transport of indinavir, a protease inhibitor, in the presence of two pure phytochemical compounds and crude extracts of *A. sativum*, *C. limonum* and *B. vulgaris* respectively, across excised porcine intestinal tissue. Pigs and humans share comparative anatomic and physiologic characteristics, ensuring that porcine tissue has been used as an appropriate animal model during the development of biomedical research models during the last two decades (Swindle & Smith, 1998:11). In a comparative transport study between Caco-2 cell monolayers and porcine jejunum tissue, Tarirai *et al.* (2012:262) concluded that the Caco-2 cells were in general more sensitive to the crude extracts than the excised porcine jejunum tissue. This sensitivity of Caco-2 cell monolayers may cause overestimation of the effect of co-administrated plant extracts during transport studies. Therefore, the conclusion was made that the excised porcine jejunum model gives a more realistic estimation of the herb-drug interactions than the Caco-2 model (Tarirai *et al.*, 2012:262).

4.2 Materials

The cloves of *A. sativum* L. (Liliaceae), fruits of *C. limonum* L. (Rutaceae), and bulbs of *B. vulgaris* (Chenopodiaceae) were purchased from a local supplier in South Africa. The plants were identified as such by the A.P. Goossens Herbarium, North-West University, South Africa and voucher specimens (*A. sativum* PUC 11432, *C. limonum* PUC 11157 and *B. vulgaris* PUC 11214) of the respective plants were stored on location at the herbarium (PUC). Indinavir (batch number 0042180) has been donated generously by Merck®, USA. Krebs-Ringer bicarbonate (KRB) buffer (product no. K4002), L-Aliin (product no. 74264), hesperidin (product no. H5254), eriocitrin (product no. 45714), β -carotene (product no. 22040) and betaine monohydrate (product no. 14300) were purchased from Sigma-Aldrich®, Kempton Park, South Africa. Diallyl disulphide (product no. 293320250) was purchased from Acros Organics®, New Jersey, USA (see Annexure C for certificates of analysis).

4.3 Preparation and characterisation of crude plant extracts

The method described here is an adaptation of the methodology followed by Tarirai *et al.* (2012:255). The cloves of *A. sativum* (98 g) and bulbs of *B. vulgaris* (465 g) were cut into smaller pieces and liquidised in 200 ml and 800 ml distilled water respectively, using a food blender. The fruit pulp of *C. limonum* (700 ml) was liquidised in a food blender together with 200 ml distilled water. For the preparation of the extracts, the liquidised plant material was stirred on a magnetic stirrer for 2 h, then ultra-sonificated (Model LLMC 5, Integral Systems) for 30 min and subsequently ultra-centrifuged (Beckman-Coulter® Optima® L-100XP, Indiana, USA) at $150\,000 \times g$ (*A. sativum*), $50\,000 \times g$ (*C. limonum*) and $100\,000 \times g$ (*B. vulgaris*) for 10 min. Following ultra-centrifugation, the supernatant from each plant extract was frozen at $-80\text{ }^{\circ}\text{C}$ and freeze-dried (Virtis®, New York, USA). The respective freeze-dried powders were micronized with a mortar and pestle, labelled and stored in a moisture-free, air-tight desiccator until used in following experiments.

The following tests were performed with the crude plant extracts:

4.3.1 Loss on drying test

The loss on drying test is a method used to determine the loss in mass of the sample when dried under specific conditions. This method is used to determine the amount of water (including water of crystallisation) or volatile matter in the sample which should be removed during drying (JP XIV, General tests: 51). The Shimadzu® DTG-60 differential thermal gravimetric (DTG) analyser is also a differential thermogravimetric analyser (TGA) which combines a heat-flux type DTG with a *Roberval* type TGA which measures changes in both the physical state and the weight of sample as a function of temperature over time. In this

process, both the reference standard and the sample are heated at the same rate. The temperatures of each side are compared and the difference in output is documented ($T_s - T_r = DT$, where T_s is the temperature of the sample, T_r is temperature of the reference and DT is the relative change in heat [μV]). As a rule, the sample will either absorb or radiate heat. As the sample weight changes, it is being read by the *Roberval* balance. The changes in weight are then recorded in mg (Greenbeck). These tests were done to confirm that the extracted powders of *A. sativum*, *C. limonum* and *B. vulgaris* were indeed dry after they were subjected to freeze drying.

4.3.2 Chemical fingerprinting of crude plant extracts and pure phytoconstituents

The chromatographic profiles of the freeze dried crude extracts (see section 3.3) of *A. sativum*, *B. vulgaris* and *C. limonum* and pure phytoconstituents (L-alliin, diallyl disulphide, β -carotene, betaine monohydrate, hesperidin and eriocitrin) were obtained by means of HPLC.

In order to obtain chemical fingerprinting chromatographic profiles of the extracts, 10 mg of each plant extract was dissolved in 500 μl of ethanol after which the solution was made up to a volume of 10 ml with distilled water. For the pure phytoconstituents, 1 mg of each compound was used and prepared in the same way. The samples were injected into the HPLC at a rate of 1 ml/min and the injection volume was 50 μl . The conditions for this HPLC analysis are summarised in Table 4.1.

Table 4.1: Chromatographic conditions for chemical fingerprinting of plant extracts and phytochemical compounds

Apparatus and conditions	Information
Column	Column #63, Venusil XBP C18(2), 5 μ m, 100Å, 4.6 x 150mm Cat no: VX951505-2 SIN: V9510515BK 0016K
Mobile phase	Acetonitrile containing 0.2% triethylamine, pH 7.45/55
Sample volume injection	50 μ l
Detection wavelength	210.4 nm
Solvents for sample preparation	Distilled water and ethanol

4.4 Validation of analytical method

4.4.1 Specificity

Specificity is the ability to clearly assess the analyte in the presence of components which are also expected to be present (ICH, 1996:4). All possible components that may be present were individually analysed to ensure there would be no interference with the key compound, indinavir. A placebo sample (negative control) was prepared by filling a HPLC vial with KRB buffer (used as solvent during this study). A positive control sample of indinavir was constituted by dissolving 16.42 mg of indinavir in 200 ml of fresh distilled water to obtain a final concentration of 82.1 μ g/ml. This sample was diluted to a ratio of 1:1 with distilled water, 0.1M hydrochloric acid, 0.1M sodium hydroxide and 10% hydrogen peroxide respectively. These solutions were stored overnight in closed test tubes. The nine test compounds were also prepared by dissolving 1 mg of each compound in 10 ml distilled water. The samples were injected into the HPLC hardware and the respective chromatographs were recorded (Figures 4.1 to 4.14). From these chromatograms it is clear that none of the components interfered with the peak area of indinavir.

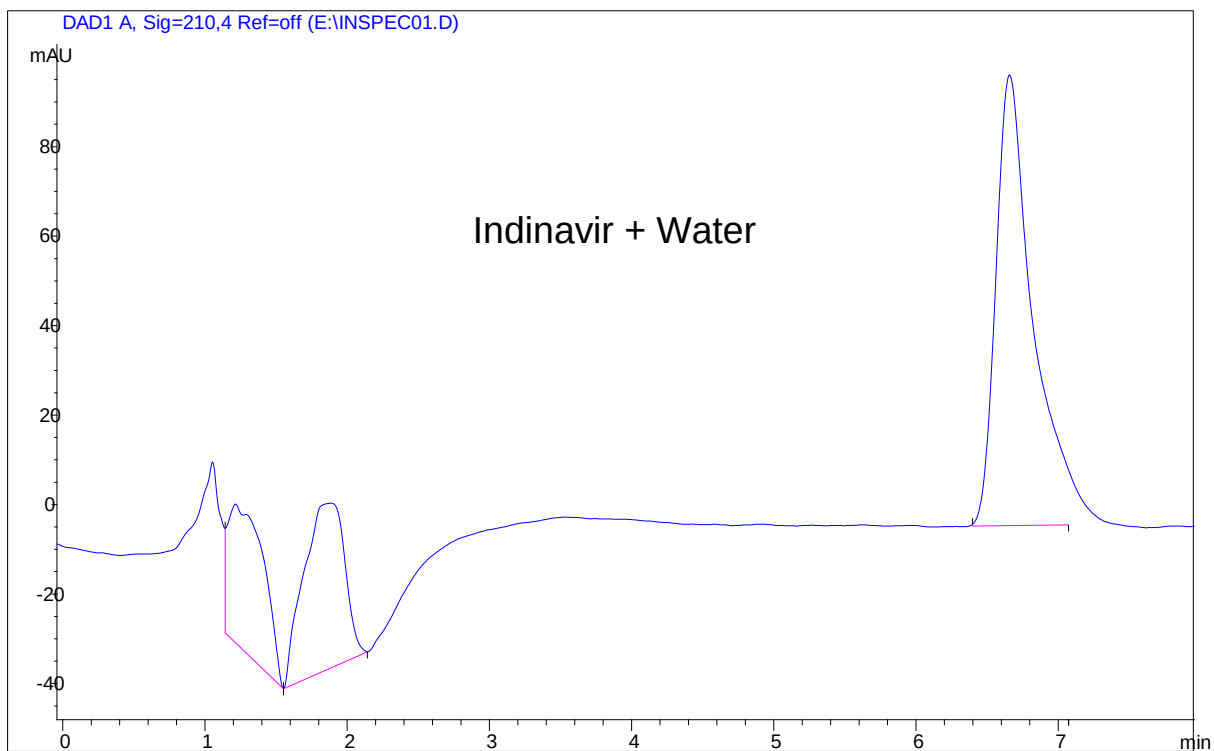


Figure 4.1: Chromatogram of indinavir stressed in water for 24 hours

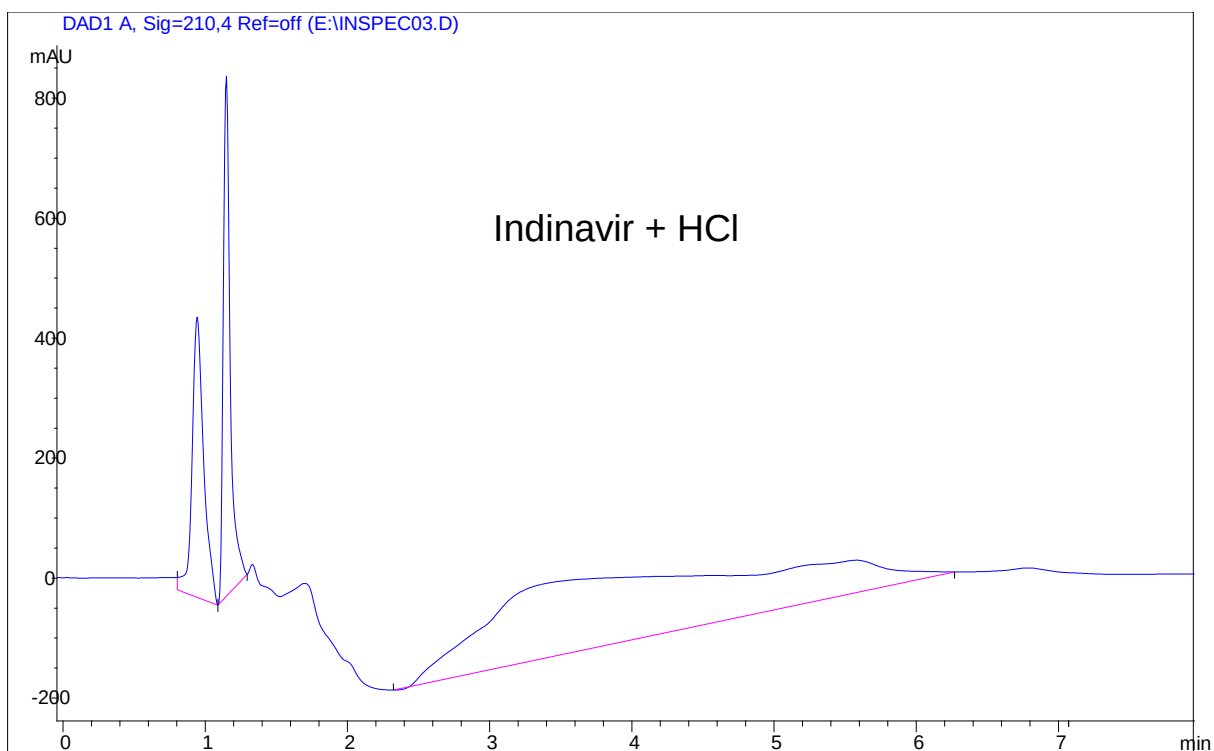


Figure 4.2: Chromatogram of a sample solution stressed in 0.1 M hydrochloric acid for 24 hours

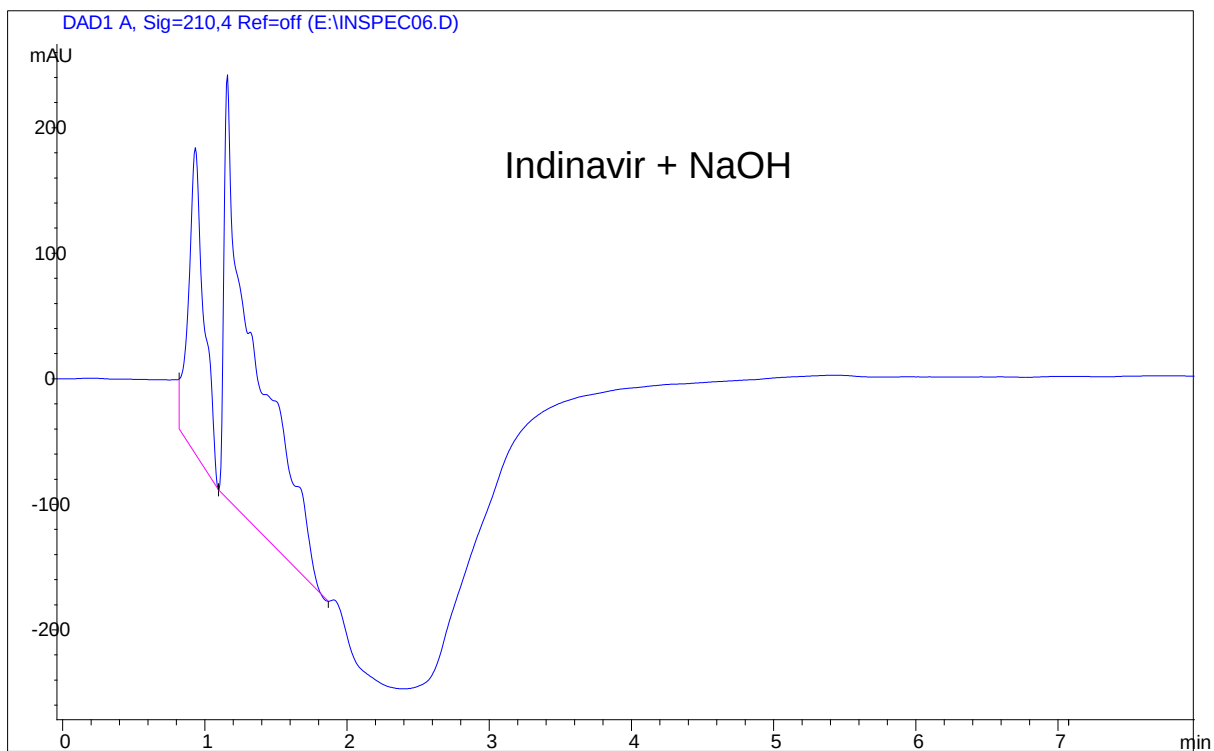


Figure 4.3: Chromatogram of a sample solution stressed in 0.1 M sodium hydroxide for 24 hours

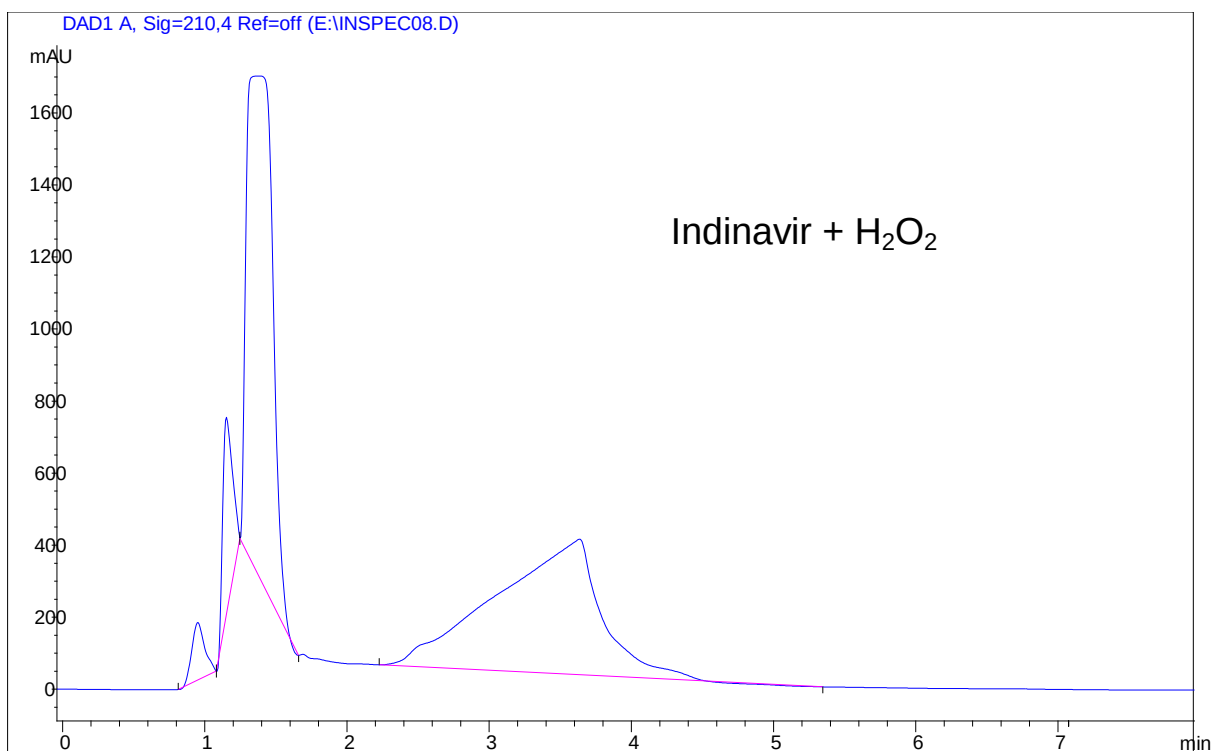


Figure 4.4: Chromatogram of a sample solution stressed in 0.1 M hydrogen peroxide for 24 hours

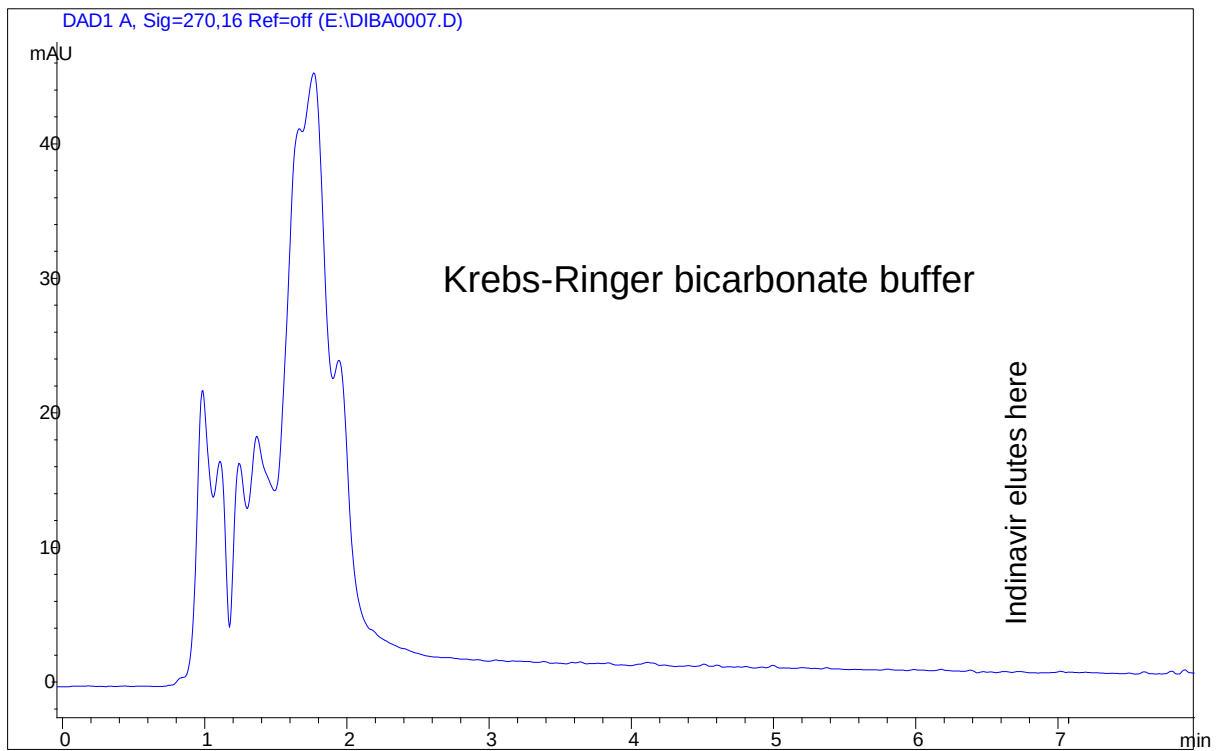


Figure 4.5: Chromatogram of a placebo with Krebs-Ringer bicarbonate buffer

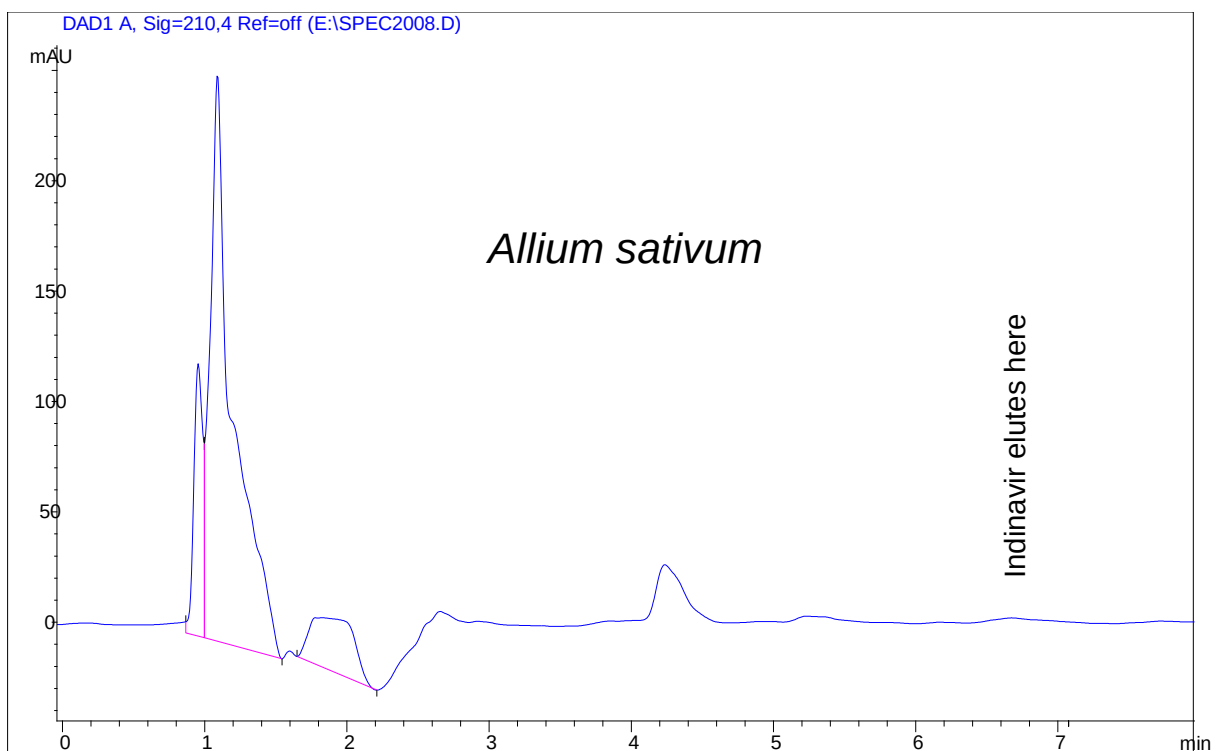


Figure 4.6: Chromatogram of *Allium sativum* crude extract

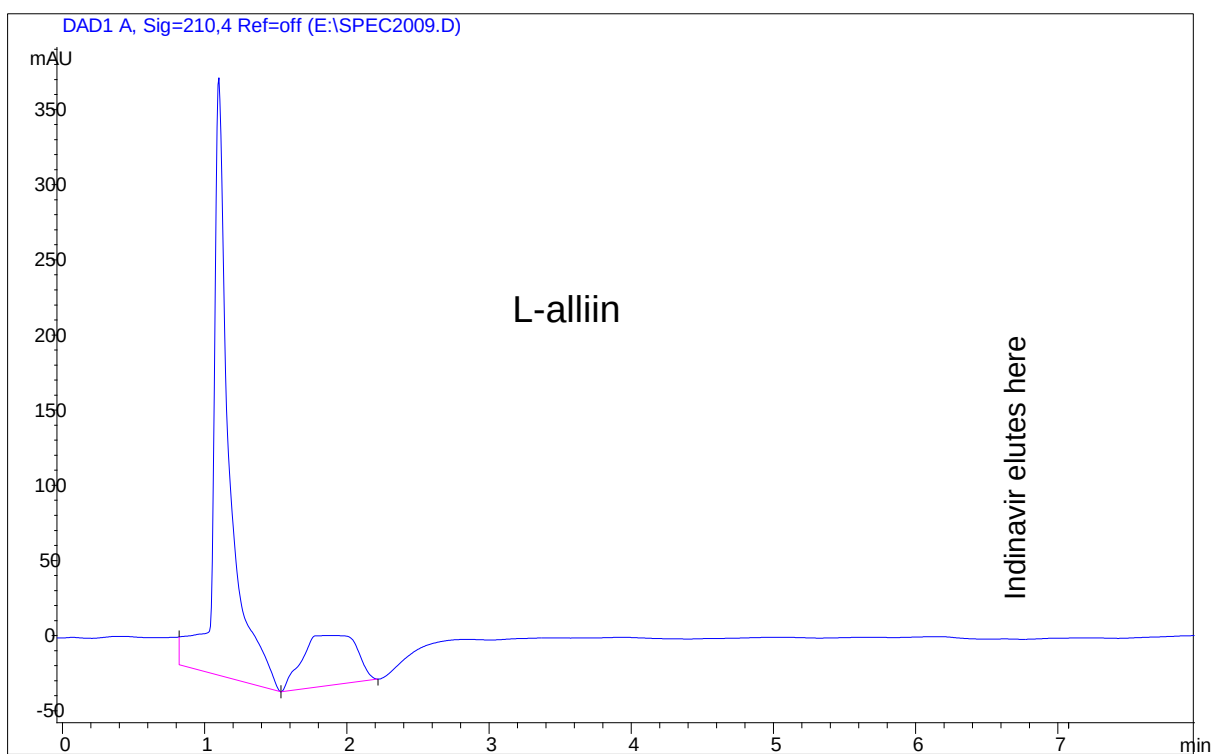


Figure 4.7: Chromatogram of L-alliin

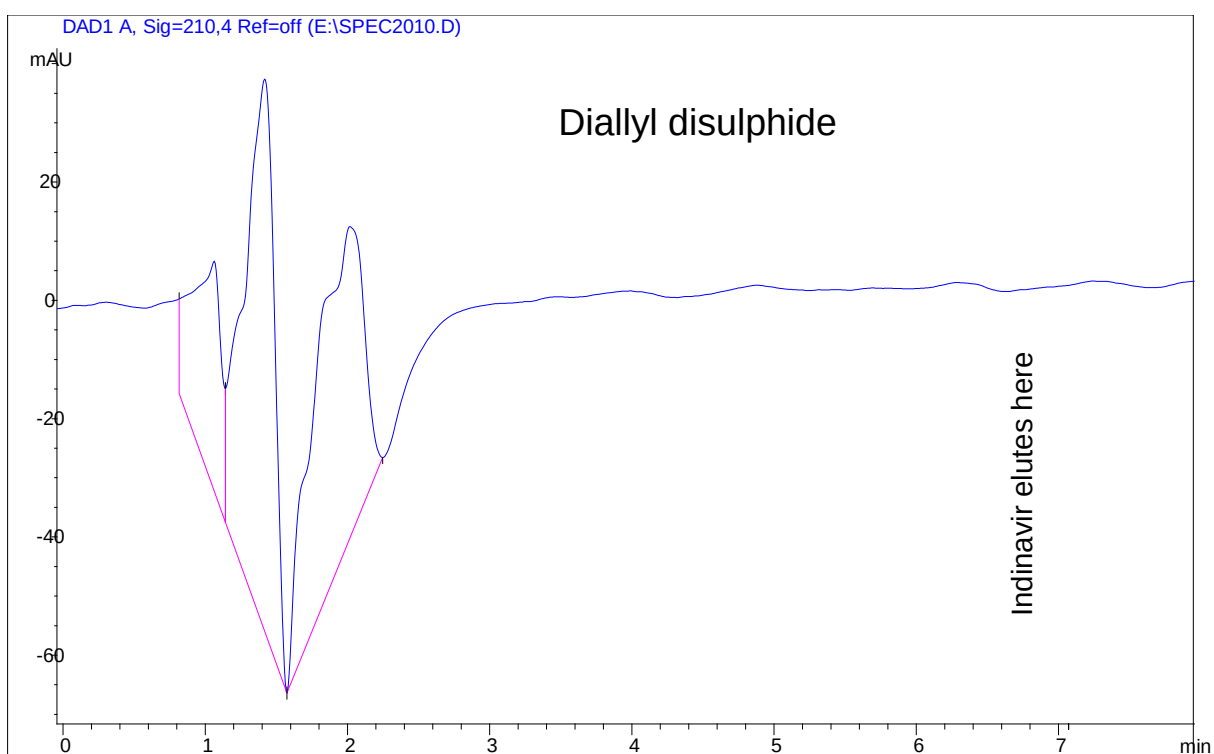


Figure 4.8: Chromatogram of diallyl disulphide

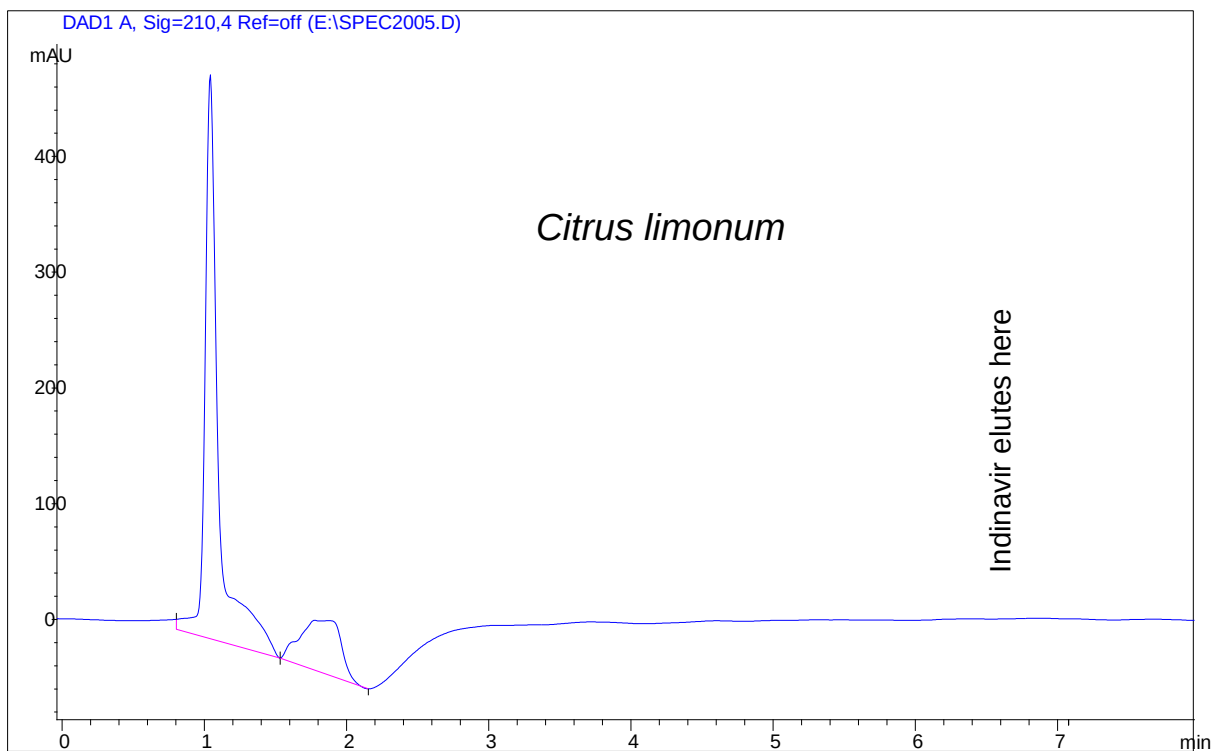


Figure 4.9: Chromatogram of *Citrus limonum* crude extract

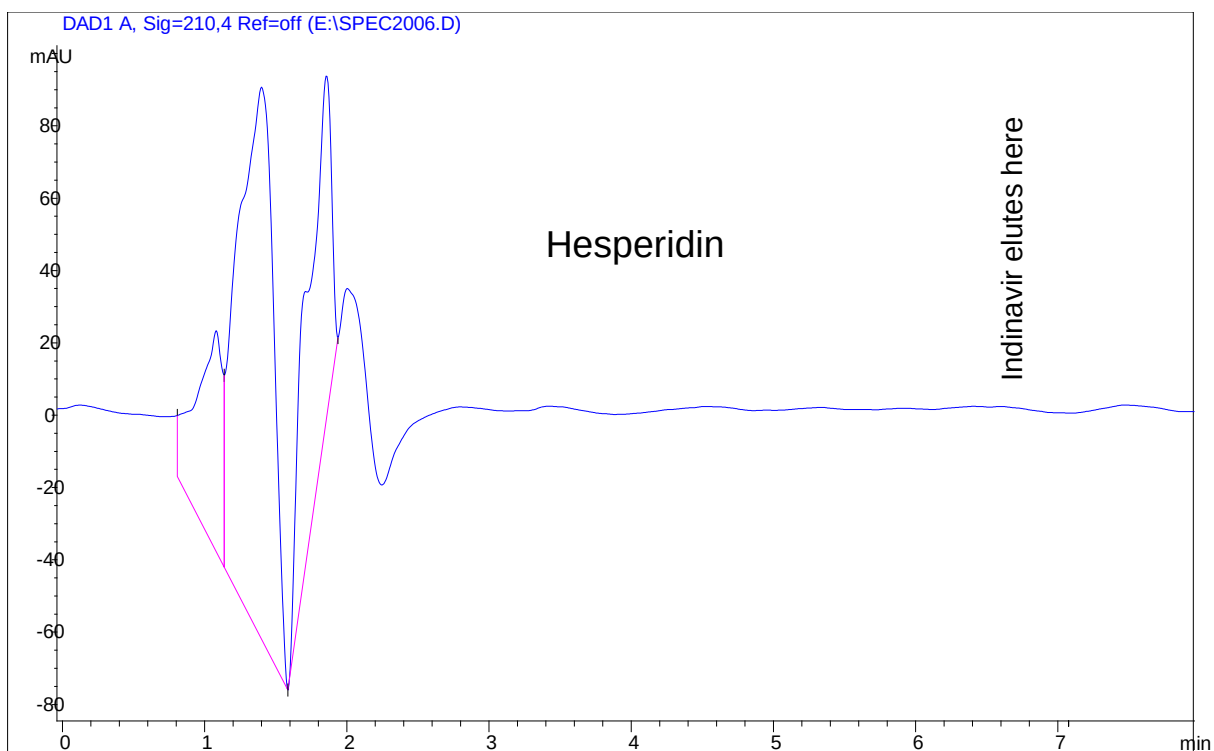


Figure 4.10: Chromatogram of hesperidin

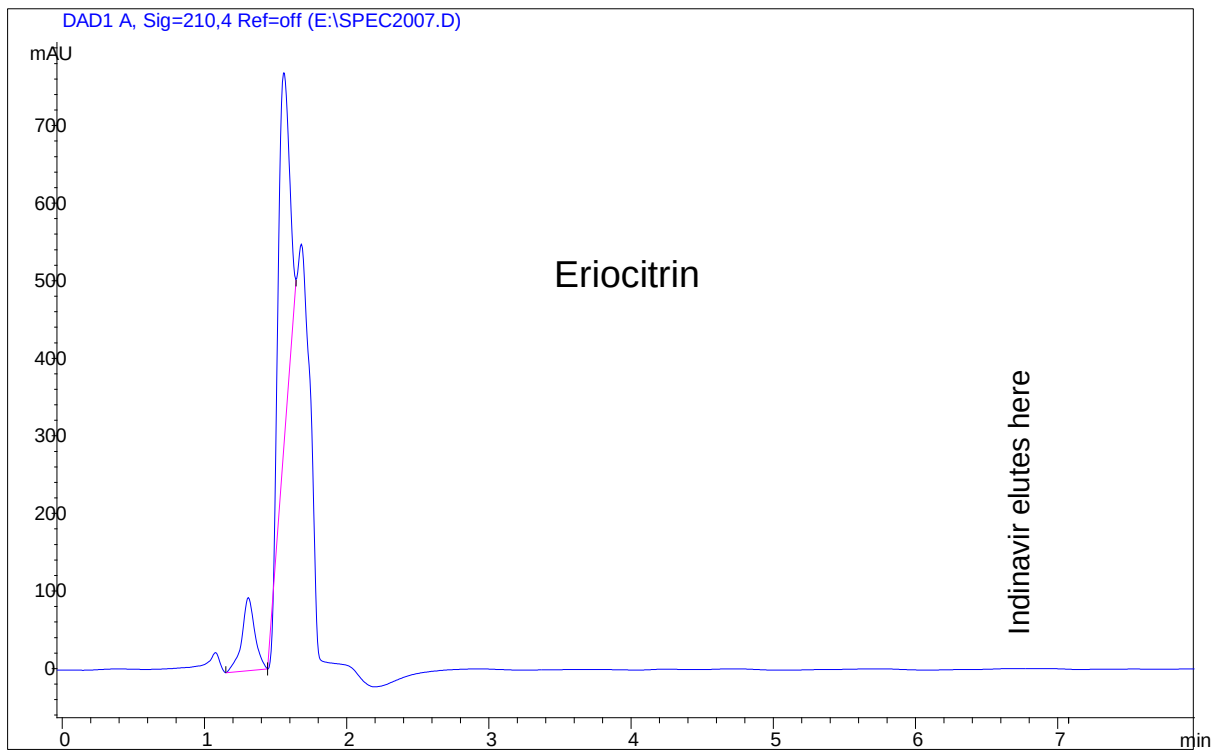


Figure 4.11: Chromatogram of eriocitrin

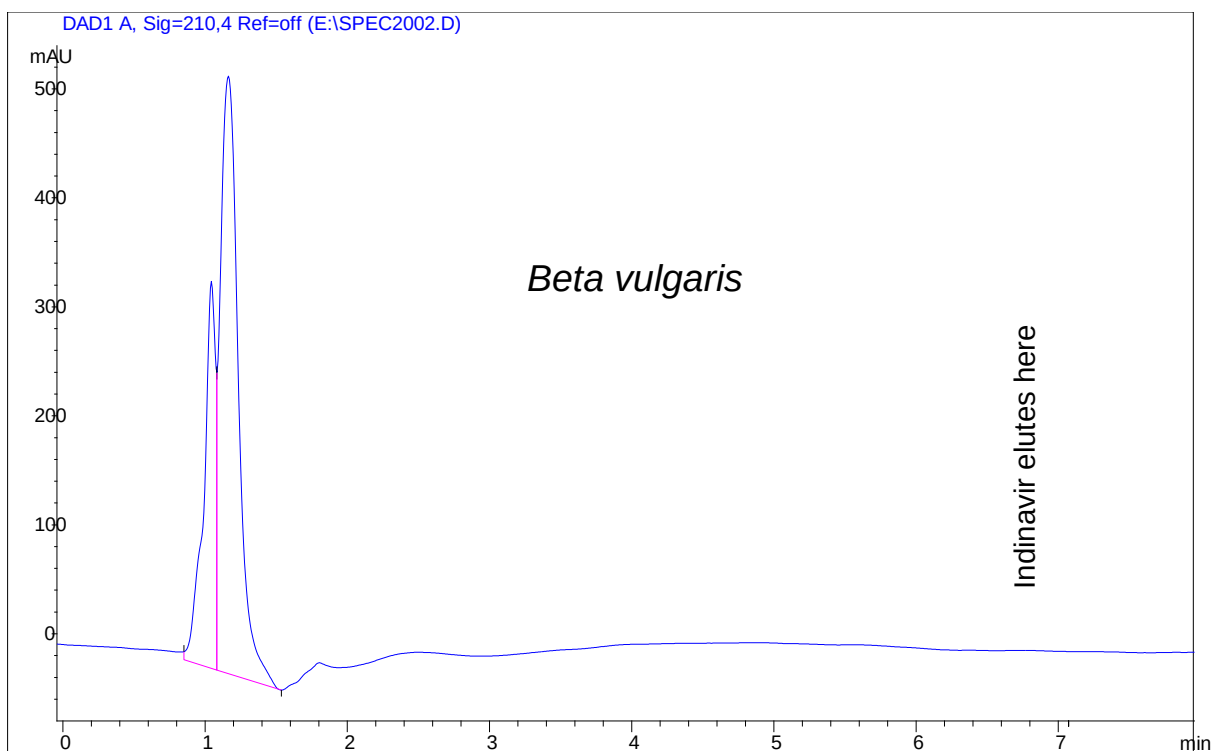


Figure 4.12: Chromatogram of *Beta vulgaris* crude extract

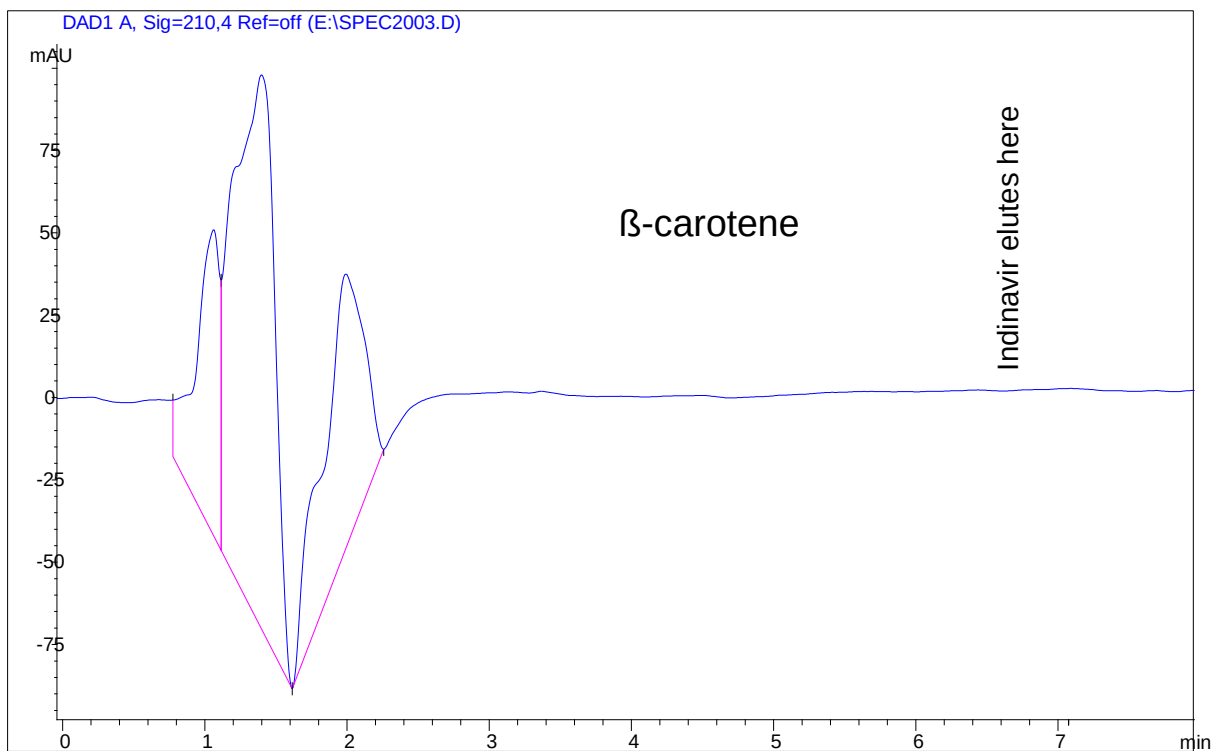


Figure 4.13: Chromatogram of β -carotene

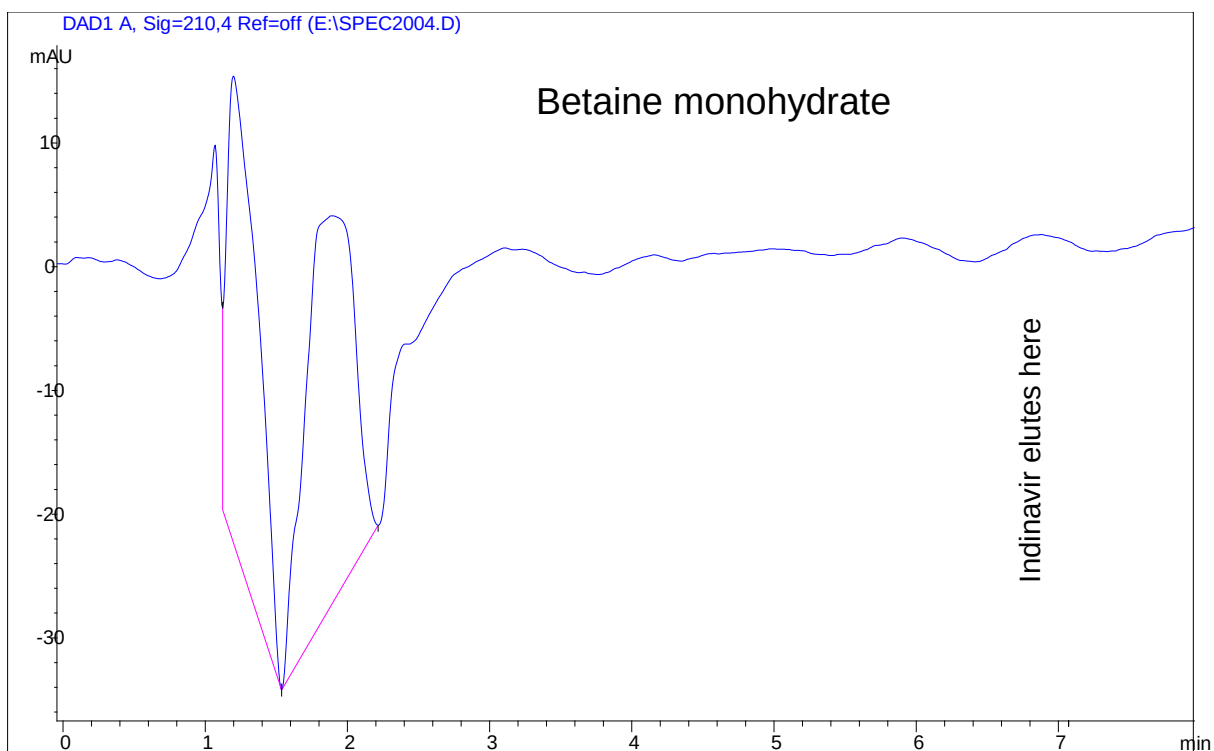


Figure 4.14: Chromatogram of betaine monohydrate

4.4.2 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample analysed (ICH, 1996:5). A concentrated standard solution was prepared by dissolving 1.74 mg of accurately weighed indinavir in distilled water with volume of 10 ml. This solution was diluted again by taking 2 ml and adding distilled water to obtain a final volume of 20 ml. Different volumes of these solutions (2.5 µl, 5 µl, 10µl, 15 µl, 20 µl and 25 µl) were withdrawn in duplicate and placed into the chromatograph. The linearity data and regression statistics for indinavir are summarised in Tables 4.2 and 4.3, while the linear regression graph for indinavir is illustrated in Figure 4.15.

Table 4.2: Data for linearity of indinavir

µg/ml	Area 1	Area 2	Mean
1.74	82.0	84.3	83.1
3.48	153.7	162.8	158.3
6.96	317.6	334.2	325.9
10.44	484.9	489.8	487.4
13.92	669.0	654.7	661.9
17.40	816.9	823.9	820.4
20.88	1006.6	960.7	983.7
17.40	820.7	809.6	815.2
34.80	1595.2	1607.5	1601.4
69.60	3149.5	3192.0	3170.8
104.40	4672.9	4762.5	4717.7
139.20	6248.0	6341.9	6295.0
174.00	7761.2	7780.4	7770.8
208.80	9342.5	9200.7	9271.6

Table 4.3: Regression statistics

R Squared	0.999988	Lower 95%	Upper 95%
Intercept	-29.4375	-62.9177	4.042721
Slope	55.93089	55.39163	56.47015

The results were examined and a realistic concentration range was determined. The realistic and measurable concentration range was plotted and linear regression was performed. Linear regression analysis should yield a regression coefficient (R squared) of ≥ 0.99 . This method displayed linearity over a concentration range of 1.74 to 208.8 $\mu\text{g/ml}$. Therefore, the method was declared suitable for single point calibration.

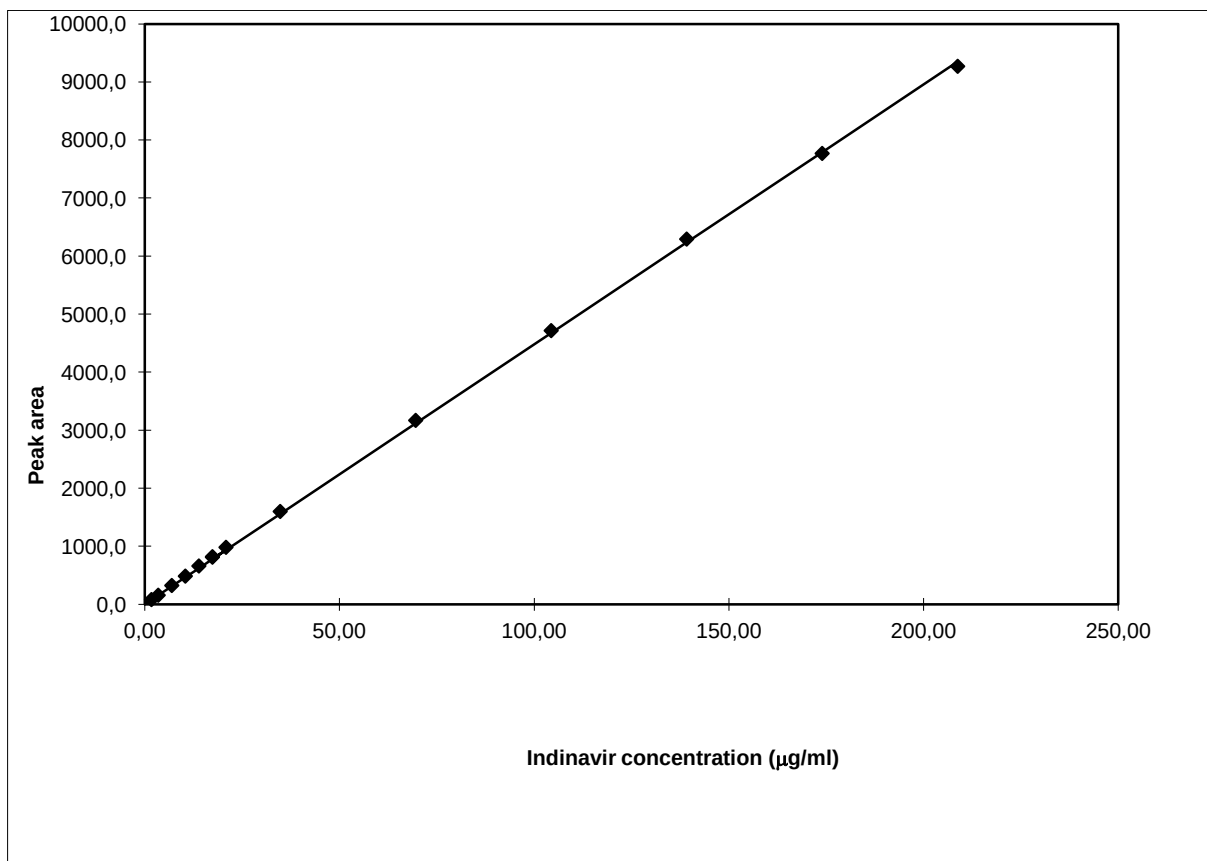


Figure 4.15: Linear regression graph for indinavir

4.4.3 Accuracy

The accuracy (also termed trueness) of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the experimental value obtained (ICH, 1996:4). The accuracy results are summarised in Tables 4.4 and 4.5.

Approximately 15.94 mg of indinavir was weighed and decanted into a 100 ml volumetric flask with distilled water and filled to volume. From this solution 10 ml was transferred into a 20 ml volumetric flask and made up to volume with distilled water. Thereafter, 10 ml of the latter solution was transferred into a 50 ml volumetric flask and filled to volume with distilled water. This yielded solutions contained 159.4, 79.7 and 15.9 $\mu\text{g/ml}$ indinavir respectively. These solutions were subsequently transferred into auto sampler vials and analysed.

Table 4.4: Data for accuracy of indinavir

Conc. spiked				Recovery	
µg/ml	Area 1	Area 2	Mean	µg/ml	%
15.9	804.4	845.5	825	16.1	100.7
15.9	826.2	820.2	823	16.0	100.5
15.9	821.4	803	812	15.8	99.1
79.7	4106.4	4101.7	4104	81.9	102.8
79.7	4113.9	4071.3	4093	81.7	102.5
79.7	4109.9	4097.2	4104	81.9	102.7
159.4	7863.6	7797.5	7831	156.7	98.3
159.4	8124	8003.2	8064	161.4	101.3
159.4	8126.6	8127.4	8127	162.7	102.0

Table 4.5: Accuracy statistical analysis

Statistical analysis	
Mean	101.1
Stdev (standard deviation)	1.5
RSD %	1.5
95% confidence intervals	
Lower limit	101.2
Upper limit	101.9
Estimated median	101.6
Confidence Level	0.3

Recovery must be between 98 to 102%. Over the range of 80 to 120% of the sample concentration, the method yielded a mean recovery of 101.1%.

4.4.4 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained following multiple sampling of the same homogeneous sample (ICH, 1996:4). Precision consists of two categories namely intra-day and inter-day precision.

4.4.4.1 Intra-day precision

Repeatability expresses the precision under the same operating conditions over a short interval of time (ICH, 1996:4). Since no sample preparation is performed during analysis, the data obtained for the accuracy experiment can also be used for precision. Intra-day precision thus gave a value of 1.5% for the relative standard deviation (RSD) which is better than the specified value of 2% ($n = 9$).

4.4.4.2 Inter-day precision

Inter-day precision was performed by analysing two replicates of three different indinavir concentrations on three consecutive days of which the data is summarised in Table 4.6. Three samples of the 79.9 $\mu\text{g/ml}$ concentration (prepared as described above for intra-day precision) were analysed on two more days to determine the between-day variability of the method. For day 1 the solution in a concentration of 79.7 $\mu\text{g/ml}$ was used. For the solution used on day 2, 16.38 mg of indinavir was dissolved in a 200 ml volumetric flask and made up to volume with distilled water to obtain a final concentration of 81 $\mu\text{g/ml}$. On day 3 a solution was prepared by dissolving 16.42 mg of indinavir in a 200 ml volumetric flask and made up to volume to render a concentration of 82.1 $\mu\text{g/ml}$. The one-way analyses of variance (ANOVA) single factor statistics and the ANOVA statistics are summarised in Tables 4.7 and 4.8.

Table 4.6: Data for inter-day precision for indinavir

	Day 1	Day 2	Day 3	Between days
	102.8	103.4	103.3	
	102.5	102.6	101.5	
	102.7	102.7	102.2	
Mean	102.65	102.93	102.34	102.64
Stdev	0.13	0.36	0.74	0.24
RSD %	0.13	0.35	0.73	0.23

Table 4.7: ANOVA single factor statistics

SUMMARY				
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Day 1	3	307.955	102.652	0.027
Day 2	3	308.783	102.928	0.197
Day 3	3	307.013	102.338	0.831

Table 4.8: ANOVA statistics

ANOVA					
Source of Variation	SS	df	MS	F	p-value
Inter day	0.52263	2	0.261315	0.743096	0.514838
Intra day	2.109942	6	0.351657		
Total	2.632572	8.0			

In Table 4.8, SS denotes the sum of squares, df the degrees of freedom, MS the mean squares and F the ratio. Inter-day precision must be better than 5% ($n = 9$). The inter-day variance was not significantly different. The repeatability is within acceptable limits, and it was accepted that the assay should perform well, even when executed by a different laboratory.

4.4.5 Conclusion

The method performed well and will be suitable to analyse indinavir in the samples for stability testing, quality control and batch release purposes. No interference was encountered from stressed samples or known related substances. Therefore, the method can be regarded as being stability-indicating.

4.5 Analysis of transport samples

A HPLC system was used to analyse the validity of indinavir. An analytical method was developed for this analysis. The apparatus and conditions are summarised in Table 4.9.

Table 4.9: Apparatus and chromatographic conditions

Apparatus and conditions	Information
Analytical instrument	HP1100 series HPLC equipped with a pump, autosampler, UV detector and Chemstation Rev. A.10.01 [1653], Agilent® Technologies data acquisition and analysis software
Column	Column #63, Venusil XBP C18(2), 5 µm, 100Å, 4.6x150 mm Cat no: VX951505-2 SIN: V9510515BK 0016K
Mobile phase	50:50, acetonitrile : 0.1% ammonium formate, pH 7, 50/50
Flow rate	1 ml/min
Sample volume injection	100 µl
Detection wavelengths	210.8 nm; 254.4 nm; 350.50 nm
Retention time	4.6 min
Stop time	8 min
Solvent	Krebs-Ringer bicarbonate

4.5.1 Preparation of *in vitro* drug transport solutions

4.5.1.1 Negative and positive control

The transport medium for all the *in vitro* transport experiments was KRB buffer. Where the concentrations of the phytoconstituents used in the experiments were too small to ensure accurate weighing, stock solutions were prepared. The negative control was prepared by dissolving 7.1 mg of indinavir in 500 µl methanol. KRB buffer was added to constitute a volume of 50 ml with a final indinavir concentration of 200 µM. For the positive control,

7.1 mg of indinavir (200 μ M) and 2.455 mg of verapamil (100 μ M) were dissolved in 500 μ l of methanol and made up to a volume of 50 ml with KRB buffer.

4.5.1.2 L-alliin and diallyl disulphide

L-alliin (1.46 mg) was weighed and a stock solution prepared by adding transport medium to constitute a volume of 10 ml. From this solution 1.2 ml was withdrawn and, together with 7.1 mg indinavir, mixed in 500 μ l methanol. This solution was made up to a volume of 50 ml with the transport medium to obtain a final concentration of 20 μ M for L-alliin and 200 μ M for indinavir.

A stock solution was prepared for diallyl disulphide by dissolving 10.03 mg of the compound in 10 ml transport medium. A final solution with a concentration of 20 μ M diallyl disulphide was prepared by adding 146 μ l of the stock solution to 7.1 mg indinavir in 500 μ l methanol. This solution was made up to 50 ml with the transport medium to obtain a final concentration of 20 μ M for diallyl disulphide and 200 μ M for indinavir.

4.5.1.3 Hesperidin and eriocitrin

Hesperidin (0.61 mg) was weighed and dissolved in 500 μ l of methanol together with 7.1 mg indinavir and made up to 50 ml with transport medium to obtain a final concentration of 20 μ M for hesperidin and 200 μ M for indinavir.

Eriocitrin (0.597 mg) was weighed and dissolved together with 7.1 mg indinavir in 500 μ l of methanol and made up to 50 ml with transport medium to obtain a final concentration of 20 μ M for eriocitrin and 200 μ M for indinavir.

4.5.1.4 Betaine monohydrate and β -carotene

A stock solution was prepared for betaine monohydrate by dissolving 3.38 mg of the compound in 10 ml transport medium. A solution was prepared to constitute a concentration of 20 μ M betaine monohydrate by mixing 400 μ l of the stock solution with 7.1 mg indinavir in 500 μ l of methanol. Again, this solution was made up to 50 ml with the transport medium to obtain a final concentration of 20 μ M for betaine monohydrae and 200 μ M for indinavir.

For β -carotene, a stock solution was prepared by dissolving 2.13 mg of the compound in 10 ml transport medium. A solution with a final concentration of 20 μ M β -carotene was prepared by mixing 2.52 ml of the stock solution with 7.1 mg indinavir in 500 μ l methanol. This solution was made up to 50 ml with the transport medium with a final concentration of 20 μ M for β -carotene and 200 μ M for indinavir.

4.5.1.5 Crude plant extracts

An amount of 250 mg of each crude plant extract was dissolved in 500 μ l of methanol together with 7.1 mg of indinavir and made up to a volume of 50 ml with the transport medium resulting in a concentration 5 mg/ml of the crude extracts.

The pH of all the solutions mentioned above were adjusted to 7.4 using prepared 1.0 M NaOH or 1.0 M HCl solution, if necessary.

4.6 Transport studies

4.6.1 Sweetana-Grass diffusion apparatus

The Sweetana-Grass diffusion apparatus, derived from the Ussing chamber, which was developed and patented by Grass and Sweetana (1988:372), was used for the transport studies in this project. The Sweetana-Grass diffusion model is based on laminar flow across excised gastro-intestinal tissue, making it appropriate for the analysis of drug transport across epithelial barriers. The design of the cells is simple which makes it possible to be manufactured in a variety of sizes to ensure the best surface area volume (Grass & Sweetana, 1988:372). The apparatus consists of a front and back plate which holds six cell blocks linearly. Water from a heated reservoir is circulated in the plate in order to maintain the desired temperature in the cells. Each cell consists of two half-cells clamped side-by-side with the excised tissue in between. Integrated metallic mounting pins keep the tissue in position between the half cells. In each half cell a circulation path is presented by means of gas flow parallel to the tissue surface for maximum exposure of the fluid to the membrane and to prevent stagnant layer formation (Sweetana & Grass, 1993:1).

The Sweetana-Grass diffusion apparatus (Costar NaviCyte Snapwell[®], USA) used during this study is illustrated in Figure 4.16.

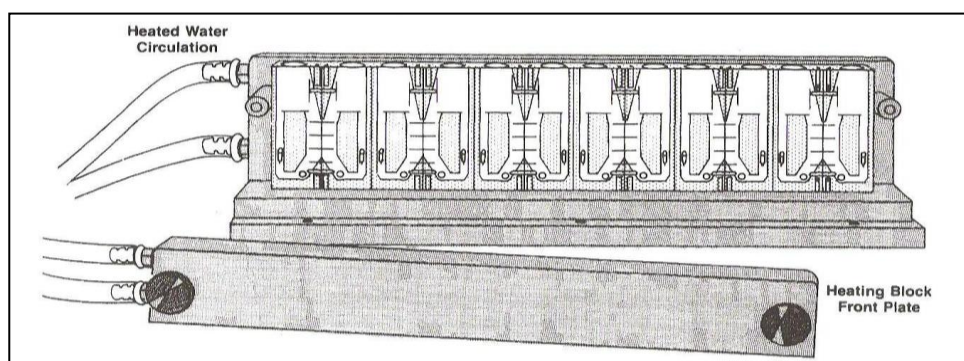


Figure 4.16: Schematic illustration of the Sweetana-Grass diffusion apparatus used for the transport studies (Grass & Sweetana, 1988:374)

4.6.2 Preparation of porcine tissue for transport studies

Pieces of porcine jejunum were collected from a local abattoir (Potch Abattoir, Potchefstroom, South Africa) directly after the pigs have been slaughtered. A veterinary surgeon was consulted to identify the different parts of the porcine gastro-intestinal tract. The proximal 30% of the small intestine consists of the duodenum, followed by the jejunum (60%) and the ileum (distal 10%). A piece of jejunum (approximately 40 cm) was collected from the gastro-intestinal tract of each pig. The tissue was rinsed with ice cold KRB buffer and placed in a cooler box filled with cold buffer and transported to the laboratory where the transport studies were conducted. The cold chain was never interrupted.

In the laboratory the jejunum was rinsed thoroughly with ice cold KRB buffer for a second time and cut into shorter pieces with surgical scissors (Figure 4.17: Slides 1 and 2). The tissue samples were kept in cold KRB buffer throughout the experiment and all dissecting procedures were performed on a glass plate positioned on ice.



Slide 1



Slide 2

Figure 4.17: Photos demonstrating the rinsing and cutting of the porcine jejunum tissue obtained from the abattoir

The excised piece of porcine jejunum was gently pulled over a glass test tube. The serosa and overlaying longitudinal and circular muscle layers were stripped by blunt dissection using surgical tweezers (Figure 4.18: Slides 3 and 4).



Slide 3



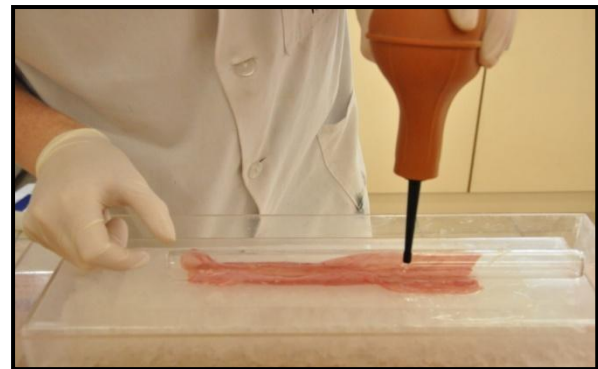
Slide 4

Figure 4.18: Photos demonstrating the excised porcine jejunum being stretched over the glass tube and stripping of the overlaying longitudinal and circular muscle layers

A piece of heavy duty filter paper moistened with the cold KRB buffer was placed underneath the glass tube over which the porcine jejunum was stretched. The jejunum was cut along the mesenteric border with a scalpel blade and the porcine jejunum was washed from the glass tube with the cold KRB buffer onto the filter paper, resulting in the apical side of the membrane facing upwards (Figure 4.19: Slide 5 and 6).



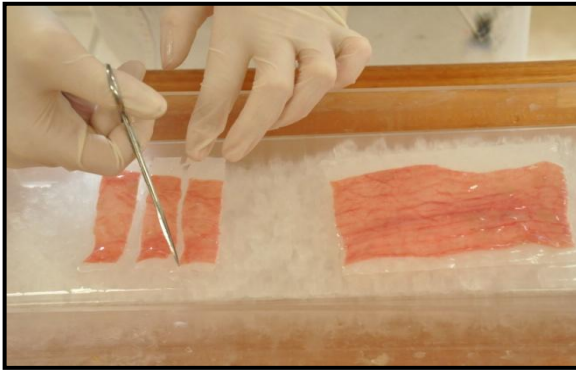
Slide 5



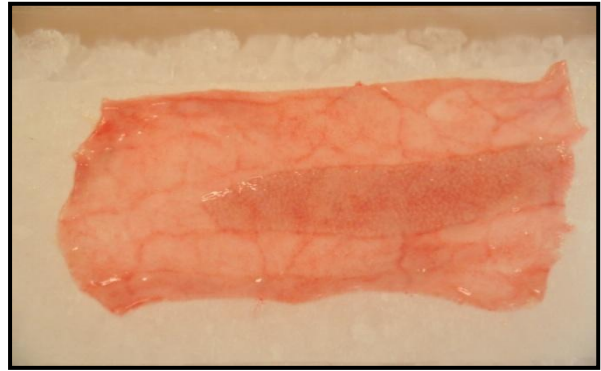
Slide 6

Figure 4.19: Photos demonstrating the cutting of the porcine jejunum along the mesenteric border and washing from the glass tube onto the filter paper

Following the above steps, the jejunum and filter paper were cut together into smaller pieces of approximately 2 cm wide (Figure 4.20: Slide 7). The filter paper protects the jejunum segments from being damaged and ensures appropriate handling. The segments were kept moist with cold KRB buffer throughout the procedure. Strips lined with Peyer's patches (Figure 4.20: Slide 8) were discarded as variation in the transport rate might occur across these areas (Norris *et al.*, 1998:141).



Slide 7



Slide 8

Figure 4.20: Photos demonstrating the cutting of the porcine jejunum into smaller pieces and Peyer's patches which should be avoided

4.6.3 Transport studies

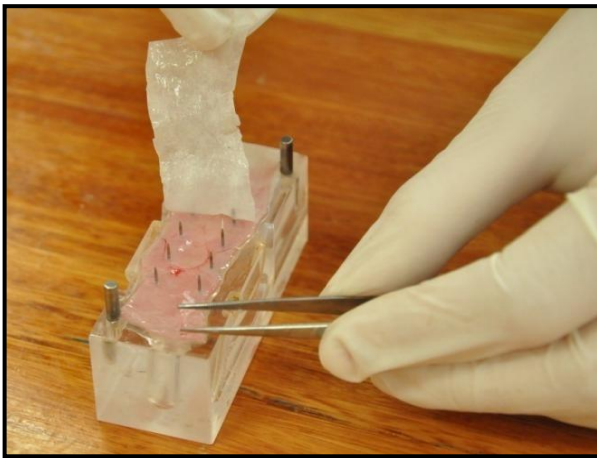
The excised segments of the porcine intestinal tissue were carefully mounted onto the pins of the half-cells. The filter paper faced upwards and the apical side of the jejunum tissue faced downwards (Figure 4.21: Slides 9 and 10; next page). The filter paper was removed carefully and the matching half-cells clamped together with metal rings (Figure 4.21: Slides 11, 12 and 13; next page). The assembled chambers were then placed in the heating block (Figure 4.22). Each chamber section was filled simultaneously with 7 ml heated (37 °C) KRB buffer.



Slide 9



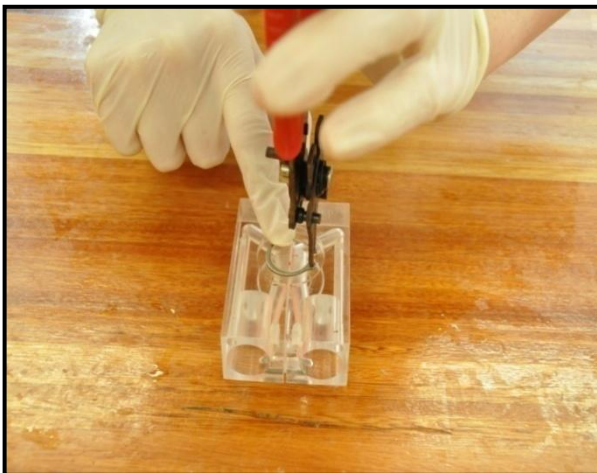
Slide 10



Slide 11



Slide 12



Slide 13

Figure 4.21: Photos demonstrating the mounting of the porcine jejunum and clamping of the half-cells

Each half cell was connected to parallel gas flow (95% O₂: 5% CO₂) at a flow rate of 15 to 20 ml/min to ensure circulation of the buffer inside the cells and simulate intestinal conditions. The cells were left for 30 min to stabilise and reach a state of equilibrium before commencement of the transport studies. The whole procedure, from collecting the jejunum until commencing the transport studies (including the time to reach equilibrium), was performed within a maximum period of 60 minutes. After stabilisation, the total volume of the buffer in all the cells was aspirated with an Integra Vacusafe vacuum system (Vacusafe®, model 158 3XX). Immediately following the removal of the buffer, the test solutions were added to the different chambers.

Before commencement of the transport studies, the transepithelial electrical resistance was measured with a Millicel ERS-2 meter (Millipore® Corporation, USA) (as per the method described in Tarirai *et al.*, 2012:256). The following procedure was executed to perform the transport studies of the nine experimental groups. The transport of indinavir was determined for the AP-BL direction as well as for the BL-AP direction across the jejunum segments. Indinavir alone in solution (200 µM) served as the negative control group. For the AP-BL analysis, 7 ml of KRB buffer was added to the acceptor chambers (basolateral side) and 7 ml of indinavir solution was added to the donor chambers (apical side). For the BL-AP analysis, 7 ml of indinavir solution was added to the donor chambers (basolateral side) and 7 ml of KRB buffer was added to the acceptor chambers (apical side). Every 20 minutes samples (200 µl) were withdrawn from the acceptor chambers over a total period of 180 minutes, while the relevant chambers were immediately replenished by an equal volume of fresh KRB buffer solution.

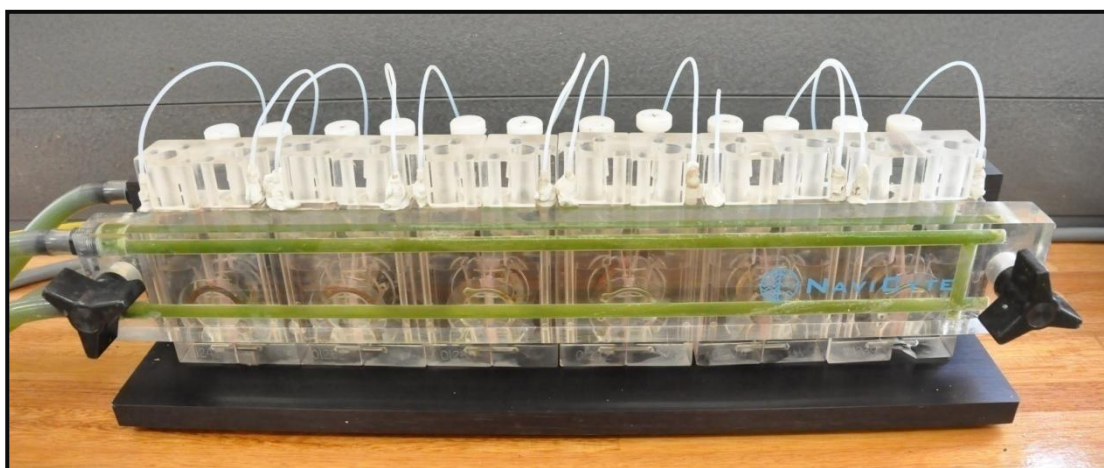


Figure 4.22: Photo demonstrating the assembled Sweetana-Grass diffusion apparatus during a transport study

For the positive control, the transport of indinavir was determined in the presence of verapamil (100 μ M), which is a known P-gp inhibitor (Bansal *et al.*, 2009:55). Again, for the AP-BL analysis, 7 ml of KRB buffer was added to the acceptor chambers (basolateral side) while 7 ml of a mixture of 200 μ M indinavir and 100 μ M verapamil was added to the donor chambers (apical side). The opposite scenario was true for the BL-AP analysis. The 7 ml mixture of 200 μ M indinavir and 100 μ M verapamil was added to the donor chambers (basolateral side) and 7 ml of KRB buffer was added to the acceptor chambers (apical side) and again samples (200 μ l) were withdrawn every 20 minutes over a total period of 180 min and the volume replenished.

For the AP-BL analysis with the nine test compounds (*A. sativum*, diallyl disulphide, L-alliin, *C. limonum*, hesperidin, eriocitrin, *B. vulgaris*, β -carotene and betaine monohydrate), 7 ml of KRB buffer was added to the acceptor chambers (basolateral side) and 7 ml of a mixture of indinavir (200 μ M) and test compound (20 μ M) or crude plant extract (5 mg/ml) solution was added to donor chambers (apical side). For the BL-AP analysis, 7 ml of KRB buffer was added to the acceptor chambers (apical side) and 7 ml of a mixture of indinavir (200 μ M) and test compound (20 μ M) or crude plant extract (5 mg/ml) solution was added to donor chambers (basolateral side). Samples (200 μ l) were withdrawn over 180 minutes at the same rate as for the control experiments. Following sample extraction, HPLC analysis was performed immediately (see section 4.3.3).

4.7 Data analysis and statistics

4.7.1 Apparent permeability coefficient (P_{app})

The apparent permeability coefficient is an index commonly used and is an integral part of the drug screening process during absorption studies. It is defined as the initial flux of compound through the membrane, normalised by membrane surface area and donor concentration (Palumbo *et al.*, 2008:235) and is calculated by means of Equation 1.

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A.C_0} \quad \text{Equation 1}$$

In Equation 1, dQ/dt (μ g/s) represents the increase in the amount of drug in the acceptor chamber over time, A (cm^2) denotes the effective surface area of the excised porcine jejunum tissue segment exposed to the transport medium and C_0 (μ g/ml) represents the initial drug concentration in the donor chamber (Hayeshi *et al.*, 2006:72; Tarirai *et al.*, 2012:257).

4.7.2 Efflux ratio (ER)

The efflux ratio (ER) was calculated to determine any asymmetry in the transport of indinavir under the influence of each of the extracts using Equation 2.

$$ER = \frac{P_{app}(BL - AP)}{P_{app}(AP - BL)} \quad \text{Equation 2}$$

P_{app} (BL-AP) and P_{app} (AP-BL) denote the apparent permeability in the secretory and absorptive directions, respectively (Tarirai *et al.*, 2012:257). The efflux ratio was calculated to determine any changes in the transport of indinavir from the BL-AP direction relative to the AP-BL direction due to the influence of the named test compounds. In principle, compounds exhibiting inhibitory effects on indinavir efflux would demonstrate net absorptive transport in the AP-BL direction (Tarirai *et al.*, 2012:257-258).

4.7.3 Transepithelial flux (J)

Equation 3 was used to calculate the transepithelial indinavir flux in each direction across the excised porcine jejunum tissue (Hansen & Nilsen, 2009:88).

$$J = \frac{V(dC/dt)}{A} \quad \text{Equation 3}$$

In Equation 3, J ($\mu\text{g}/\text{cm}^2/\text{min}$) is the transepithelial flux, V (mL) the volume of the receiver chamber, dC ($\mu\text{g}/\text{ml}$) the indinavir concentration at a specific time in the receiving chamber, dt (min) the overall specific transport time and A (cm^2) the effective membrane surface area of the porcine jejunum tissue segment exposed to the transport medium (Tarirai *et al.*, 2012:258).

The net flux was determined from the difference in flux in both directions using Equation 4 (Hansen & Nilsen, 2009:88).

$$J_{net} = J_{BL-AP} - J_{AP-BL} \quad \text{Equation 4}$$

In this equation, J_{BL-AP} and J_{AP-BL} represent the indinavir flux in the secretory and absorptive directions, respectively. A negative J_{net} value indicates net absorptive transport of indinavir resulting from the inhibition of P-gp mediated indinavir efflux by the test compound (Tarirai *et al.*, 2012:257).

4.7.4 Statistical analysis of results

The following statistical analyses were performed on the transport results from the porcine intestinal tissue data.

ANOVA tests were done to determine if statistically significant differences exist between the mean ER and J_{net} values of the test compounds and each of the positive and negative control groups. Levene's tests were performed for each ANOVA analysis to ensure equality of variances. In cases of equality of variances, Welch tests were performed. Normal probability plots on the residuals were done for each analysis to ensure that the data was distributed fairly (Tabachnick & Fidell, 2001). Dunnett's tests were performed to determine which of the mean ER and J_{net} values of the test compounds differed with statistical significance from the mean ER and J_{net} values of each of the standard compounds. Tukey's comparison tests were performed to classify the test compounds and the positive and negative controls into homogeneous groups according to their mean ER and J_{net} values. All these analyses were applied to the mean ER and J_{net} values for subgroup *A. sativum*, L-alliin and diallyl disulphide using *A. sativum* crude extract as the reference group, *C. limonum*, hesperidin and eriocitrin using *C. limonum* crude extract as the reference group and *B. vulgaris*, β -carotene and betaine monohydrate, using *B. vulgaris* crude extract as the reference group. These procedures were carried out using Statistica (Statsoft®, 2007). Statistical significance was set at $p < 0.05$.

CHAPTER 5

5 RESULTS AND DISCUSSION

5.1 Introduction

The potential of crude extracts and pure phytoconstituents of *A. sativum*, *C. limonum* and *B. vulgaris* to modulate the transport of indinavir was investigated in the present study. Transport analyses were conducted in the AP-BL as well as the BL-AP direction across excised porcine jejunum segments using the Sweetana-Grass diffusion apparatus.

Two control groups were included to validate that the effect of the experimental compounds on the transport of indinavir occurred due to their own inherent actions and not as a result of experimental interferences. In the negative control group, the transport of indinavir alone (200 μ M) was determined with no modulator added. In the positive control group, the transport of indinavir was determined in the presence of 100 μ M verapamil, a known P-gp inhibitor. The amount of indinavir reaching the acceptor chamber as a function of time after incubation with the plant extracts (5 mg/ml) and pure phytoconstituents (20 μ M) was measured in the test groups. To ensure membrane integrity, the TEER was measured at the beginning and end of each transport study.

All samples collected at the specified time intervals were analysed by means of a validated HPLC method and the cumulative transport was expressed as the apparent permeability coefficient (P_{app}) and the transepithelial flux (J) from which the efflux ratio (ER) and the net flux (J_{net}) were calculated. Statistical analysis was conducted on the transport results to compare results from the test groups with the control groups (section 5.7).

5.2 Loss on drying

The loss on drying profiles for each of the crude extract powders of *A. sativum*, *C. limonum* and *B. vulgaris* are depicted in Figures 5.1 to 5.3. The DTG-60 apparatus (Shimadzu®, Japan) recorded a 1.769%, 1.775% and 1.628% weight loss for the crude extracts. These values indicate that the amount of water or volatile substance present in the freeze dried powders were within an acceptable range (5 to 8 %) (Ajazuddin & Saraf, 2010:322).

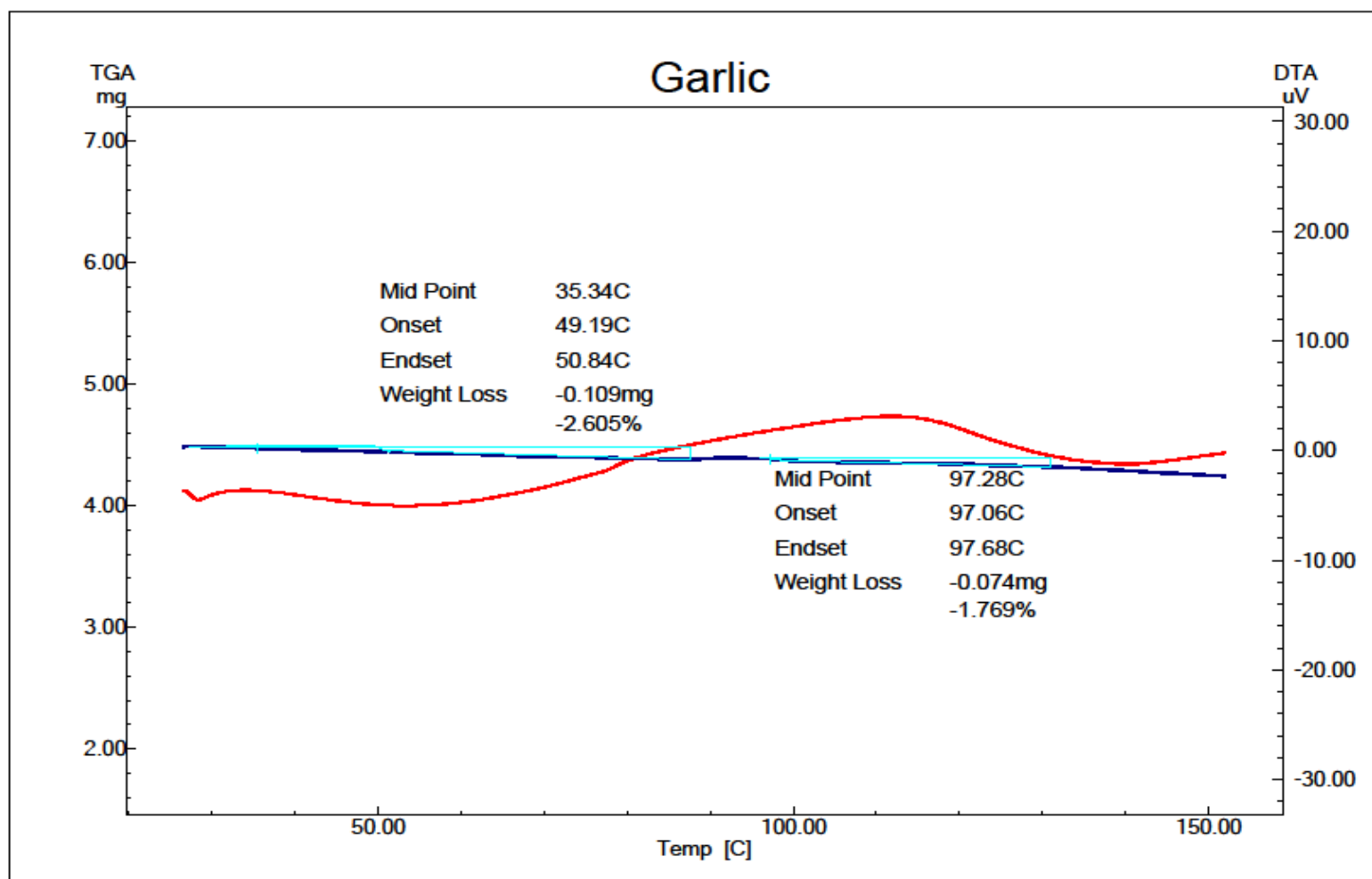


Figure 5.1: Loss on drying profile for *Allium sativum* crude extract

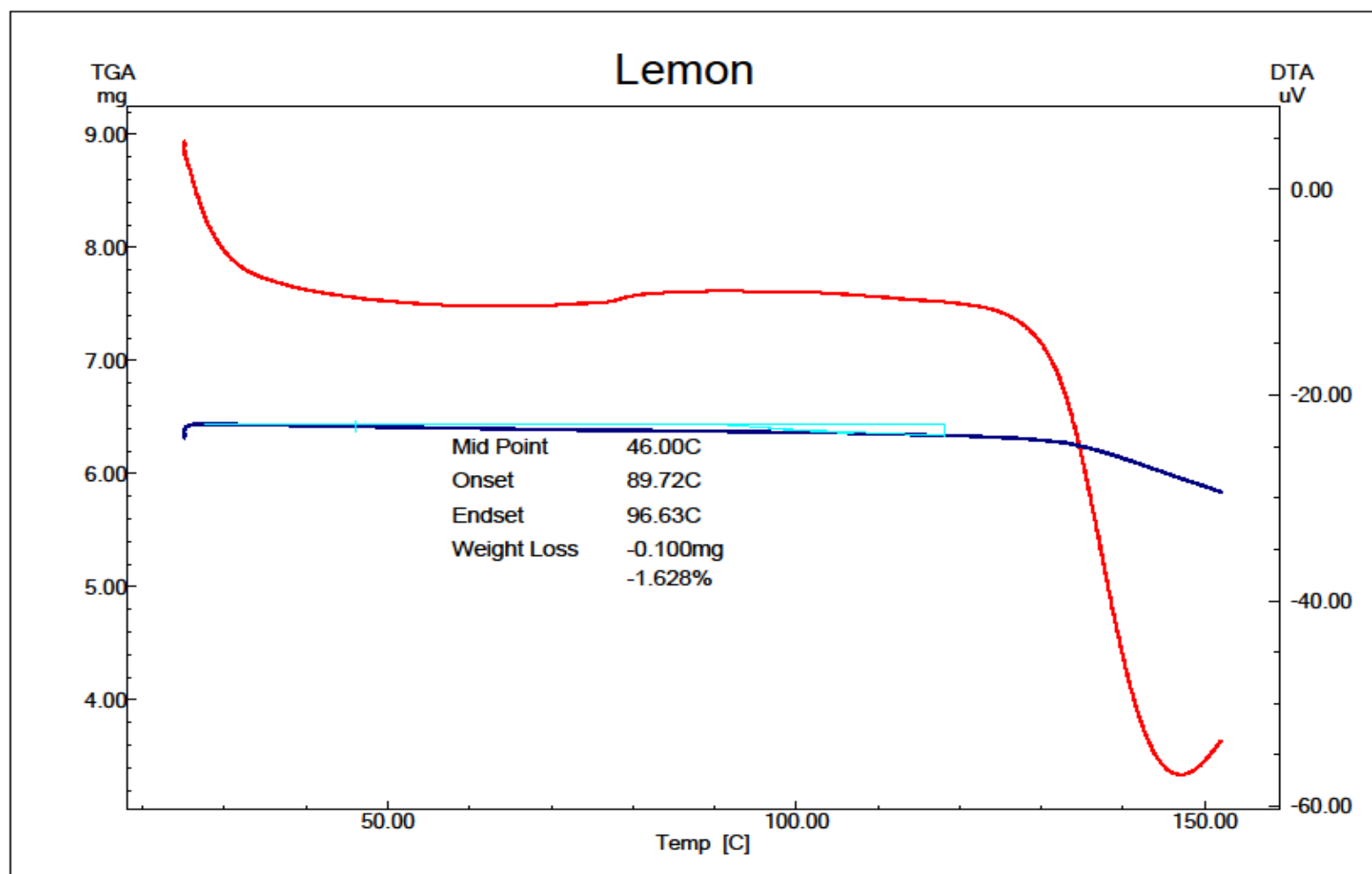


Figure 5.2: Loss on drying profile for *Citrus limonum* crude extract

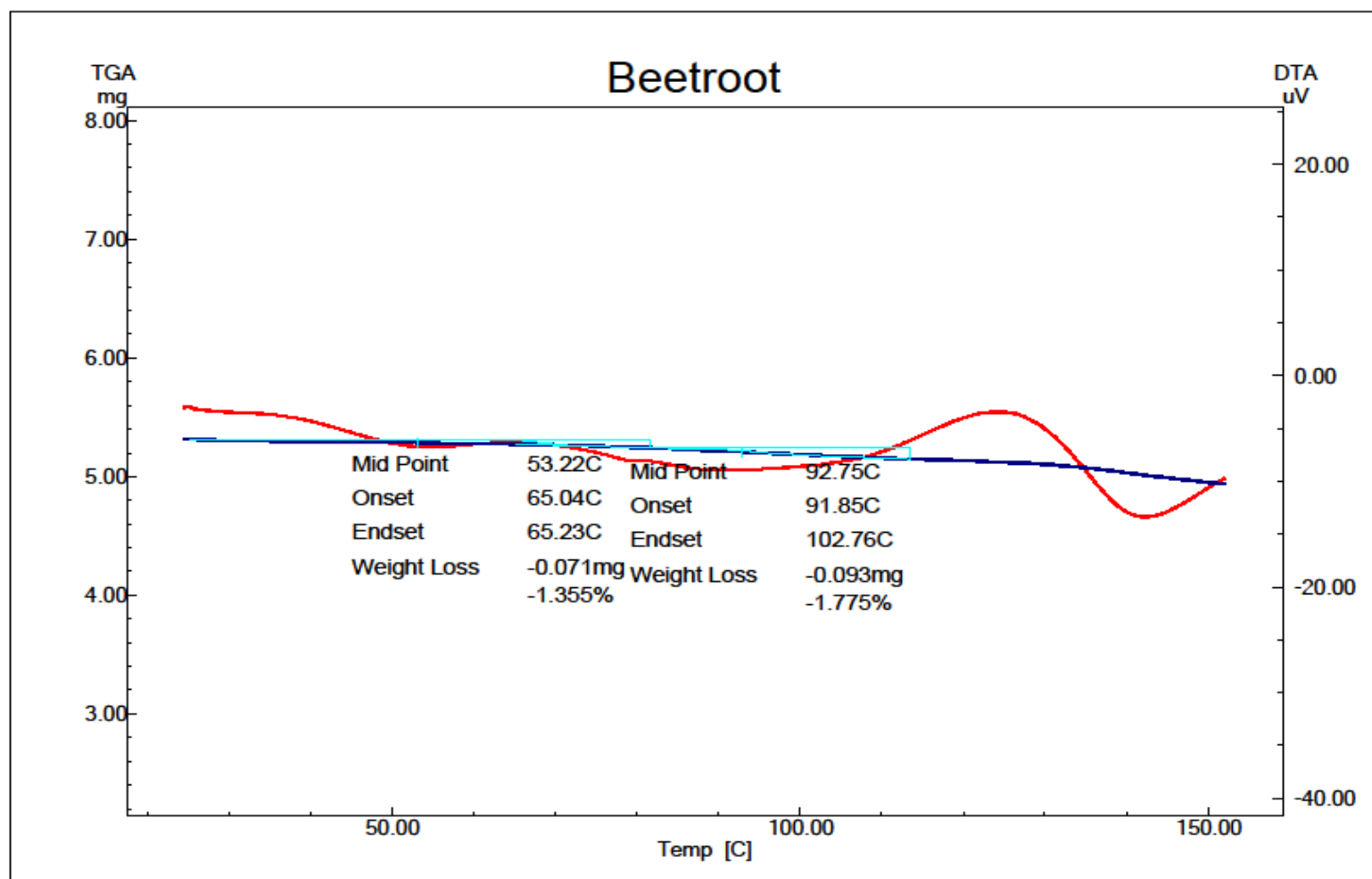


Figure 5.3: Loss on drying profile for *Beta vulgaris* crude extract

5.3 Chemical fingerprinting of crude plant extracts and pure phytoconstituents

The chromatographic profiles for the freeze dried crude extracts of *A. sativum*, *B. vulgaris* and *C. limonum* and the selected pure phytoconstituents (L-alliin, diallyl disulphide, hesperidin, eriocitrin β -carotene and betaine monohydrate) are shown in Figures 5.4 to 5.11. The objective of this analytical experiment was to qualitatively indicate if the selected phytoconstituents are present in the crude plant extracts.

5.3.1 Chromatographic profiles for *Allium sativum* crude extract, L-alliin and diallyl disulphide

The chromatographic profiles of *A. sativum* crude extract at three different wavelengths are shown in Figure 5.4. Several phytoconstituents of *A. sativum* were separated and detected by the HPLC method employed, some of which absorb light at more than one wavelength (e.g. the compound at a retention time of 6.157 min is visible at 254 nm and 270 nm).

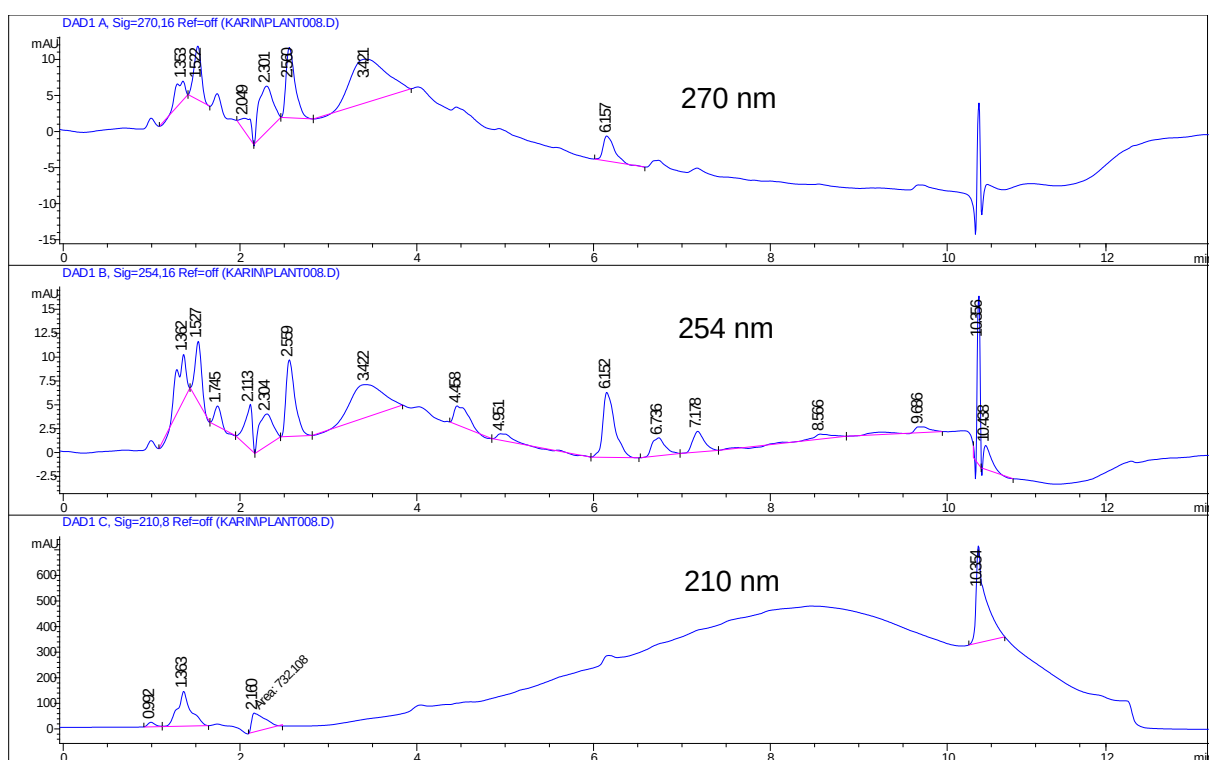


Figure 5.4: Chromatographic profile for *Allium sativum* crude extract at wavelengths 270 nm, 254 nm and 210 nm

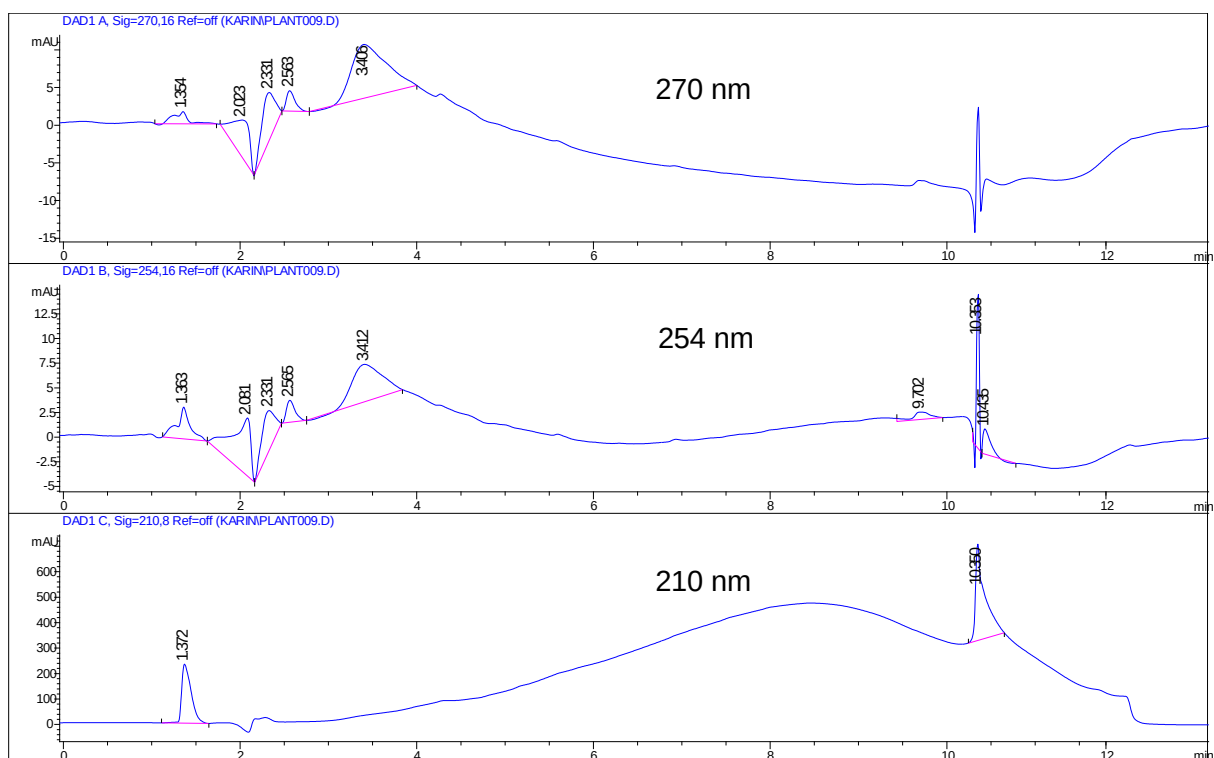


Figure 5.5: Chromatographic profile for L-alliin at wavelengths 270 nm, 254 nm and 210 nm

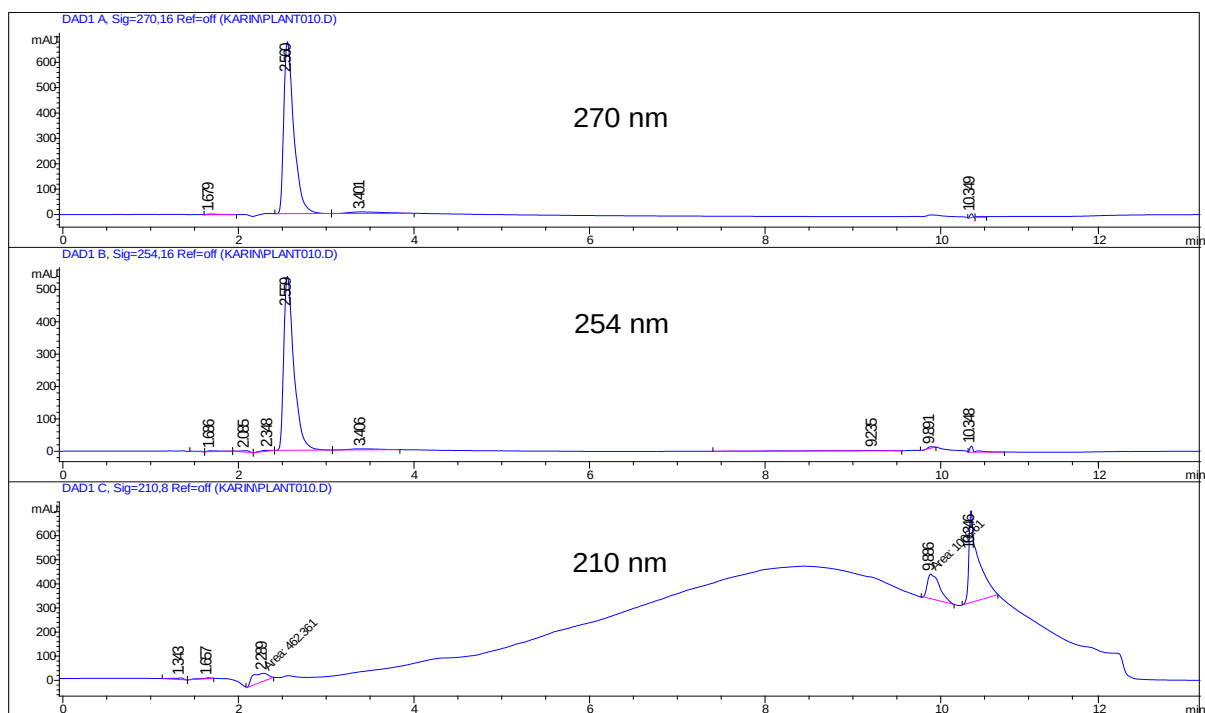


Figure 5.6: Chromatographic profile for diallyl disulphide at wavelengths 270 nm, 254 nm and 210 nm

The peak for diallyl disulphide appears at a retention time of 2.559 min (Figure 5.6) and the presence of this compound in the *A. sativum* crude extract is clearly visible at a retention time of 2.559 min (Figure 5.4).

5.3.2 Chromatographic profiles for *Citrus limonum* crude extract, hesperidin and eriocitrin

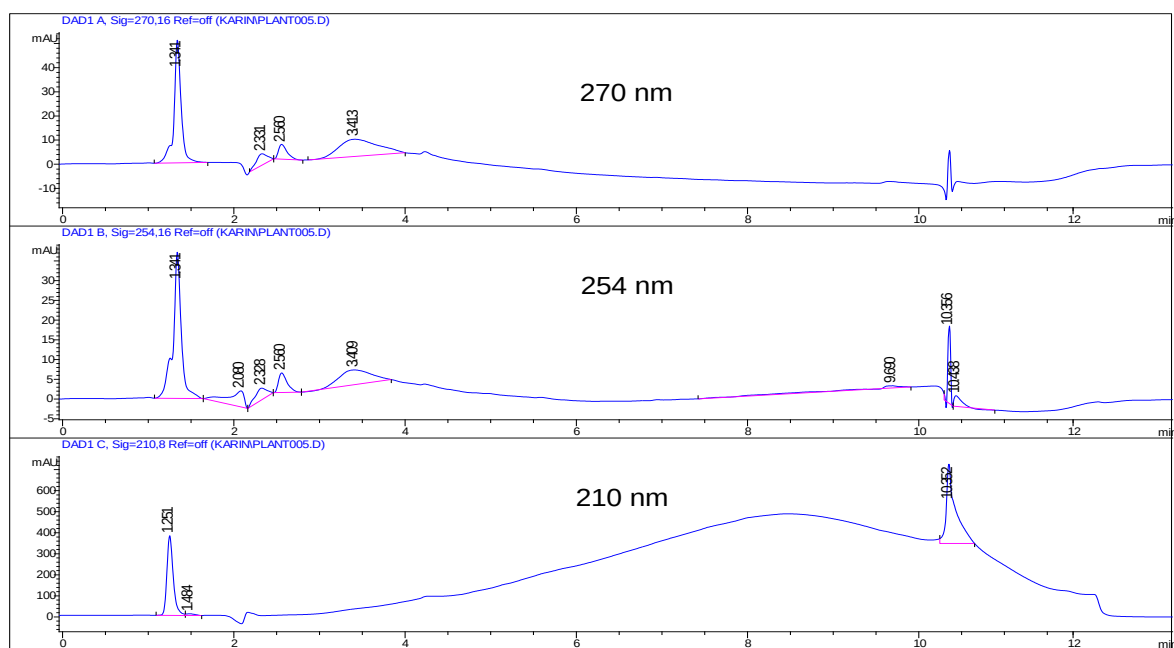


Figure 5.7: Chromatographic profile for *Citrus limonum* crude extract at wavelengths 270 nm, 254 nm and 210 nm

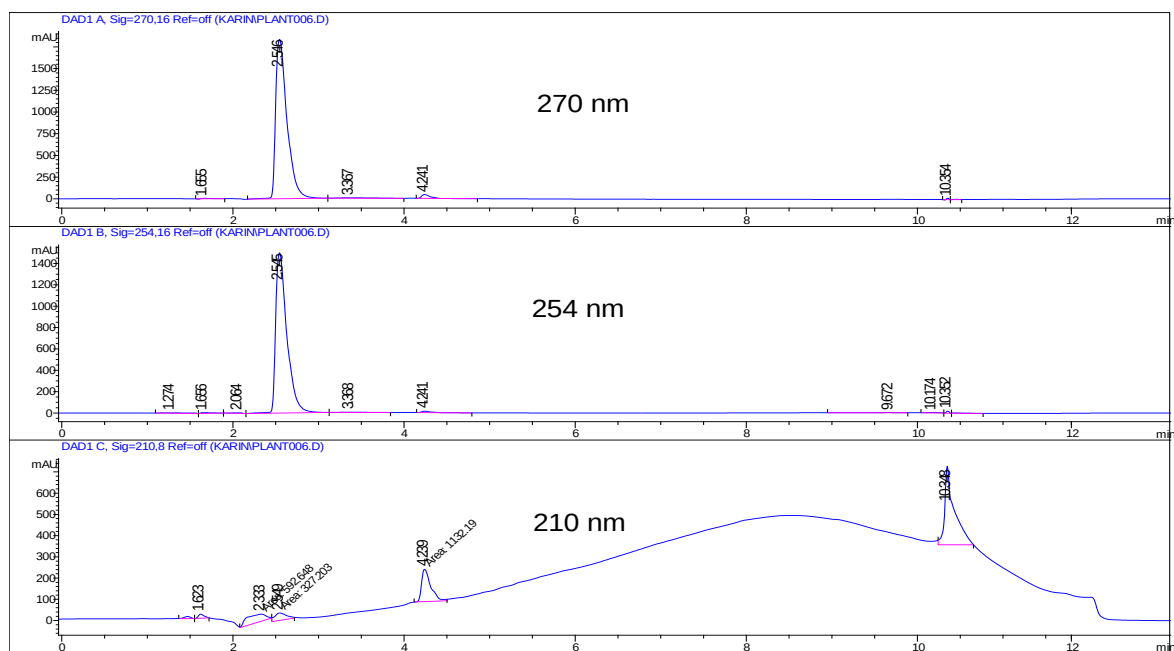


Figure 5.8: Chromatographic profile for hesperidin at wavelengths 270 nm, 254 nm and 210 nm

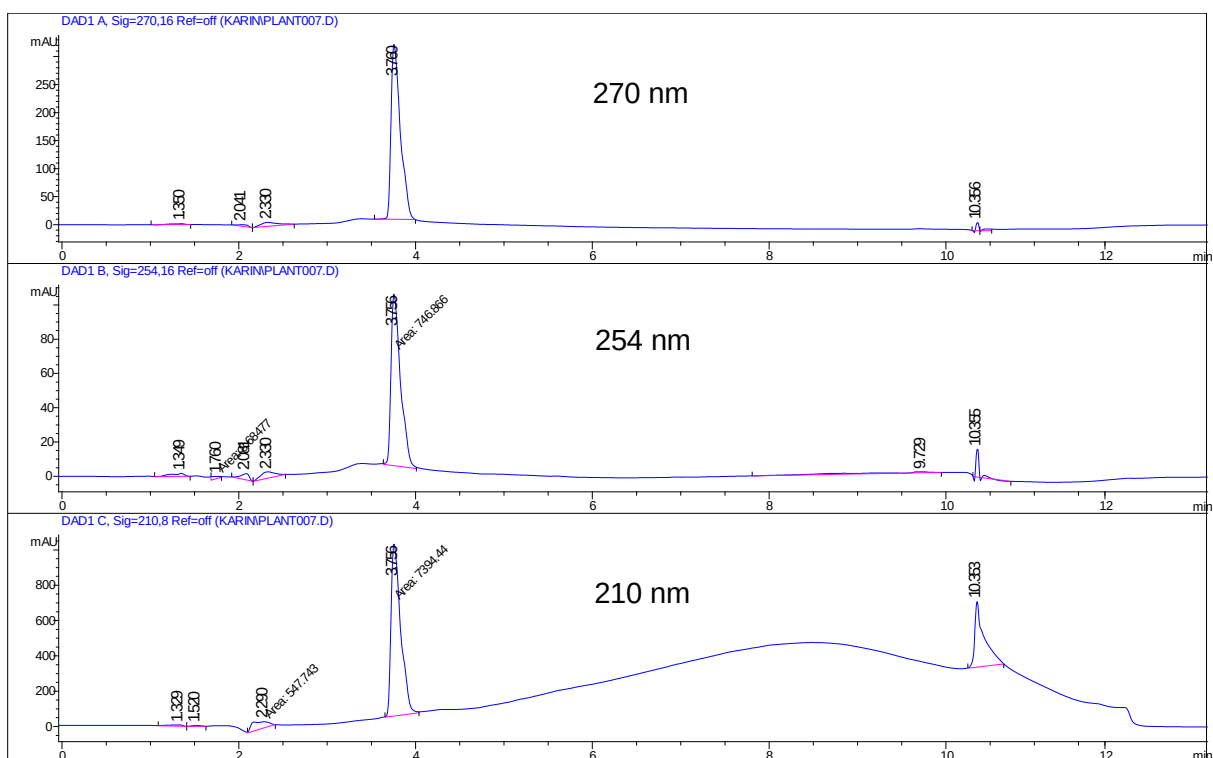


Figure 5.9: Chromatographic profile for eriocitrin at wavelengths 270 nm, 254 nm and 210 nm

The peak for hesperidin occurs at a retention time of 2.545 min (Figure 5.8) and when compared to the chromatographic profile of the *C. limonum* crude extract, a peak is visible at a retention time of 2.560 (Figure 5.7). The concurrent retention times of these peaks indicate that the hesperidin is present in the *C. limonum* crude extract.

The peak for eriocitrin occurs at a retention time of 3.756 min (Figure 5.9) and thus the presence of this compound in the freeze dried extract of *C. limonum* could not be verified as no prominent peak is visible at the specific retention time for the crude extract (Figure 5.7).

5.3.3 Chromatographic profiles for *Beta vulgaris* extract, betaine monohydrate and β -carotene

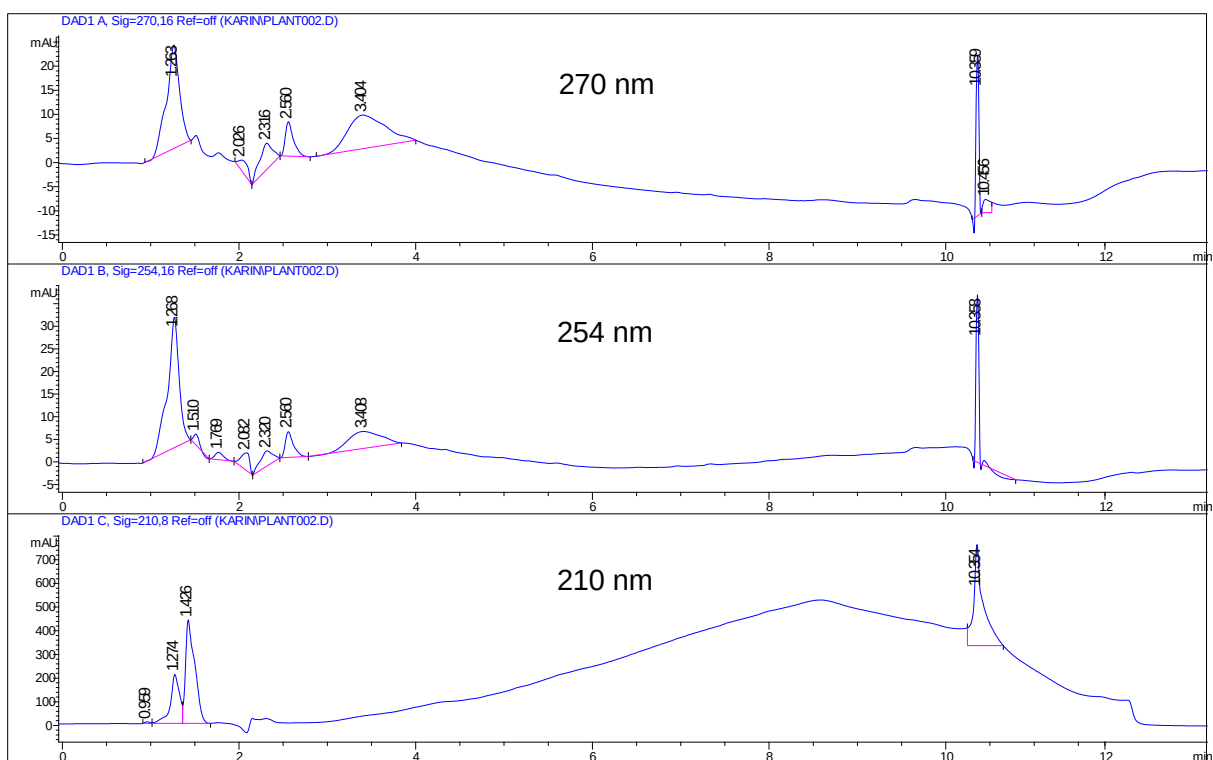


Figure 5.10: Chromatographic profile for *Beta vulgaris* crude extract at wavelengths 270 nm, 254 nm and 210 nm

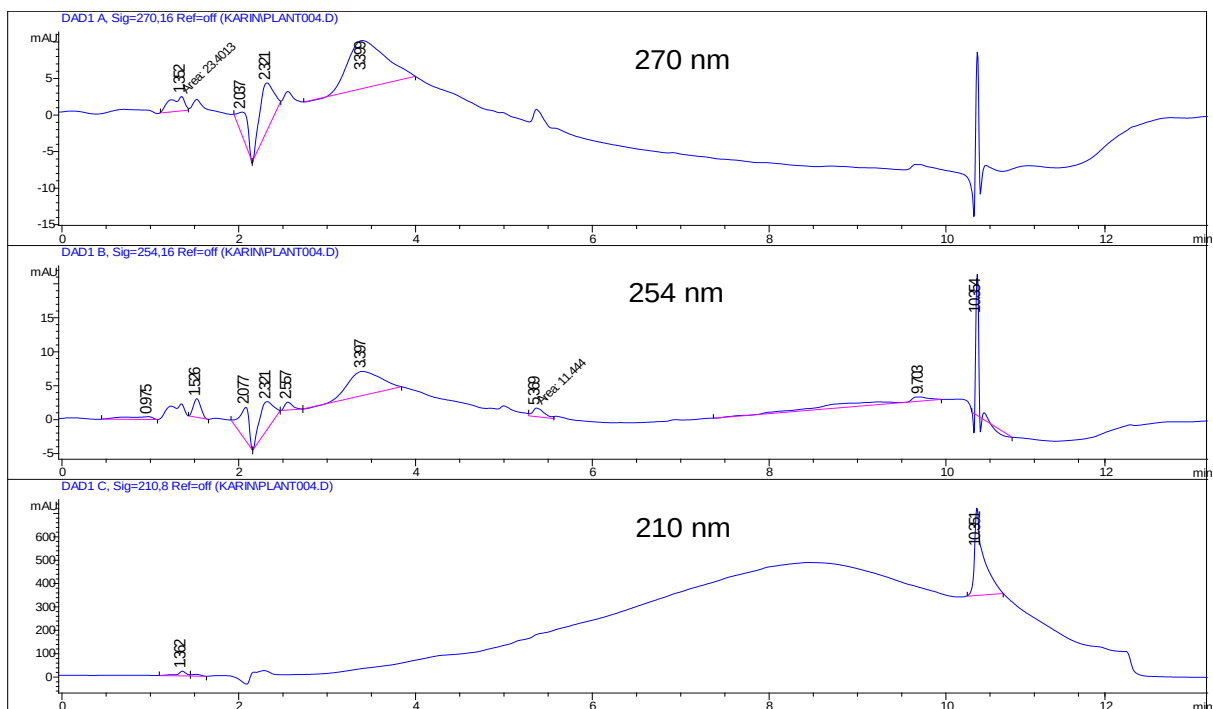


Figure 5.11: Chromatographic profile for betaine monohydrate at wavelengths 270 nm, 254 nm and 210 nm

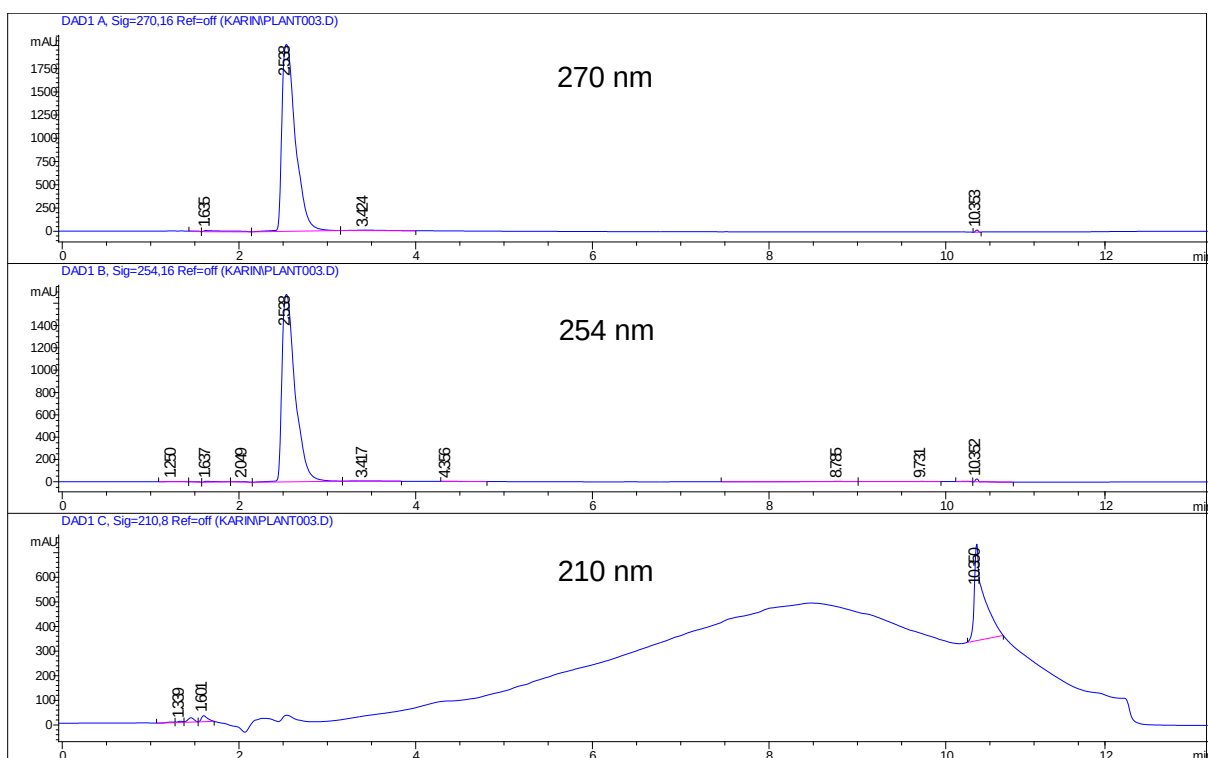


Figure 5.12: Chromatographic profile for β -carotene at wavelengths 270 nm, 254 nm and 210 nm

The peak for betaine monohydrate occurs at a retention time of 3.397 min (Figure 5.11) and when comparing it to the chromatographic profile of the *B. vulgaris* crude extract, a peak is visible at a retention time of 3.404 min (Figure 5.10). The concurrent retention times of these peaks indicate that the betaine monohydrate is present in the *B. vulgaris* crude extract.

The peak for β -carotene occurs at a retention time of 2.568 min (Figure 5.12). The *B. vulgaris* crude extract exhibits a peak at 2.560 min, which could possibly indicate the presence of this compound in the freeze dried *B. vulgaris* extract (Figure 5.9).

5.4 *In vitro* transport of indinavir across excised porcine intestinal tissue

Several studies have demonstrated that PIs (e.g. indinavir) are substrates for P-gp related efflux, which limits their bioavailability after oral administration. Efflux may also limit the distribution of the PI into several therapeutically relevant compartments, and thus reduce the chance of achieving effective concentrations at the site of action (Huisman *et al.*, 2000:237).

5.4.1 *In vitro* transport of indinavir alone (negative control group)

The bi-directional transport data of indinavir alone across excised porcine intestinal tissue into the acceptor chamber expressed as a percentage relative to the initial concentration in the donor chamber (200 μ M) over a period of 180 min is presented in Table 5.1 for each time interval.

Table 5.1: The bi-directional cumulative transport (%) of indinavir alone across excised porcine intestinal tissue

Time (min)	Cell 1		Cell 2		Cell 3		Cell 4	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
40	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
60	0.00262	0.00000	0.07016	0.00000	0.07127	0.00000	0.06063	0.00000
80	0.10007	0.26559	0.10304	0.18740	0.11453	0.16856	0.05760	0.31007
100	0.18303	0.30048	0.16642	0.21865	0.14658	0.23875	0.16851	0.24984
120	0.21626	0.28242	0.27470	0.20721	0.22668	0.22869	0.31305	0.38694
140	0.40257	0.50364	0.44058	0.48579	0.30553	0.56419	0.44126	0.59037
160	0.28453	0.63828	0.47120	0.65091	0.31110	0.68607	0.58562	0.76078
180	0.62941	0.67246	0.65599	0.80999	0.50795	0.66057	0.54911	0.75446

The bi-directional cumulative transport (%) of indinavir across excised porcine intestinal tissue plotted as a function of time for the negative control group (indinavir alone) is shown in Figure 5.13.

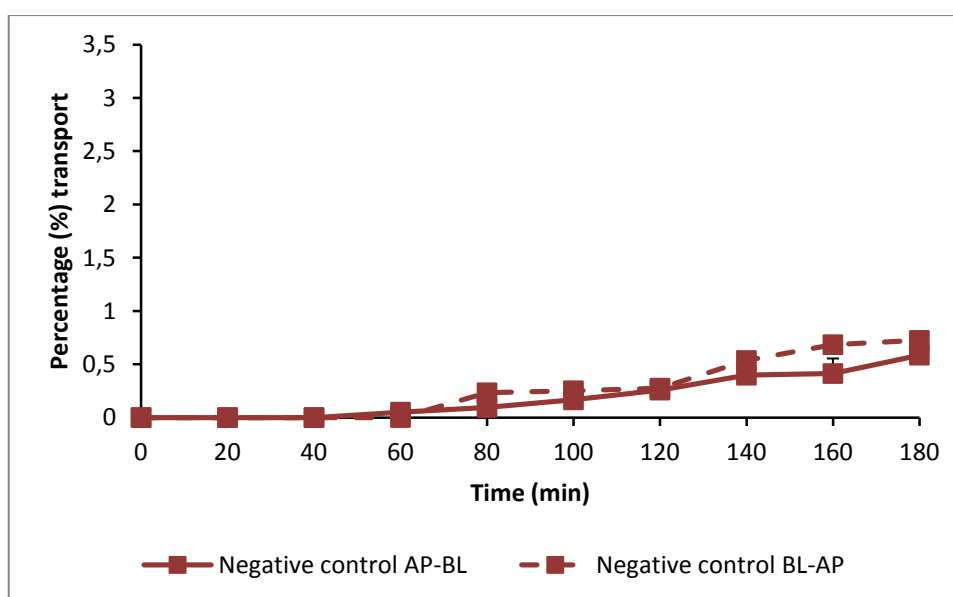


Figure 5.13: Bi-directional cumulative percentage transport of indinavir alone plotted as a function of time

The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values calculated from the cumulative transport of indinavir alone (negative control group) are presented in Table 5.2.

A higher transport of indinavir in the BL-AP direction as opposed to the AP-BL direction was demonstrated in the negative control group, indicating active transport of indinavir in the secretory direction. In the intestine, efflux transporters such as P-gp are localised on the apical domain of the epithelial cells and actively pump compounds from the cell back into the intestinal lumen in the secretory direction (Tarirai *et al.*, 2012:225).

Table 5.2: P_{app} and ER values of indinavir alone (negative control group) across excised porcine intestinal tissue

Cell	P_{app} ($\times 10^{-7}$)		Efflux Ratio (ER)
	AP-BL	BL-AP	
1	2.71000	3.94974	1.457467
2	3.42815	4.23563	1.235544
3	2.48981	4.06448	1.632445
4	3.43768	4.58748	1.334469
Mean	3.016410	4.209333	1.414981
Stdev	0.489283	0.278123	0.171051

The mean apparent permeability coefficient (P_{app}) and the efflux ratio (ER) values for indinavir transport with no modulator added are illustrated in Figure 5.14.

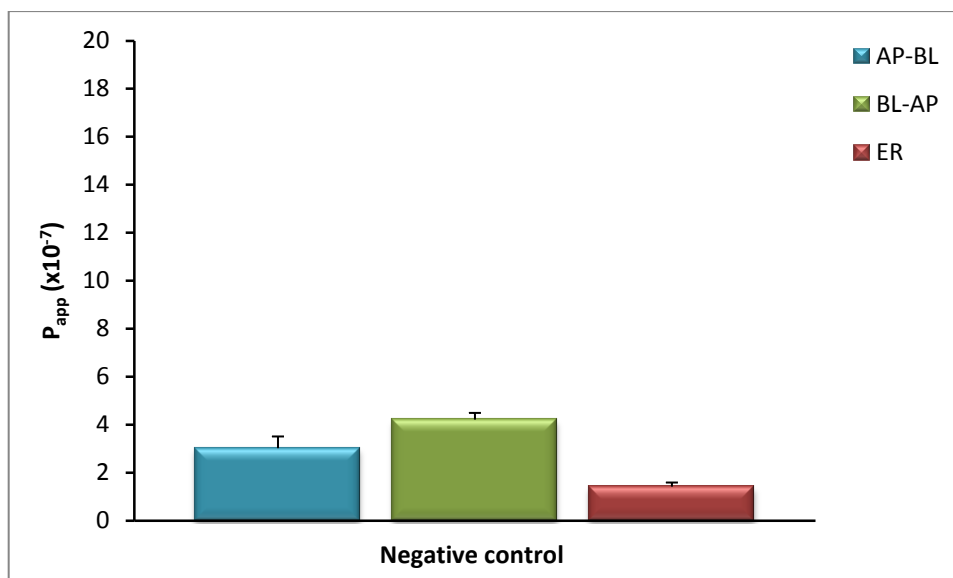


Figure 5.14: Mean P_{app} and ER values of indinavir transport (negative control group) across excised porcine intestinal tissue

The transepithelial flux (J) and net flux (J_{net}) values for indinavir alone (negative control group) are presented in Table 5.3 and compared in Figure 5.15.

Table 5.3: The J and J_{net} values of indinavir alone (negative control group) across excised porcine intestinal tissue

Cell	J ($\times 10^{-2}$)		Net Flux (J_{net}) ($\times 10^{-2}$)
	AP-BL	BL-AP	
1	1.1377	1.65889	0.52119
2	1.43982	1.77896	0.33914
3	1.04572	1.70708	0.66136
4	1.44383	1.92674	0.48291
Mean	1.266768	1.767918	0.50115
Stdev	0.205604	0.116811	0.132475

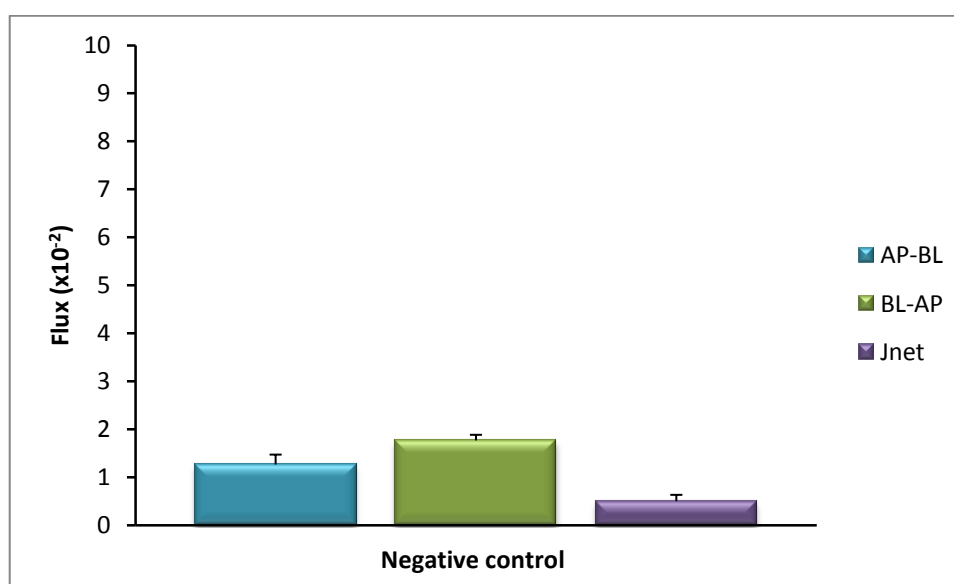


Figure 5.15: Mean J and J_{net} values for indinavir alone (negative control group) across excised porcine intestinal tissue

The results obtained from the negative control group confirms the suitability of indinavir to serve as a model compound to investigate the pharmacokinetic interactions of co-administered compounds (postulated to be P-gp inducers or inhibitors) in the excised porcine tissue model. The results are congruent with previous studies which demonstrated that PIs such as indinavir are substrates for efflux transporters such as P-gp, resulting in limited intestinal absorption (Brown *et al.*, 2008:589).

5.4.2 *In vitro* transport of indinavir in the presence of verapamil (positive control group)

Verapamil is a known P-gp inhibitor and therefore should be capable of reducing the transport of indinavir in the BL-AP direction (Brown *et al.*, 2008:589). The bi-directional transport of indinavir in the presence of verapamil (positive control group) across excised porcine intestinal tissue, expressed as a percentage relative to the initial concentration in the donor chamber (200 μ M) over a period of 180 min is presented in Table 5.4 for each time interval.

Table 5.4: The bi-directional cumulative transport (%) of indinavir in the presence of verapamil across excised porcine intestinal tissue

Time (min)	Cell 1		Cell 2		Cell 3		Cell 4	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
40	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
60	0.17998	0.12682	0.21897	0.20480	0.31332	0.20254	0.23509	0.11109
80	0.38110	0.22400	0.37811	0.12236	0.41940	0.30993	0.31052	0.21851
100	0.52730	0.28410	0.54581	0.40592	0.65647	0.51605	0.57981	0.30473
120	0.76469	0.41964	0.79099	0.50803	0.92296	0.77731	0.79968	0.52764
140	1.01490	0.63315	0.99158	0.79740	1.21198	0.98444	0.98586	0.57842
160	1.45093	0.75061	1.42117	0.70692	1.52040	0.77700	1.20362	0.57540
180	1.57221	0.77224	1.59609	0.86976	1.37886	0.77429	1.42946	0.76495

The bi-directional cumulative transport (%) of indinavir in the presence of verapamil (positive control group) plotted as a function of time across excised porcine intestinal tissue is shown in Figure 5.16.

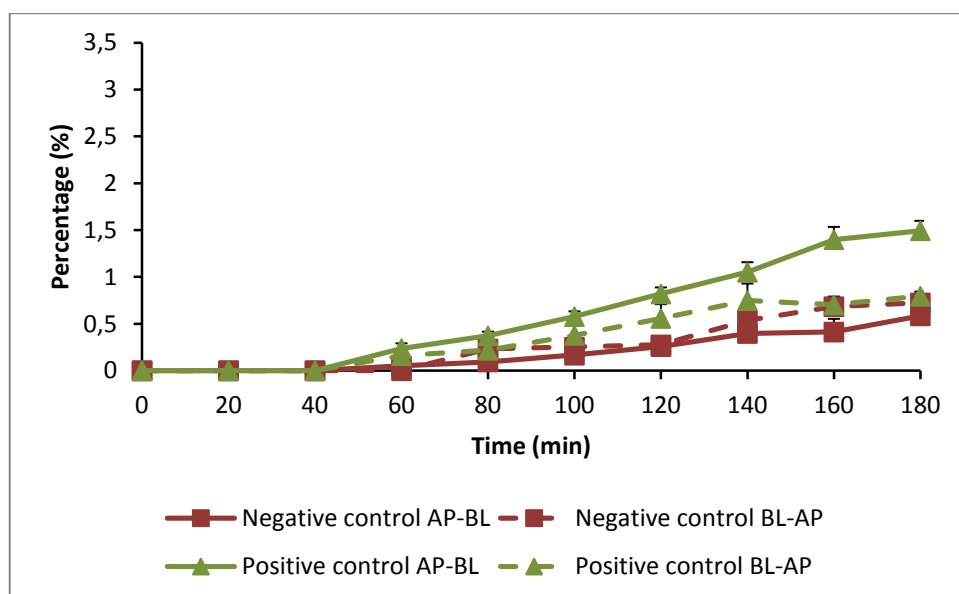


Figure 5.16: Bi-directional cumulative percentage transport of indinavir in the presence of verapamil plotted as a function of time

The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values calculated from the cumulative transport of indinavir in the presence of verapamil (positive control group) are presented in Table 5.5.

Table 5.5: P_{app} and ER values of indinavir transport in the presence of verapamil (positive control group) across excised porcine intestinal tissue

Cell	$P_{app} (x10^{-7})$		Efflux Ratio (ER)
	AP-BL	BL-AP	
1	8.87562	4.62739	0.521359
2	8.83972	5.0949	0.576364
3	8.84645	5.46483	0.617743
4	7.99649	4.29584	0.537216
Mean	8.63957	4.87074	0.563171
Stdev	0.429003	0.514108	0.043105

The mean apparent permeability coefficient (P_{app}) and the efflux ratio (ER) values for indinavir transport in the presence of verapamil are illustrated in Figure 5.17.

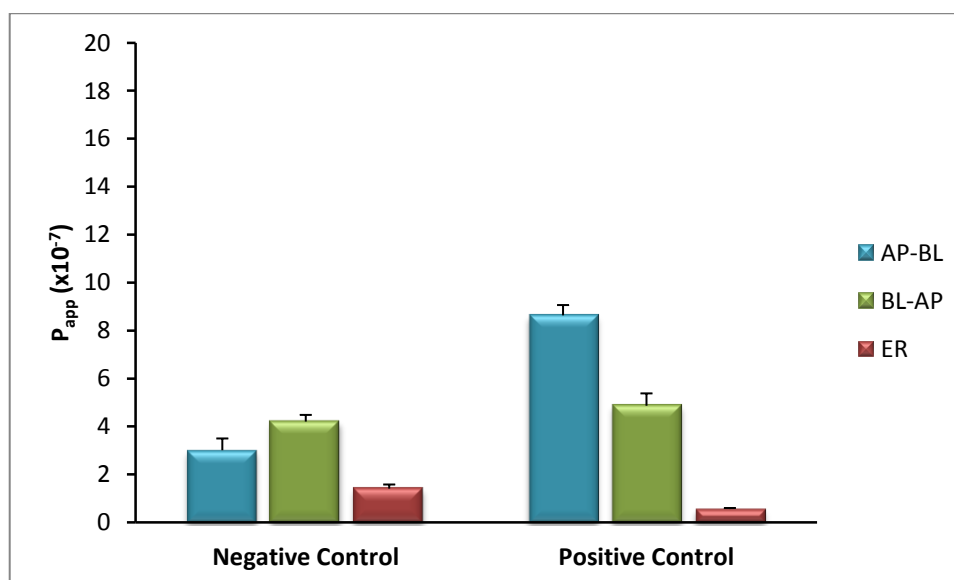


Figure 5.17: Mean P_{app} and ER values of indinavir transport in the presence of verapamil (positive control group) across excised porcine intestinal tissue

The transepithelial flux (J) and net flux (J_{net}) values for indinavir in the presence of verapamil (positive control) are presented in Table 5.6 and compared in Figure 5.18.

Table 5.6: The J and J_{net} values of indinavir transport in the presence of verapamil (positive control group) across excised porcine intestinal tissue

Cell	$J (\times 10^{-2})$		Net flux $J_{\text{net}} (\times 10^{-2})$
	AP-BL	BL-AP	
1	3.72776	1.9435	-1.78426
2	3.71268	2.13986	-1.57282
3	3.71551	2.29523	-1.42028
4	3.35853	1.80425	-1.55428
Mean	3.62862	2.04571	-1.58291
Stdev	0.180179	0.215928	0.150457

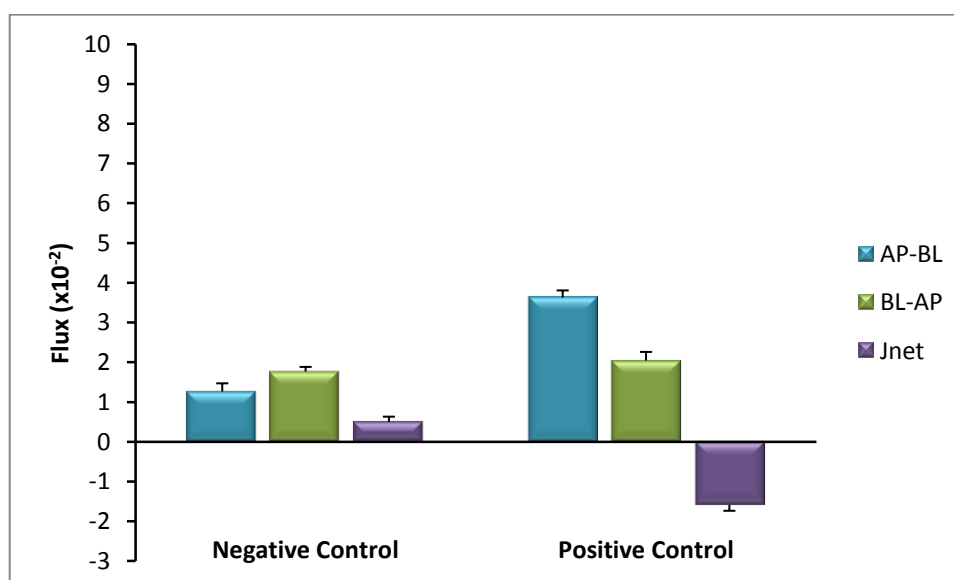


Figure 5.18: Mean J and J_{net} values for indinavir transport in the presence of verapamil (positive control) across excised porcine intestinal tissue

From the results obtained for the bi-directional transport of indinavir in the presence of verapamil, it is clear that a higher transport of indinavir occurred in the AP-BL direction (i.e. absorptive transport) compared to the transport in the BL-AP direction (i.e. secretory transport). The mean J_{net} value for indinavir transport changed from a positive value in the negative control group (0.005 ± 0.001) to a negative value in the positive control group (-0.158 ± 0.001) demonstrating that the net transport of indinavir occurred in the absorptive direction. Thus it can be concluded that verapamil was confirmed an appropriate compound to serve as the positive control to measure inhibition of efflux of indinavir across excised porcine jejunum. The results are in congruence with the results obtained by Bansal *et al.*

(2009:55), which indicated that verapamil is a P-gp inhibitor and will change the transport of P-gp substrates from the secretory direction to the absorptive direction.

5.4.3 *In vitro* transport of indinavir in the presence of crude extract and phytoconstituents of *Allium sativum*

5.4.3.1 Transport in the presence of *Allium sativum* crude extract

Bi-directional transport of indinavir in the presence of 5 mg/ml *A. sativum* crude extract across porcine intestinal tissue into the acceptor chamber expressed as a percentage relative to the initial concentration in the donor chamber (200 μ M) over a period of 180 min is presented in Table 5.7 for each time interval.

Table 5.7: The bi-directional cumulative transport (%) of indinavir in the presence of *Allium sativum* crude extract across excised porcine intestinal tissue

Time (min)	Cell 1		Cell 2		Cell 3		Cell 4	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20	0.00000	0.29085	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
40	0.00000	0.33131	0.00000	0.00000	0.00000	0.00000	0.00000	0.11482
60	0.14162	0.30697	0.16672	0.12643	0.21865	0.15730	0.25251	0.29422
80	0.22048	0.38480	0.23913	0.33000	0.33380	0.22114	0.26916	0.44255
100	0.35399	0.46876	0.35481	0.49899	0.53900	0.31493	0.39467	0.54053
120	0.42560	0.77359	0.26896	0.84219	0.69053	0.54358	0.52739	0.98213
140	0.61920	0.36579	0.81559	1.01960	0.90348	0.76809	0.67364	1.07592
160	0.72092	0.84009	0.86460	1.38485	1.07262	0.95728	0.80753	1.48470
180	0.97898	0.46225	1.09978	1.84063	1.28514	1.26307	1.06162	1.93537

The bi-directional cumulative transport (%) of indinavir across excised porcine intestinal tissue plotted as a function of time in the presence of *A. sativum* crude extract is shown in Figure 5.19.

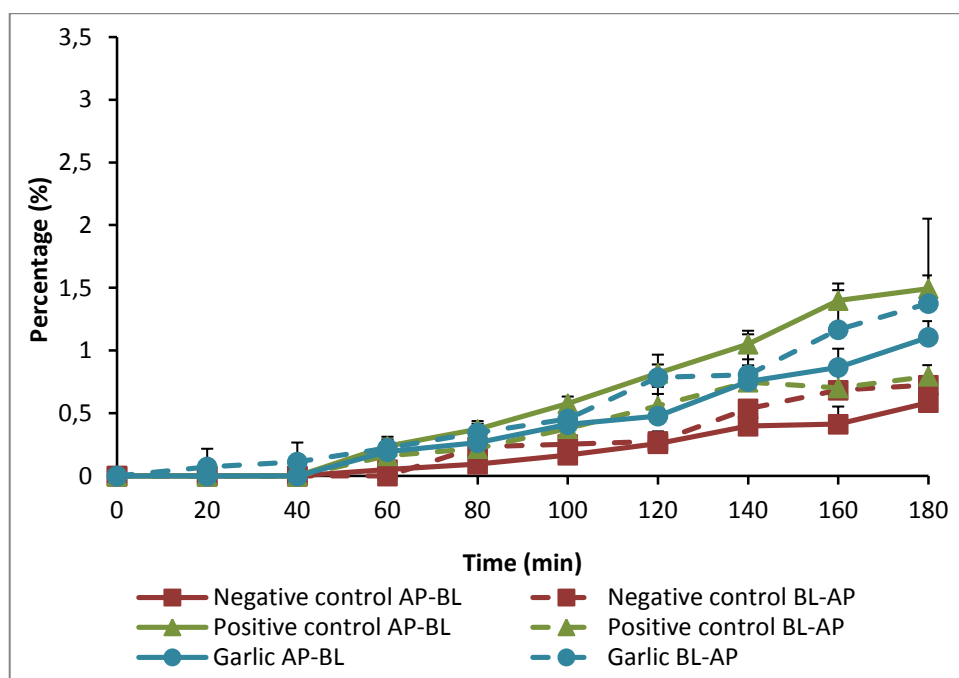


Figure 5.19: Bi-directional cumulative percentage transport of indinavir in the presence of *Allium sativum* crude extract plotted as a function of time

The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values calculated from the cumulative transport of indinavir in the presence of *A. sativum* crude extract are presented in Table 5.8.

Table 5.8: P_{app} and ER values of indinavir transport in the presence of *Allium sativum* crude extract across excised porcine intestinal tissue

Cell	P_{app} ($\times 10^{-7}$)		Efflux Ratio (ER)		
	AP-BL	BL-AP	<i>A. sativum</i>	Negative control	Positive control
1	5.08987	8.81773	1.732405	1.457467	0.521359
2	5.80257	9.55450	1.646598	1.235544	0.576364
3	4.15000	6.57180	1.583566	1.632445	0.617743
4	5.54013	9.86788	1.781163	1.334469	0.537216
Mean	5.145643	8.702978	1.685933	1.414981	0.563171
Stdev	0.726083	1.487411	0.088043	0.171051	0.043105

The mean apparent permeability coefficient (P_{app}) and efflux ratio (ER) values for indinavir transport in the presence of *A. sativum* crude extract are illustrated in Figure 5.20.

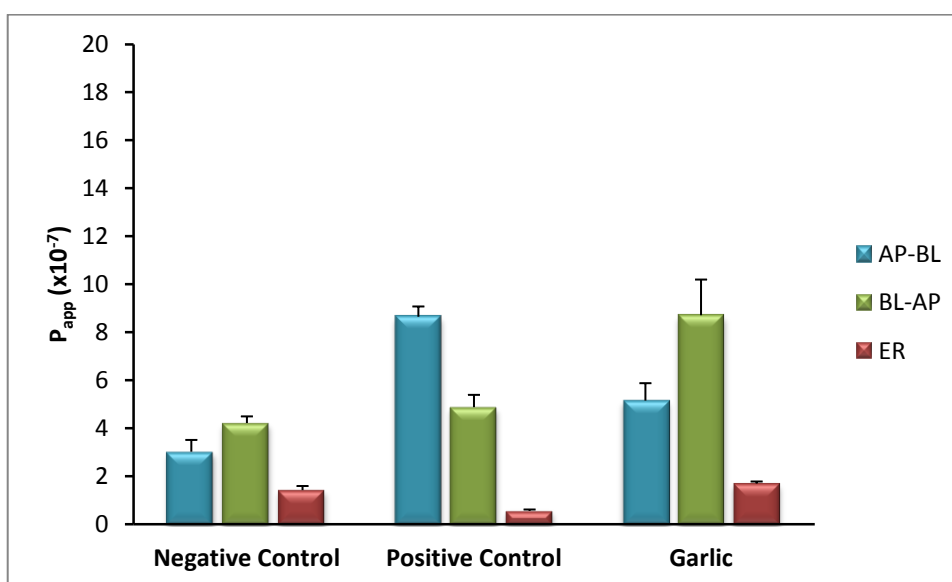


Figure 5.20: Mean P_{app} and ER values of the indinavir transport in the presence of *Allium sativum* crude extract across excised porcine intestinal tissue

The transepithelial flux (J) and net flux (J_{net}) values for indinavir transport in the presence of *A. sativum* crude extract are presented in Table 5.9 and compared in Figure 5.21.

Table 5.9: The J and J_{net} values of indinavir transport in the presence of *Allium sativum* crude extract across excised porcine intestinal tissue

Cell	$J (\times 10^{-2})$		Net Flux (J_{net}) ($\times 10^{-2}$)		
	AP-BL	BL-AP	<i>A. sativum</i>	Negative control	Positive control
1	2.13775	3.70345	1.56570	0.52119	-1.78426
2	2.43708	4.01289	1.57581	0.33914	-1.57282
3	1.74260	2.76016	1.01756	0.66136	-1.42028
4	2.32685	4.14451	1.81766	0.48291	-1.55428
Mean	2.16107	3.655253	1.494183	0.50115	-1.58291
Stdev	0.305137	0.624711	0.338420	0.132475	0.150457

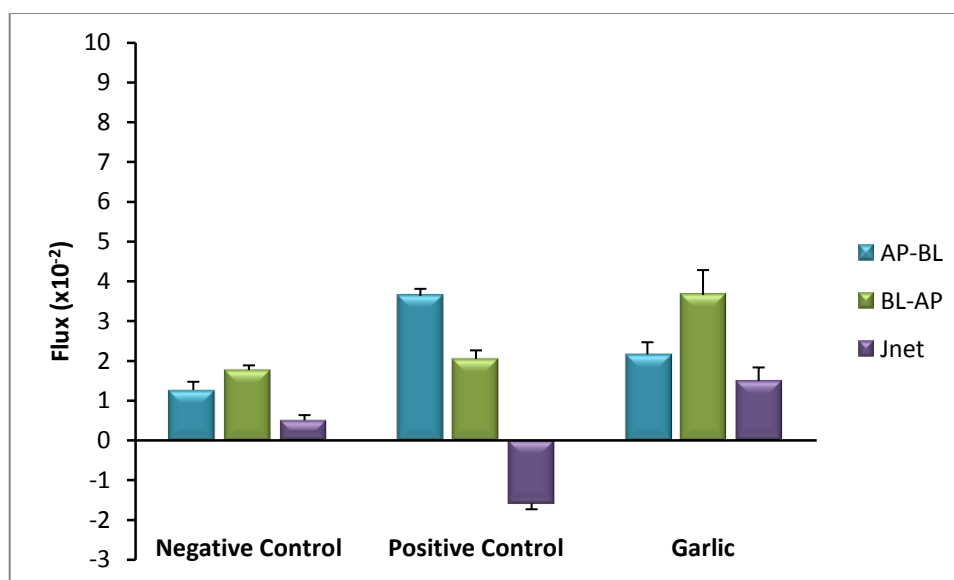


Figure 5.21: Mean J and J_{net} values for indinavir transport in the presence of *Allium sativum* crude extract across excised porcine intestinal tissue

From Tables 5.8 and 5.9 it is evident that although not statistically significant, the mean ER and J_{net} values for indinavir transport in the presence of *A. sativum* crude extract were higher than the values for the negative control group. Both the mean ER and the J_{net} values for indinavir transport in the presence of *A. sativum* crude extract correspond with the values of the negative control group, which indicate that the efflux of indinavir was not affected by the inclusion of *A. sativum* crude extract. These results were verified by applying Tukey's comparison test which classified *A. sativum* crude extract in a homogeneous group with the negative control group with respect to the ER and J_{net} values (Tables 5.35 and 5.38).

This signifies that no inhibition or promotion of P-gp related efflux of indinavir occurred. Conflicting data has been obtained during previous studies as to whether raw garlic inhibits P-gp mediated efflux (Foster *et al.*, 2001:176,183). A number of reports indicated that P-gp inhibition does occur, albeit the fact that this inhibition was low or moderate if compared to the inhibiting effects of the known P-gp inhibitor, verapamil (Zhou *et al.*, 2004:63). Clearly, future systematic studies are needed to confirm this phenomenon (Foster *et al.*, 2001:176,183).

5.4.3.2 Transport in the presence of L-alliin

Allicin does not exist in *A. sativum* until it is cut or crushed. This is due to the activation of the enzyme allinase, metabolising alliin to allicin. Alliin is a non-protein amino acid with four isomers of which one is present in *A. sativum* (Omar & Al-Wabel, 2010:52).

The bi-directional transport of indinavir in the presence of 20 μ M L-alliin across excised porcine intestinal tissue into the acceptor chamber expressed as a percentage relative to the initial concentration in the donor chamber (200 μ M) over a period of 180 min is presented in Table 5.10 for each time interval.

Table 5.10: The bi-directional cumulative transport (%) of indinavir in the presence of L-alliin across excised porcine intestinal tissue

Time (min)	Cell 1		Cell 2		Cell 3		Cell 4	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20	0.00000	0.00000	0.00000	0.33876	0.00000	0.33432	0.00000	0.14380
40	0.00000	0.11475	0.00000	0.38185	0.00000	0.39020	0.00000	0.19020
60	0.03447	0.21457	0.25336	0.53912	0.06061	0.79885	0.33439	0.17129
80	0.11032	0.29320	0.29744	0.61430	0.28945	0.70589	0.58164	0.40416
100	0.74462	0.53333	0.67146	0.68450	0.61553	0.93745	0.68697	0.37313
120	1.08767	0.61712	1.37845	0.80713	0.88882	0.84184	1.08046	0.30617
140	1.32234	0.97649	1.30769	0.33174	1.29397	0.97773	1.61099	0.46567
160	1.79498	1.28064	1.50066	0.67962	1.71524	0.54831	1.67542	0.38120
180	2.23150	1.81737	1.77646	0.83296	1.79264	0.78259	1.99124	0.71828

The bi-directional cumulative transport (%) of indinavir across excised porcine intestinal tissue plotted as a function of time in the presence of L-alliin is shown in Figure 5.22.

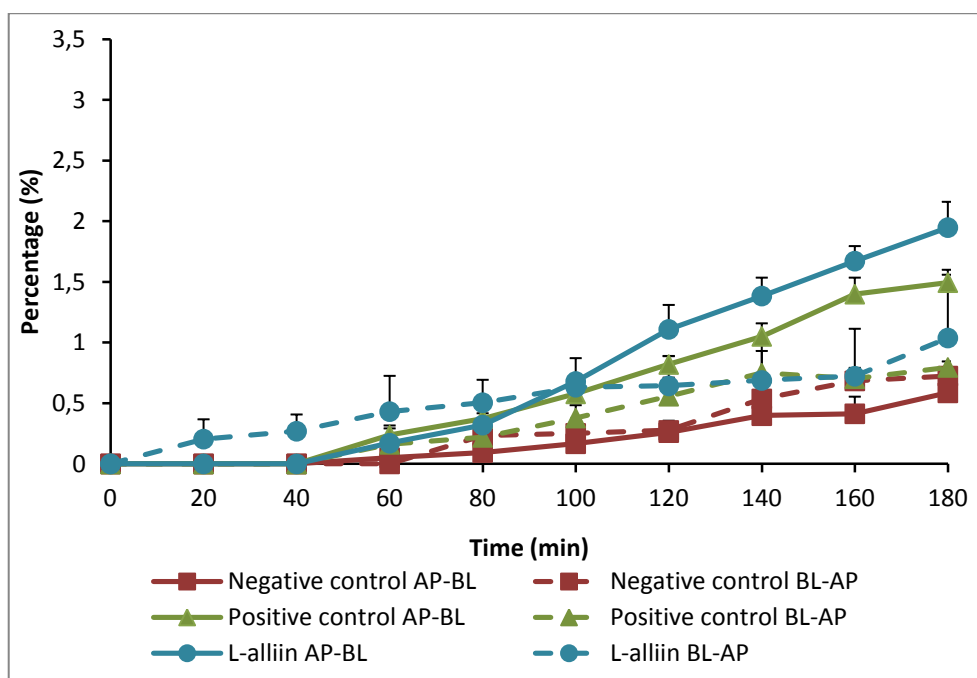


Figure 5.22: Bi-directional cumulative percentage transport of indinavir in the presence of L-alliin plotted as a function of time

The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values calculated from the cumulative transport of indinavir in the presence of L-alliin are presented in Table 5.11.

Table 5.11: P_{app} and ER values of indinavir transport in the presence of L-alliin across excised porcine intestinal tissue

Cell	Papp ($\times 10^{-7}$)		Efflux Ratio (ER)		
	AP-BL	BL-AP	L-alliin	Negative control	Positive control
1	12.216	3.32000	0.271775	1.457467	0.521359
2	10.436	2.98102	0.285648	1.235544	0.576364
3	10.6177	3.35928	0.316385	1.632445	0.617743
4	11.3629	2.80255	0.246640	1.334469	0.537216
Mean	11.15815	3.115713	0.280112	1.414981	0.563171
Stdev	0.811286	0.269117	0.029076	0.171051	0.043105

The mean apparent permeability coefficient (P_{app}) and the efflux ratio (ER) values for indinavir transport in the presence of L-alliin are illustrated in Figure 5.23.

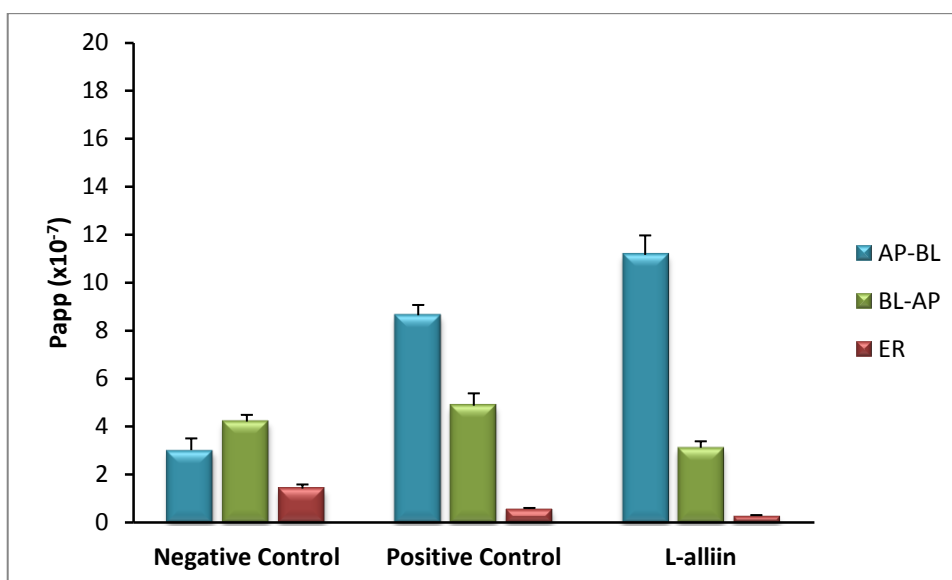


Figure 5.23: Mean P_{app} and ER values of indinavir transport in the presence of L-alliin across excised porcine intestinal tissue

The transepithelial flux (J) and net flux (J_{net}) values for indinavir transport in the presence of L-alliin are presented in Table 5.12 and compared in Figure 5.24.

Table 5.12: The J and J_{net} values of indinavir transport in the presence of L-alliin across excised porcine intestinal tissue

Cell	J ($\times 10^{-2}$)		Net Flux (J_{net}) ($\times 10^{-2}$)		
	AP-BL	BL-AP	L-alliin	Negative control	Positive control
1	5.13071	1.39390	-3.73681	0.52119	-1.78426
2	4.38311	1.25200	-3.13108	0.33914	-1.57282
3	4.45943	1.41090	-3.04853	0.66136	-1.42028
4	4.77243	1.17710	-3.59536	0.48291	-1.55428
Mean	4.68642	1.308475	-3.37795	0.50115	-1.58291
Stdev	0.340741	0.112904	0.339367	0.132475	0.150457

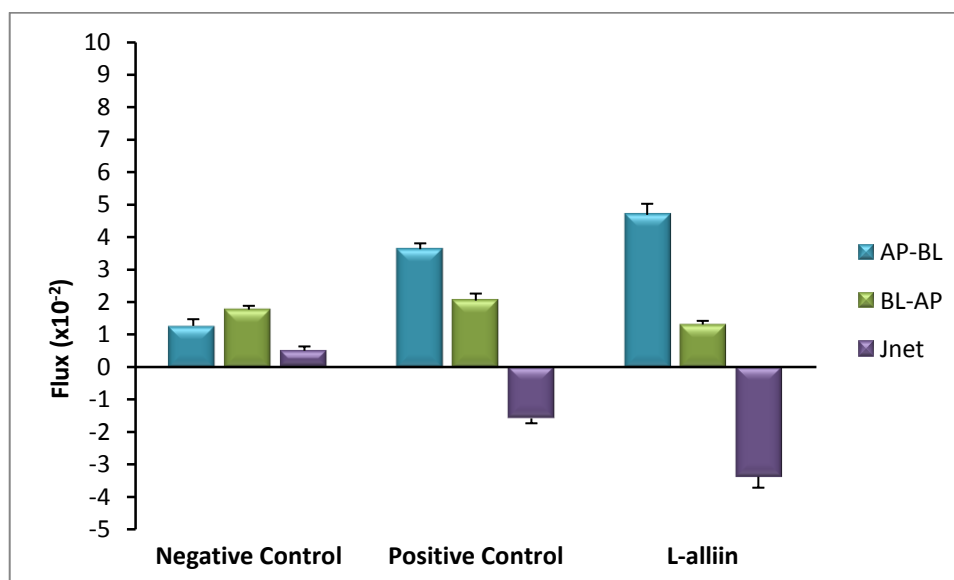


Figure 5.24: Mean J and J_{net} values of indinavir transport in the presence of L-alliin across excised porcine intestinal tissue

From Tables 5.11 and 5.12 it is evident that L-alliin was able to decrease the *in vitro* transport of indinavir in the BL-AP direction. The mean ER and J_{net} values were significantly lower ($p < 0.05$) compared to the values of the negative control group. The J_{net} value was reversed from a positive value for indinavir alone to a negative value in the presence of L-alliin resulting in a net influx of indinavir.

No statistical difference could be demonstrated between the mean ER values of the L-alliin group and the positive control group. The mean J_{net} value of the L-alliin group was

significantly lower than the value of the positive control group. This indicates that the inhibition of P-gp related efflux of indinavir by L-alliin was even greater than the effect of the positive control group with verapamil. Tukey's comparison test classified L-alliin into a homogeneous group with the positive control group for the ER value and into a group with a bigger effect than the positive control for the J_{net} value (Tables 5.36 and 5.39). These results signify the inhibition of indinavir efflux by L-alliin and indicate that L-alliin related P-gp inhibition is a more robust process than caused by verapamil. It may be concluded that L-alliin is involved in the inhibition of P-gp related efflux of indinavir as stated in a study done by Zhou *et al.* (2004:63) on *A. sativum*.

5.4.3.3 Transport in the presence of diallyl disulphide

The bi-directional transport of indinavir in the presence of 20 μ M diallyl disulphide across excised porcine intestinal tissue into the acceptor chamber expressed as a percentage relative to the initial concentration in the donor chamber (200 μ M) over a period of 180 min is presented in Table 5.13 for each time interval.

Table 5.13: The bi-directional cumulative transport (%) of indinavir in the presence of diallyl disulphide across excised porcine intestinal tissue

Time (min)	Cell 1		Cell 2		Cell 3		Cell 4	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
40	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
60	0.00000	0.00000	0.15740	0.00000	0.03897	0.00000	0.12967	0.00000
80	0.06757	0.14426	0.34581	0.12391	0.07200	0.09857	0.28983	0.13843
100	0.31940	0.18427	0.54203	0.22082	0.41797	0.20214	0.52914	0.23534
120	0.53667	0.18495	0.49670	0.30082	0.26142	0.20208	0.58059	0.21710
140	0.57429	0.28701	0.67613	0.32323	0.39764	0.33906	0.63964	0.40399
160	0.77596	0.39150	0.81255	0.39162	0.55208	0.39887	0.99132	0.42695
180	0.82103	0.49517	0.96506	0.52672	1.38855	0.51095	1.07660	0.44683

The bi-directional cumulative transport (%) of indinavir across excised porcine intestinal tissue plotted as a function of time in the presence of diallyl disulphide is shown in Figure 5.25.

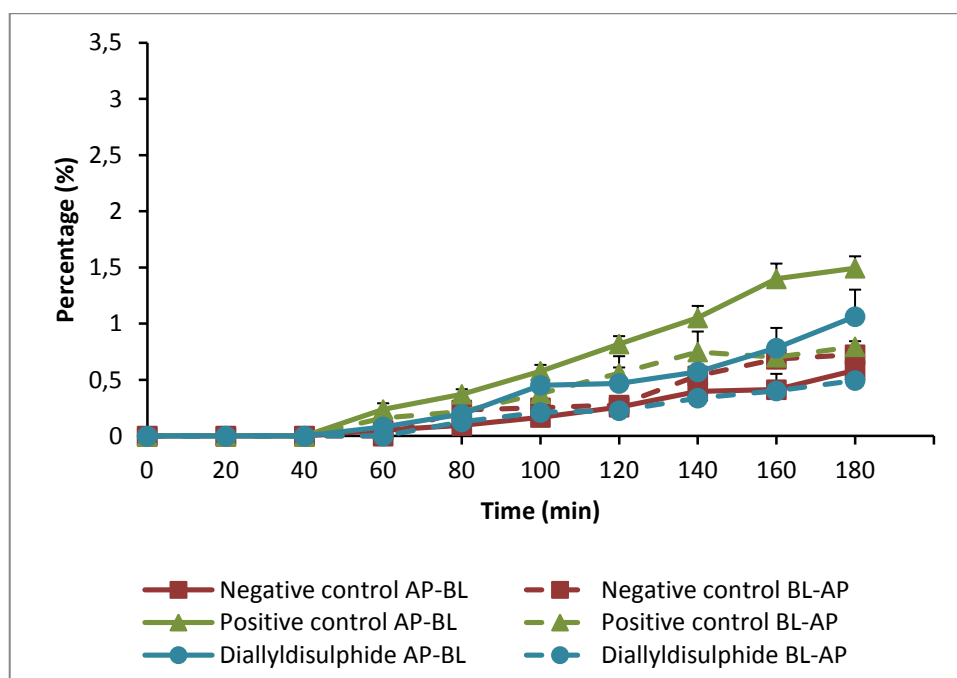


Figure 5.25: Bi-directional cumulative percentage transport of indinavir in the presence of diallyl disulphide plotted as a function of time

The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values calculated from the cumulative transport of indinavir in the presence of diallyl disulphide are presented in Table 5.14.

Table 5.14: P_{app} and ER values of indinavir transport in the presence of diallyl disulphide across excised porcine intestinal tissue

Cell	P_{app} ($\times 10^{-7}$)		Efflux Ratio (ER)		
	AP-BL	BL-AP	Diallyl disulphide	Negative control	Positive control
1	4.98081	2.61801	0.525621	1.457467	0.521359
2	5.38197	2.86499	0.532331	1.235544	0.576364
3	5.49399	2.77941	0.50590	1.632445	0.617743
4	6.07731	2.77445	0.456527	1.334469	0.537216
Mean	5.48352	2.759215	0.505095	1.414981	0.563171
Stdev	0.453036	0.102903	0.034267	0.171051	0.043105

The mean apparent permeability coefficient (P_{app}) and the efflux ratio (ER) values for indinavir transport in the presence of diallyl disulphide are illustrated in Figure 5.26.

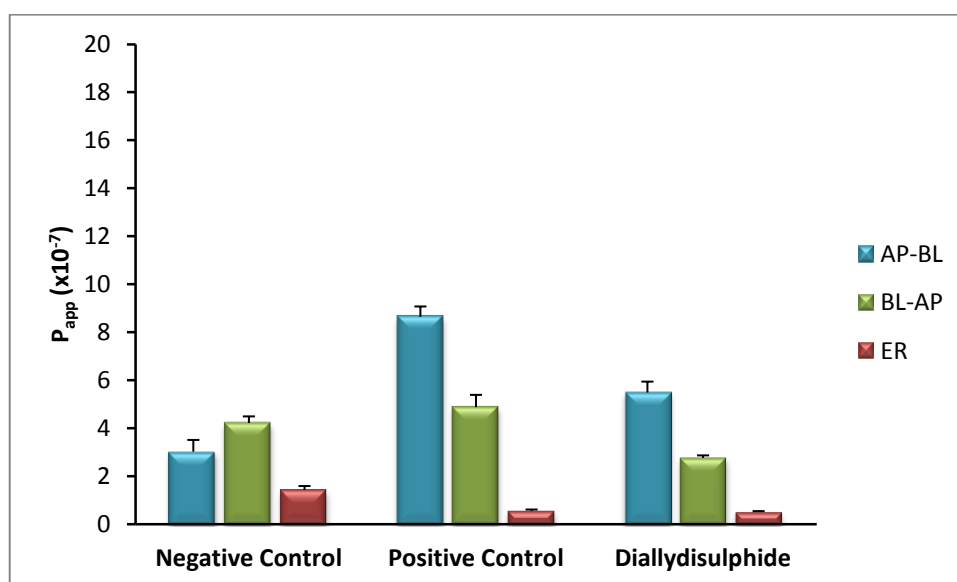


Figure 5.26: Mean P_{app} and ER values of indinavir transport in the presence of diallyl disulphide across excised porcine intestinal tissue

The transepithelial flux (J) and net flux (J_{net}) values for indinavir transport in the presence of diallyl disulphide are presented in Table 5.15 and compared in Figure 5.27.

Table 5.15: The J and J_{net} values of indinavir transport in the presence of diallyl disulphide across excised porcine intestinal tissue

Cell	J ($\times 10^{-2}$)		Net Flux (J_{net}) ($\times 10^{-2}$)		
	AP-BL	BL-AP	Diallyl disulphide	Negative control	Positive control
1	2.09194	1.09957	-0.99237	0.52119	-1.78426
2	2.26043	1.20330	-1.05713	0.33914	-1.57282
3	2.30747	1.16735	-1.14012	0.66136	-1.42028
4	2.55247	1.16527	-1.38720	0.48291	-1.55428
Mean	2.303078	1.158873	-1.14421	0.50115	-1.58291
Stdev	0.190275	0.043218	0.172915	0.132475	0.150457

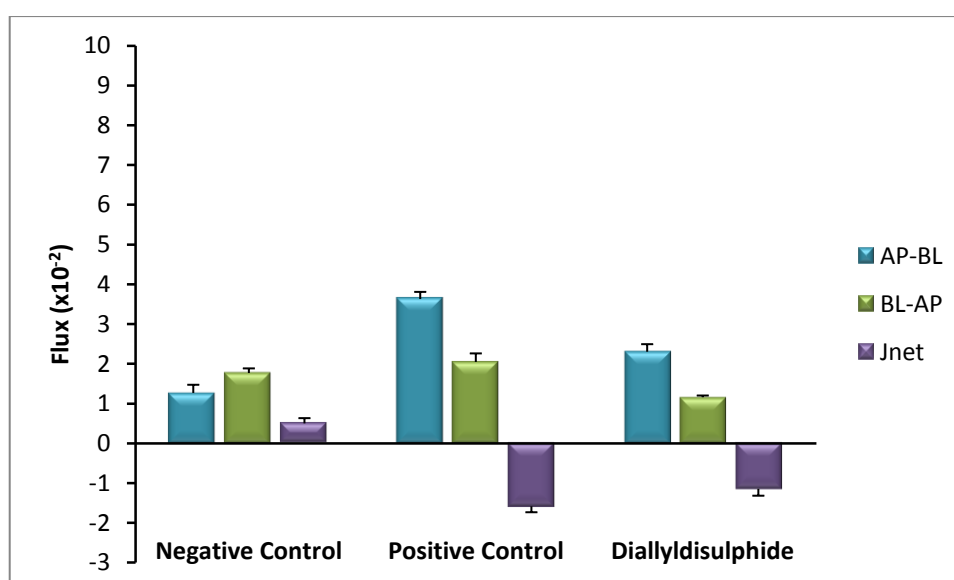


Figure 5.27: Mean J and J_{net} values for indinavir transport in the presence of diallyl disulphide across excised porcine intestinal tissue

From the P_{app} and ER values obtained for the transport of indinavir in the presence of diallyl disulphide, it is clear that the results correspond with that obtained from the positive control group. The BL-AP transport of indinavir was decreased in the presence of diallyl disulphide resulting in a net influx of indinavir. From Tables 5.14 and 5.15 it is evident that the mean ER and J_{net} values for indinavir transport in the presence of diallyl disulphide was lower compared to the values of the negative control group. Both the ER and J_{net} values differed significantly ($p < 0.05$) from the values of the negative control group.

The mean ER and J_{net} values were higher compared to the value of the positive control group, but were not statistically significant. From the above results it is clear that the results

for diallyl disulphide correspond with the results obtained for the positive control group indicating the inhibition of P-gp related efflux of indinavir by diallyl disulphide. The negative net flux value is a clear indication that diallyl disulphide decreased the BL-AP transport of indinavir. Tukey's comparison test also classified diallyl disulphide into a homogeneous group with the positive control for the ER and J_{net} values (Tables 5.36 and 5.39).

The results obtained following the concomitant administration of indinavir and diallyl disulphide signify the *in vitro* inhibition of P-gp related indinavir efflux caused by diallyl disulphide. This is in accordance with previous studies (Zhou *et al.*, 2004:63) which have demonstrated the same.

5.4.4 *In vitro* transport of indinavir in the presence of crude extract and phytoconstituents of *Citrus limonum*

5.4.4.1 Transport in the presence of *Citrus limonum* crude extract

The bi-directional transport of indinavir in the presence of 5mg/ml *C. limonum* crude extract across excised porcine intestinal tissue into the acceptor chamber expressed as a percentage relative to the initial concentration in the donor chamber (200 μ M) over a period of 180 min is presented in Table 5.16 for each time interval.

Table 5.16: Bi-directional cumulative transport (%) of indinavir in the presence of *Citrus limonum* crude extract across excised porcine intestinal tissue

Time (min)	Cell 1		Cell 2		Cell 3		Cell 4	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
40	0.00000	0.14492	0.00000	0.11993	0.00000	0.15818	0.00000	0.13506
60	0.06102	0.27644	0.06427	0.33375	0.02608	0.32525	0.00000	0.23731
80	0.11570	0.61090	0.09917	0.75150	0.09126	0.66948	0.12936	0.71405
100	0.20255	0.94936	0.17005	1.08186	0.17393	1.07213	0.18593	0.96412
120	0.26606	1.60828	0.20193	1.54681	0.13948	1.43236	0.25567	1.27589
140	0.32503	1.67078	0.24366	2.42611	0.25370	1.99088	0.27561	1.67010
160	0.41190	2.04685	0.36274	2.60163	0.41260	2.66704	0.41099	2.03669
180	0.52757	2.54491	0.57410	2.83178	0.57551	2.63463	0.55905	3.12494

The bi-directional cumulative transport (%) of indinavir across excised porcine intestinal tissue plotted as a function of time in the presence of *C. limonum* crude extract is shown in Figure 5.28.

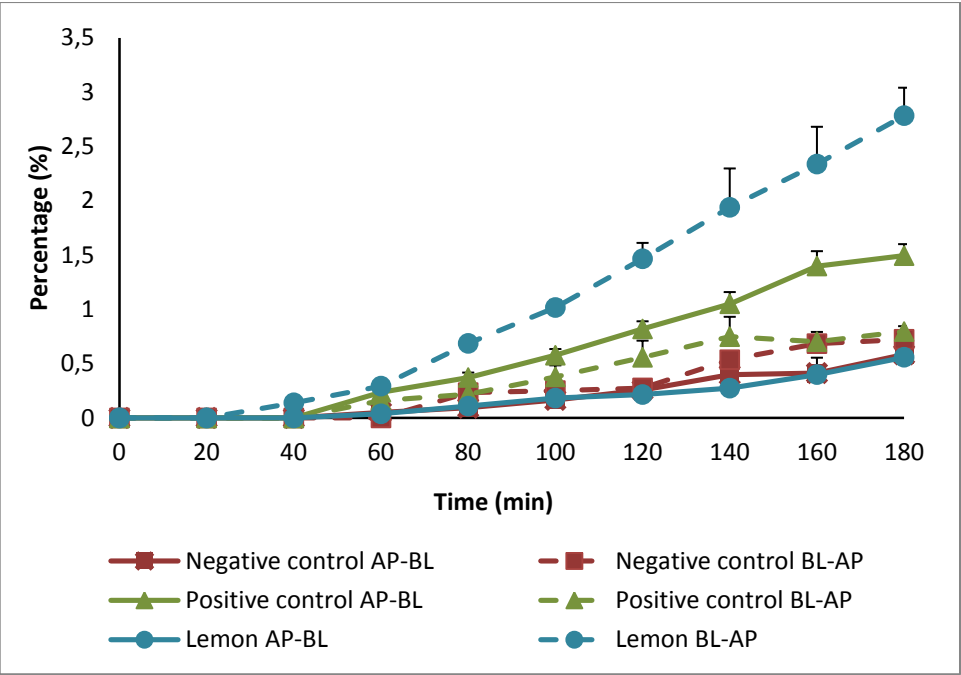


Figure 5.28: Bi-directional cumulative percentage transport of indinavir in the presence of *Citrus limonum* crude extract plotted as a function of time

The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values calculated from the cumulative transport of indinavir in the presence of *C. limonum* crude extract are presented in Table 5.17.

Table 5.17: P_{app} and ER values of indinavir transport in the presence of *Citrus limonum* crude extract across excised porcine intestinal tissue

Cell	P_{app} ($\times 10^{-7}$)		Efflux Ratio (ER)		
	AP-BL	BL-AP	<i>C. limonum</i>	Negative control	Positive control
1	2.82559	13.9585	4.940030	1.457467	0.521359
2	2.66946	16.7966	6.292134	1.235544	0.576364
3	2.76904	15.6817	5.663226	1.632445	0.617743
4	2.86858	15.1578	5.284078	1.334469	0.537216
Mean	2.783168	15.39865	5.544867	1.414981	0.563171
Stdev	0.086070	1.178477	0.579153	0.171051	0.043105

The mean apparent permeability coefficient (P_{app}) and efflux ratio (ER) values for indinavir transport in the presence of *C. limonum* crude extract are illustrated in Figure 5.29.

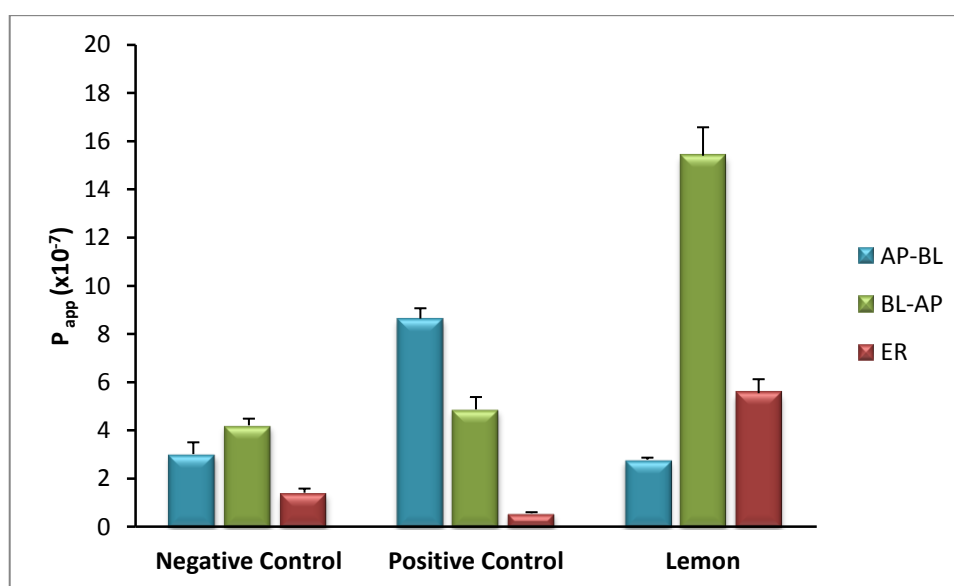


Figure 5.29: Mean P_{app} and ER values of indinavir transport in the presence of *Citrus limonum* crude extract across excised porcine intestinal tissue

The transepithelial flux (J) and net flux (J_{net}) values for indinavir transport in the presence of *C. limonum* crude extract are presented in Table 5.18 and compared in Figure 5.30.

Table 5.18: The J and J_{net} values of indinavir transport in the presence of *Citrus limonum* crude extract across excised porcine intestinal tissue

Cell	$J (\times 10^{-2})$		Net Flux (J_{net}) ($\times 10^{-2}$)		
	AP-BL	BL-AP	<i>C. limonum</i>	Negative control	Positive control
1	1.18675	5.86258	4.67583	0.52119	-1.78426
2	1.12117	7.05457	5.93340	0.33914	-1.57282
3	1.16300	6.58630	5.42330	0.66136	-1.42028
4	1.20480	6.36629	5.16149	0.48291	-1.55428
Mean	1.16893	6.467435	5.298505	0.50115	-1.58291
Stdev	0.036150	0.494953	0.524456	0.132475	0.150457

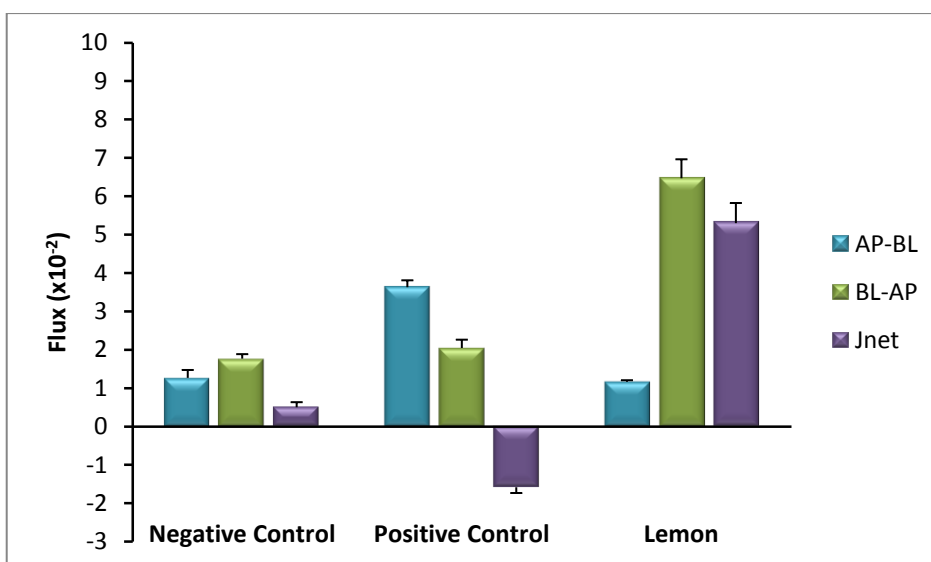


Figure 5.30: Mean J and J_{net} values for indinavir transport in the presence of *Citrus limonum* crude extract across excised porcine intestinal tissue

From above results it is clear that the transport of indinavir demonstrated a higher BL-AP flux compared to the AP-BL direction in the presence of *C. limonum* crude resulting in a net efflux of indinavir (i.e. secretory transport). Tables 5.17 and 5.18 indicate that the mean ER and J_{net} values for indinavir transport in the presence of *C. limonum* crude extract are significantly ($p < 0.05$) higher than the mean ER and J_{net} values of the negative control group. When comparing these values with the positive control group, the *C. limonum* crude extract displayed significantly higher mean ER and J_{net} values ($p < 0.05$).

The mean ER and J_{net} values obtained for *C. limonum* crude extract were therefore significantly ($p < 0.05$) higher than the values for the positive and negative control groups. It

can be concluded that the crude extract of *C. limonum* promoted P-gp related efflux of indinavir. This finding was confirmed by Tukey's comparison test (Tables 5.35 and 5.36).

Hesperidin and eriocitrin are the predominant flavonoids present in *C. limonum* and several studies demonstrated that P-gp mediated efflux of substrates can be inhibited by the concomitant administration of these compounds (González-Molina *et al.*, 2009:353). However, the promotion of active efflux, which was established in this study, is consistent with a study done by Lim *et al.* (2006:48) that demonstrated enhanced efflux of Rhodamine 123 with the addition of *C. limonum* extract.

5.4.4.2 Transport in the presence of hesperidin

As stated above, hesperidin and eriocitrin as well as small amounts of diosmetin 6,8-di-C-glucoside and diosmin are the main flavonoids present in *C. limonum* juice (González-Molina *et al.*, 2009:353).

The bi-directional transport of indinavir in the presence of 20 μ M hesperidin across excised porcine intestinal tissue into the acceptor chamber expressed as a percentage relative to the initial concentration in the donor chamber (200 μ M) over a period of 180 min is presented in Table 5.19 for each time interval.

Table 5.19: The bi-directional cumulative transport (%) of indinavir in the presence of hesperidin across excised porcine intestinal tissue

Time (min)	Cell 1		Cell 2		Cell 3		Cell 4	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20	0.00000	0.17536	0.00000	0.24512	0.00000	0.20733	0.00000	0.22530
40	0.00000	0.40727	0.00000	0.43339	0.00000	0.35015	0.00000	0.37639
60	0.00000	1.03609	0.00000	0.85053	0.00000	0.84819	0.00000	0.70840
80	0.08326	1.15011	0.08715	1.18950	0.12812	1.23049	0.11591	0.97292
100	0.16035	1.84748	0.16178	1.74956	0.22911	1.36382	0.30269	1.49255
120	0.39166	2.20355	0.33067	1.80983	0.34174	1.89418	0.47072	1.97862
140	0.54467	2.46266	0.51199	2.15475	0.56270	2.16279	0.65417	2.38330
160	0.48635	3.08453	0.64375	2.72288	0.80409	2.64737	0.82425	2.71543
180	0.99610	3.39301	0.82734	2.82090	1.00843	2.94071	1.02401	3.11210

The bi-directional cumulative transport (%) of indinavir across excised porcine intestinal tissue plotted as a function of time in the presence of hesperidin is shown in Figure 5.31.

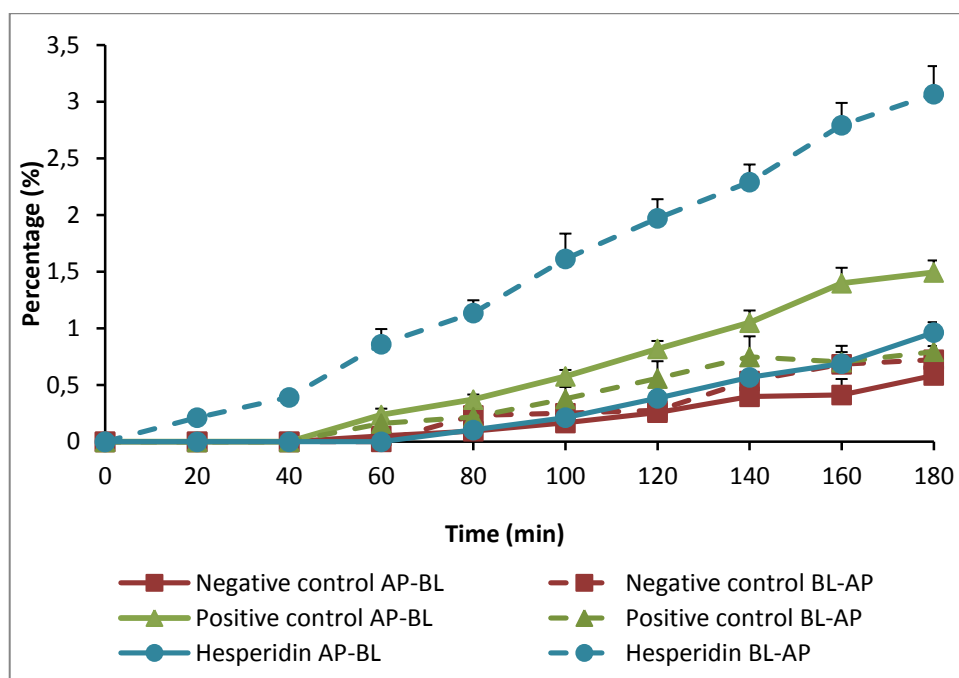


Figure 5.31: Bi-directional cumulative percentage transport of indinavir in the presence of hesperidin plotted as a function of time

The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values calculated from the cumulative transport of indinavir in the presence hesperidin are presented in Table 5.20.

Table 5.20: P_{app} and ER values of indinavir transport in the presence of hesperidin across excised porcine intestinal tissue

Cell	P_{app} ($\times 10^{-7}$)		Efflux Ratio (ER)		
	AP-BL	BL-AP	Hesperidin	Negative control	Positive control
1	4.63761	18.5501	3.999927	1.457467	0.521359
2	4.42029	15.5423	3.516127	1.235544	0.576364
3	5.29004	15.8555	2.997236	1.632445	0.617743
4	5.63377	16.9688	3.011980	1.334469	0.537216
Mean	4.995428	16.72918	3.381317	1.414981	0.563171
Stdev	0.563647	1.359555	0.477766	0.171051	0.043105

The mean apparent permeability coefficient (P_{app}) and the efflux ratio (ER) values of indinavir transport in the presence of hesperidin are illustrated in Figure 5.32.

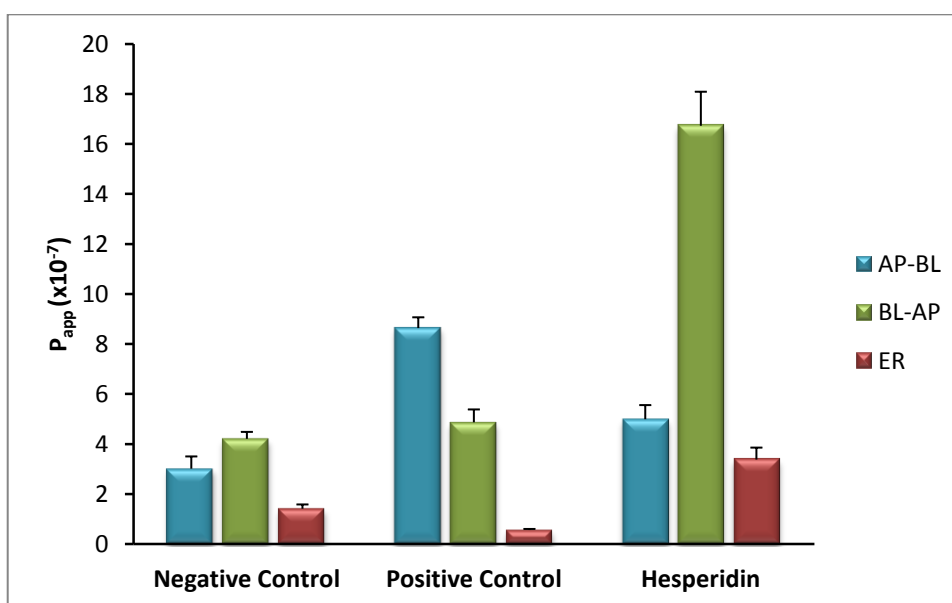


Figure 5.32: Mean P_{app} and ER values of indinavir transport in the presence of hesperidin across excised porcine intestinal tissue

The transepithelial flux (J) and net flux (J_{net}) values for indinavir in the presence of hesperidin are presented in Table 5.21 and compared in Figure 5.33.

Table 5.21: The J and J_{net} values of indinavir transport in the presence of hesperidin across excised porcine intestinal tissue

Cell	J ($\times 10^{-2}$)		Net Flux (J_{net}) ($\times 10^{-2}$)		
	AP-BL	BL-AP	Hesperidin	Negative control	Positive control
1	1.94780	7.79104	5.84324	0.52119	-1.78426
2	1.85652	6.52775	4.67123	0.33914	-1.57282
3	2.22182	6.65931	4.43749	0.66136	-1.42028
4	2.36619	7.12688	4.76069	0.48291	-1.55428
Mean	2.098083	7.026245	4.928163	0.50115	-1.58291
Stdev	0.236735	0.571016	0.625083	0.132475	0.150457

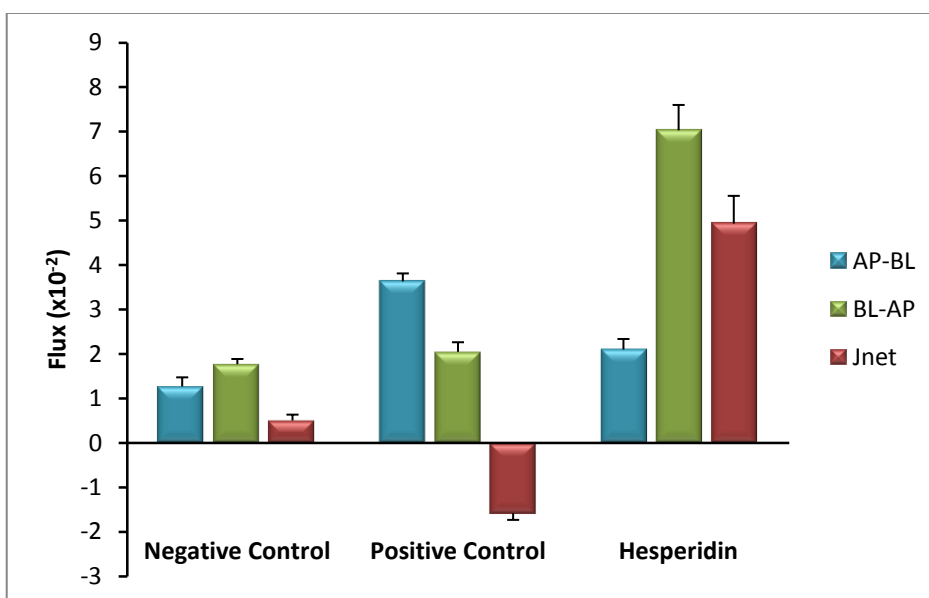


Figure 5.33: Mean J and J_{net} values for indinavir transport in the presence of hesperidin across excised porcine intestinal tissue

A significantly higher transport of indinavir in the secretory direction (BL-AP) in the presence of hesperidin has been demonstrated in the present study. From Tables 5.20 and 5.21 it is evident that both the ER and J_{net} values were significantly higher ($p < 0.05$) in this group than the values of the negative control group. Tukey's comparison test classified hesperidin in a group of its own with respect to the ER value. With regards to the J_{net} value, hesperidin was classified in a homogeneous group with *C. limonum* crude extract apart from the positive and negative control groups (Tables 5.35, 5.36, 5.38 and 5.39).

As the mean ER and J_{net} values obtained for indinavir transport in the presence of hesperidin were significantly higher compared to the positive and negative control groups, it is evident that the transport of indinavir in the presence of hesperidin, displayed net efflux. Thus it can be concluded that hesperidin acts as a potent P-gp promoter. Co-administration of hesperidin with compounds that are substrates for active efflux transporters such as P-gp could therefore result in lower plasma levels of the substrate (e.g. indinavir). Furthermore, these results are in congruence with the data obtained for the crude extract of *C. limonum* in the present study. However, the data from the present study stands in direct contrast to that demonstrated in several previous studies which indicate that flavonoids act as inhibitors, and not promoters, of P-gp (Bansal *et al.*, 2009:54). Future investigations are needed to clarify the exact mechanisms underlying the influence of hesperidin and *C. limonum* crude extract on P-gp mediated efflux.

5.4.4.3 Transport in the presence of eriocitrin

The bi-directional transport of indinavir in the presence of 20 μM eriocitrin across excised porcine intestinal tissue into the acceptor chamber expressed as a percentage relative to the initial concentration in the donor chamber (200 μM) over a period of 180 min is presented in Table 5.22 for each time interval.

Table 5.22: The bi-directional cumulative transport (%) of indinavir in the presence of eriocitrin across excised porcine intestinal tissue

Time (min)	Cell 1		Cell 2		Cell 3		Cell 4	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
40	0.10687	0.00000	0.05773	0.00000	0.09688	0.00000	0.06408	0.00000
60	0.17031	0.11810	0.11300	0.13304	0.17029	0.06081	0.14993	0.10907
80	0.21155	0.22032	0.20074	0.16201	0.21837	0.26279	0.24348	0.37288
100	0.31315	0.36740	0.27103	0.29993	0.21646	0.64466	0.33677	0.54425
120	0.36219	0.75311	0.36381	0.47980	0.39035	0.50555	0.53390	0.83095
140	0.49209	0.94252	0.58978	0.77448	0.49365	0.91368	0.91161	1.03841
160	0.61945	1.31515	0.63213	1.06150	0.66434	1.07818	0.88013	1.45482
180	0.71835	1.44281	0.86314	1.57383	0.86643	1.36803	1.05045	1.49052

The bi-directional cumulative transport (%) of indinavir across excised porcine intestinal tissue plotted as a function of time in the presence of the eriocitrin is shown in Figure 5.34.

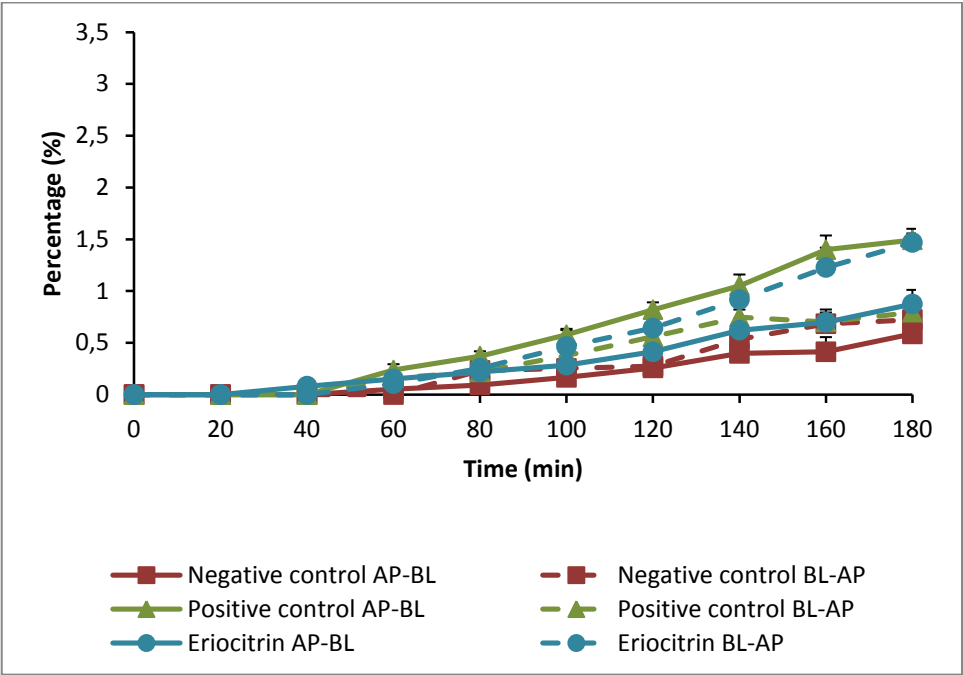


Figure 5.34: Bi-directional cumulative percentage transport of indinavir in the presence of eriocitrin plotted as a function of time

The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values calculated from the cumulative transport of indinavir in the presence of eriocitrin are presented in Table 5.23.

Table 5.23: P_{app} and ER values of indinavir transport in the presence of eriocitrin across excised porcine intestinal tissue

Cell	P_{app} ($\times 10^{-7}$)		Efflux Ratio (ER)		
	AP-BL	BL-AP	Eriocitrin	Negative control	Positive control
1	3.80339	8.21588	2.160149	1.457467	0.521359
2	4.44790	7.56030	1.699747	1.235544	0.576364
3	4.28171	7.41801	1.732489	1.632445	0.617743
4	5.98621	8.83199	1.475390	1.334469	0.537216
Mean	4.629803	8.006545	1.766944	1.414981	0.563171
Stdev	0.944644	0.650817	0.285958	0.171051	0.043105

The mean apparent permeability coefficient (P_{app}) and efflux ratio (ER) values for indinavir transport in the presence of eriocitrin are illustrated in Figure 5.35.

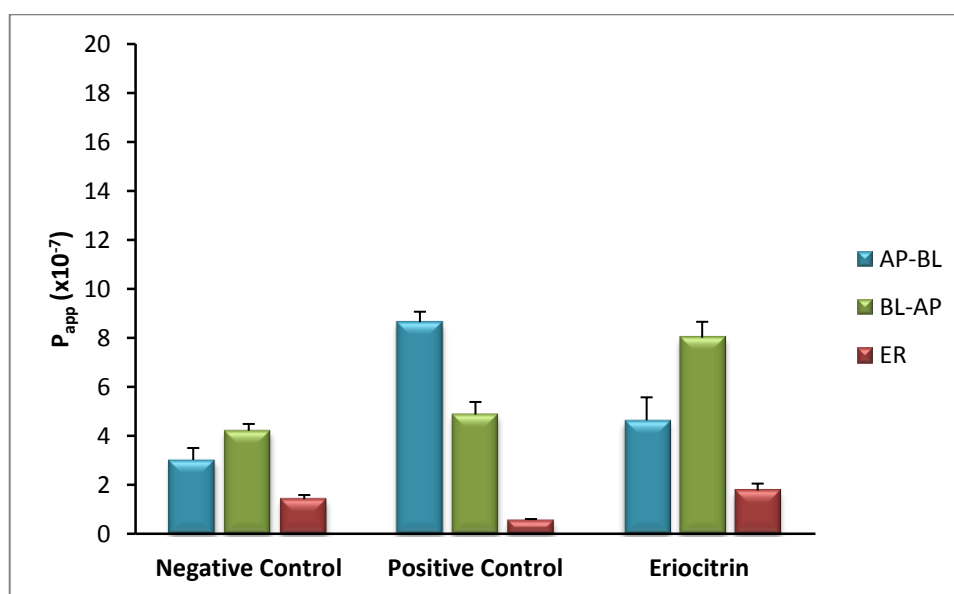


Figure 5.35: Mean P_{app} and ER values of the indinavir transport in the presence of eriocitrin across excised porcine intestinal tissue

The transepithelial flux (J) and net flux (J_{net}) values for indinavir in the presence of eriocitrin are presented in Table 5.24 and compared in Figure 5.36.

Table 5.24: The J and J_{net} values of indinavir transport in the presence of eriocitrin across excised porcine intestinal tissue

Cell	J ($\times 10^{-2}$)		Net Flux (J_{net}) ($\times 10^{-2}$)		
	AP-BL	BL-AP	Eriocitrin	Negative control	Positive control
1	1.59742	3.45067	1.85325	0.52119	-1.78426
2	1.86812	3.17532	1.30720	0.33914	-1.57282
3	1.79832	3.11556	1.31724	0.66136	-1.42028
4	2.51421	3.70944	1.19523	0.48291	-1.55428
Mean	1.944518	3.362748	1.418230	0.50115	-1.58291
Stdev	0.396752	0.273348	0.295239	0.132475	0.150457

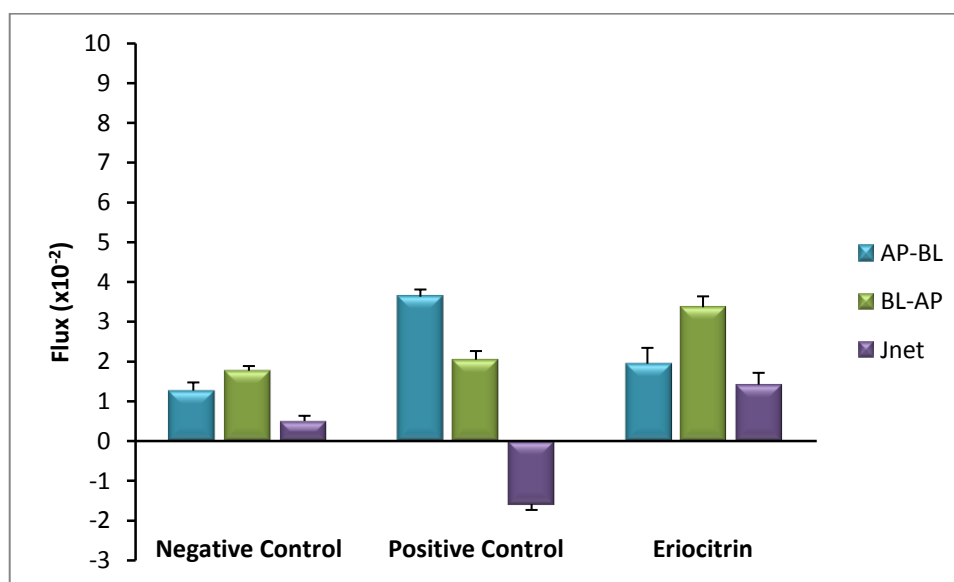


Figure 5.36: Mean J and J_{net} values of indinavir transport in the presence of eriocitrin across excised porcine intestinal tissue

As was the case with *C. limonum* crude extract and hesperidin, eriocitrin displayed P-gp promoting properties in the present study. This is clear in the results obtained from this experimental group, where the transport of indinavir was increased in the BL-AP direction in the presence of eriocitrin. Furthermore, Tables 5.23 and 5.24 indicate that the mean ER and J_{net} values of indinavir transport in the presence of eriocitrin was higher than the values for the negative control group, albeit the fact that no statistical significance could be demonstrated.

However, a significant difference between the ER and J_{net} values of this group and the positive control could be demonstrated ($p < 0.05$).

The transport of indinavir in the presence of eriocitrin corresponded with the results obtained for the negative control group (i.e. indinavir alone), which indicates that eriocitrin did not have a significant effect on P-gp related efflux. The accuracy of these results was confirmed by Tukey's comparison test which classified eriocitrin in a homogeneous group with the negative control group for both the mean ER and J_{net} values (Tables 5.35 and 5.38).

5.4.5 *In vitro* transport of indinavir in the presence of crude extract and phytoconstituents of *Beta vulgaris*

5.4.5.1 Transport in the presence of *Beta vulgaris* crude extract

The bi-directional transport of indinavir in the presence of 5 mg/ml *B. vulgaris* crude extract across excised porcine intestinal tissue into the acceptor chamber expressed as a percentage relative to the initial concentration in the donor chamber (200 μ M) over a period of 180 min is presented in Table 5.25 for each time interval.

Table 5.25: The bi-directional cumulative transport (%) of indinavir in the presence of *Beta vulgaris* crude extract across excised porcine intestinal tissue

Time (min)	Cell 1		Cell 2		Cell 3		Cell 4	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20	0.10787	0.00000	0.00000	0.00000	0.15281	0.00000	0.00000	0.00000
40	0.11726	0.22868	0.16124	0.21591	0.14220	0.25529	0.07853	0.22751
60	0.22842	0.48040	0.39677	0.54347	0.20562	0.51123	0.15694	0.47414
80	0.34421	0.82194	0.78264	0.92210	0.27540	0.67820	0.44675	0.78621
100	0.52189	1.30962	1.17936	1.32685	0.37964	1.23162	0.68394	1.21408
120	0.74838	1.69145	1.57039	1.66908	0.60023	1.33873	0.94091	1.56592
140	1.06006	2.01447	2.01582	2.01488	0.78849	1.73330	1.17764	1.73944
160	1.35075	2.40828	2.54133	2.32981	1.02616	2.01076	1.56313	2.17950
180	1.74600	2.79085	2.96841	2.68270	1.25092	2.18414	1.90549	2.60061

The bi-directional cumulative transport (%) of indinavir across excised porcine intestinal tissue plotted as a function of time in the presence of *B. vulgaris* crude extract is shown in Figure 5.37.

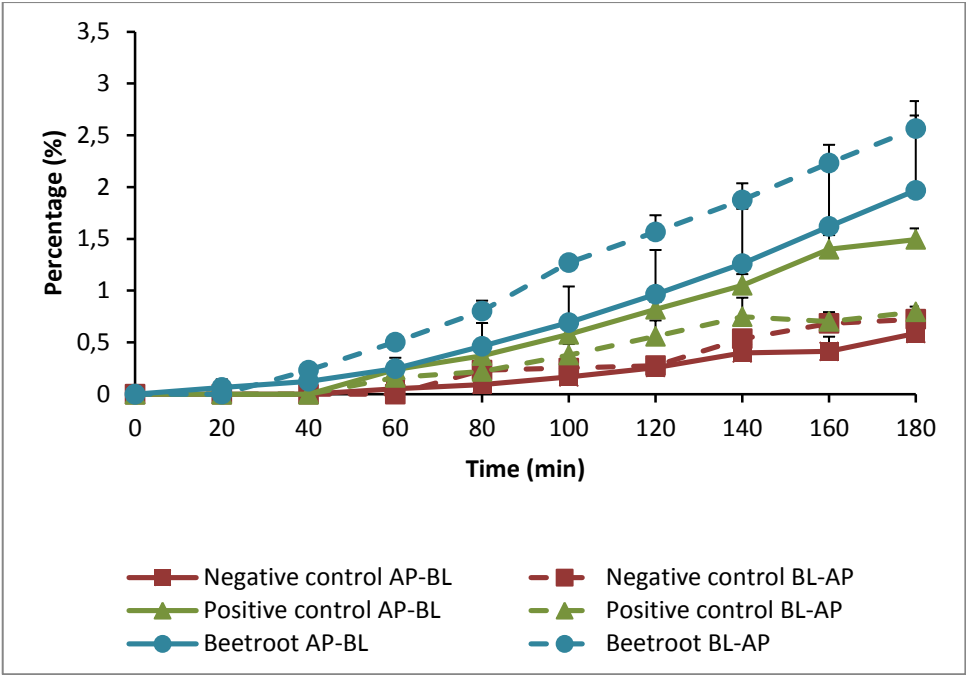


Figure 5.37: Bi-directional cumulative percentage transport of indinavir in the presence of *Beta vulgaris* crude extract plotted as a function of time

The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values calculated from the cumulative transport of indinavir in the presence the *B. vulgaris* crude extract are presented in Table 5.26.

Table 5.26: P_{app} and ER values of indinavir transport in the presence of *Beta vulgaris* crude extract across excised porcine intestinal tissue

Cell	P_{app} ($\times 10^{-7}$)		Efflux Ratio (ER)		
	AP-BL	BL-AP	<i>B. vulgaris</i>	Negative control	Positive control
1	11.6792	15.6127	1.336793	1.457467	0.521359
2	16.3703	15.1031	0.922593	1.235544	0.576364
3	11.2826	12.5294	1.110504	1.632445	0.617743
4	10.2644	14.1655	1.380054	1.334469	0.537216
Mean	12.39913	14.35268	1.187486	1.414981	0.563171
Stdev	2.713681	1.355257	0.212501	0.171051	0.043105

The mean apparent permeability coefficient (P_{app}) and the efflux ratio (ER) values for indinavir transport in the presence of *B. vulgaris* crude extract are illustrated in Figure 5.38.

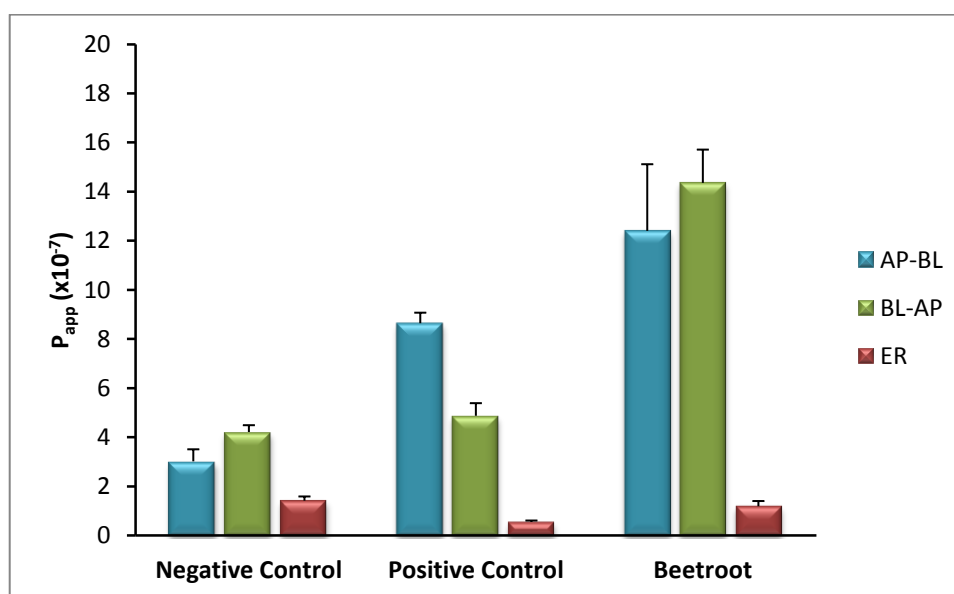


Figure 5.38: Mean P_{app} and ER values of indinavir transport in the presence of *Beta vulgaris* crude extract across excised porcine intestinal tissue

The transepithelial flux (J) and net flux (J_{net}) values for indinavir transport in the presence of *B. vulgaris* crude extract are presented in Table 5.27 and compared in Figure 5.39.

Table 5.27: The J and J_{net} values of indinavir transport in the presence of *Beta vulgaris* crude extract across excised porcine intestinal tissue

Cell	J ($\times 10^{-2}$)		Net Flux (J_{net}) ($\times 10^{-2}$)		
	AP-BL	BL-AP	<i>B. vulgaris</i>	Negative control	Positive control
1	4.90527	6.55733	1.65206	0.52119	-1.78426
2	6.87553	6.34331	-0.53222	0.33914	-1.57282
3	4.73869	5.26234	0.52365	0.66136	-1.42028
4	4.31107	5.94951	1.63844	0.48291	-1.55428
Mean	5.20764	6.028123	0.820483	0.50115	-1.58291
Stdev	1.139742	0.569212	1.045385	0.132475	0.150457

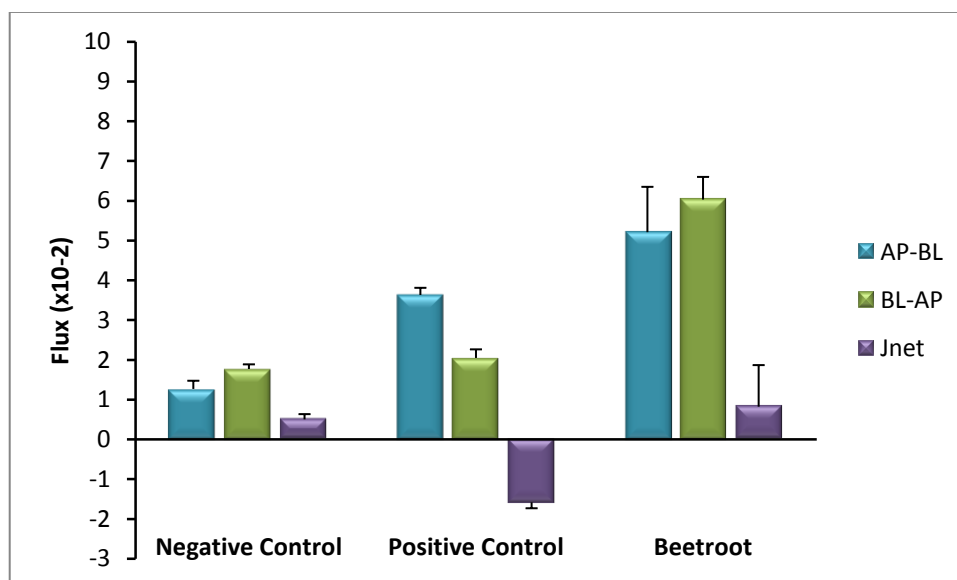


Figure 5.39: Mean J and J_{net} values for indinavir transport in the presence of *Beta vulgaris* crude extract across excised porcine intestinal tissue

Both the AP-BL and BL-AP transport of indinavir was increased in the presence of *B. vulgaris* crude extract. However, the mean ER and J_{net} values were similar to the values obtained for the negative control group.

In Tables 5.26 and 5.27 no statistically significant differences could be demonstrated between the mean ER and J_{net} values of indinavir transport in the presence of *B. vulgaris* crude extract. However, the mean ER and J_{net} values obtained in this group were significantly higher than the values of the positive control group ($p < 0.05$). It can therefore be concluded that *B. vulgaris* crude extract did not have a significant effect on P-gp mediated indinavir efflux. These results can be substantiated by Tukey's comparison test which

classified *B. vulgaris* crude extract into a homogeneous group together with the negative control for the mean ER and J_{net} values (Tables 5.35 and 5.38).

5.4.5.2 Transport in the presence of betaine monohydrate

The bi-directional transport of indinavir in the presence of 20 μM betaine monohydrate across excised porcine intestinal tissue into the acceptor chamber expressed as a percentage relative to the initial concentration in the donor chamber (200 μM) over a period of 180 min is presented in Table 5.28 for each time interval.

Table 5.28: The bi-directional cumulative transport (%) of indinavir in the presence of betaine monohydrate across excised porcine intestinal tissue

Time (min)	Cell 1		Cell 2		Cell 3		Cell 4	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20	0.15281	0.27450	0.11646	0.31089	0.00000	0.12736	0.09134	0.09804
40	0.14220	0.21549	0.12426	0.17996	0.09440	0.17092	0.15414	0.13104
60	0.20562	0.43959	0.24587	0.32717	0.22052	0.27054	0.27022	0.26086
80	0.27540	0.76047	0.34982	0.61491	0.48401	0.50007	0.41781	0.47872
100	0.37964	1.15674	0.51007	0.84726	0.74241	0.68964	0.66024	0.74918
120	0.60023	1.56031	0.82485	1.37582	1.01089	1.11793	0.92545	1.08688
140	0.78849	2.09889	1.10153	1.71965	1.42510	1.46740	1.22978	1.27455
160	1.02616	2.41637	1.46112	2.03817	1.71410	1.79403	1.48471	1.65562
180	1.25092	2.97048	1.72784	2.48958	2.05363	2.10302	1.88670	1.87886

The bi-directional cumulative transport (%) of indinavir across excised porcine intestinal tissue plotted as a function of time in the presence of betaine monohydrate is shown in Figure 5.40.

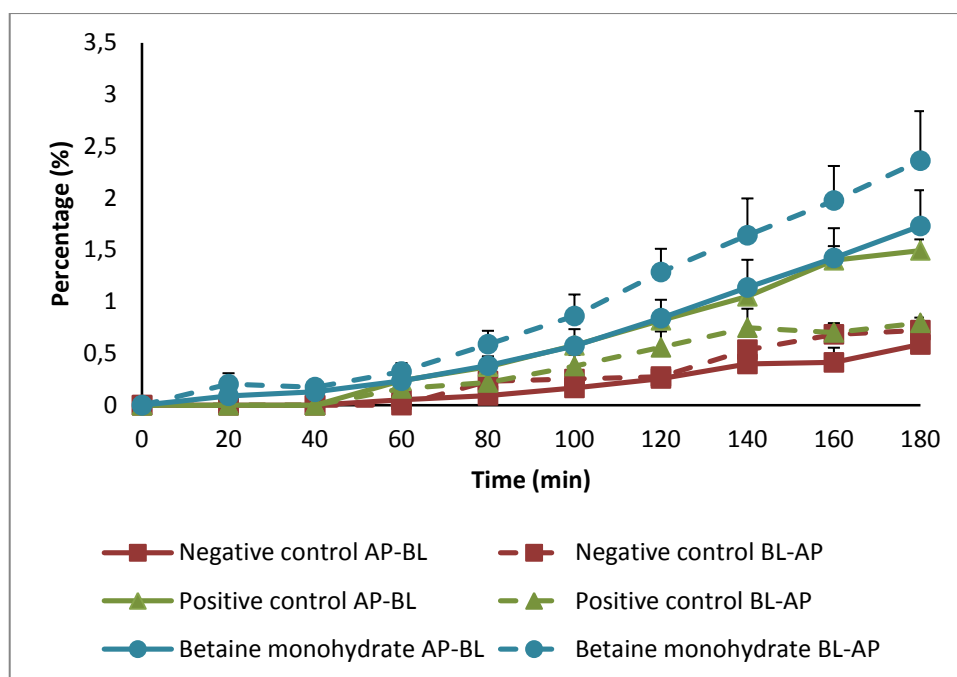


Figure 5.40: Bi-directional cumulative percentage transport of indinavir in the presence of betaine monohydrate plotted as a function of time

The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values calculated from the cumulative transport of indinavir in the presence of betaine monohydrate are presented in Table 5.29.

Table 5.29: P_{app} and ER values of indinavir transport in the presence of betaine monohydrate across excised porcine intestinal tissue

Cell	Papp ($\times 10^{-7}$)		Efflux Ratio (ER)		
	AP-BL	BL-AP	Betaine monohydrate	Negative control	Positive control
1	2.71000	3.94974	8.75771	15.5779	1.778764
2	3.42815	4.23563	9.00768	12.9310	1.435550
3	2.48981	4.06448	6.21134	11.2950	1.818447
4	3.43768	4.58748	9.73789	10.2936	1.057072
Mean	3.016410	4.209333	8.428655	12.52438	1.522458
Stdev	0.489283	0.278123	1.535592	2.307746	0.354701

The mean apparent permeability coefficient (P_{app}) and the efflux ratio (ER) values for indinavir transport in the presence of betaine monohydrate are illustrated in Figure 5.41.

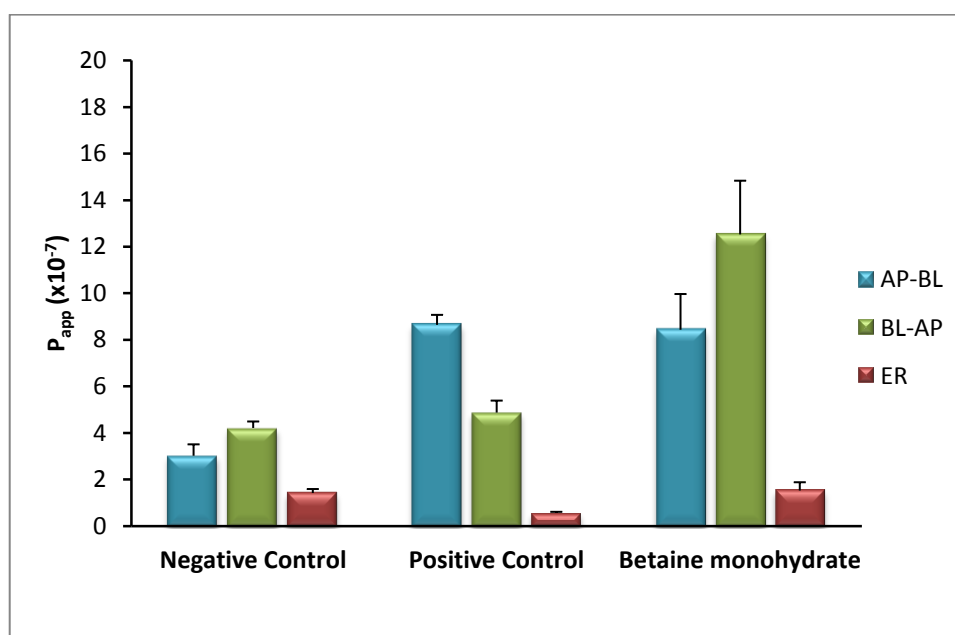


Figure 5.41: Mean P_{app} and ER values of indinavir transport in the presence of betaine monohydrate across excised porcine intestinal tissue

The transepithelial flux (J) and net flux (J_{net}) values for indinavir transport in the presence of betaine monohydrate are presented in Table 5.30 and compared in Figure 5.42.

Table 5.30: The J and J_{net} values of indinavir transport in the presence of betaine monohydrate across excised porcine intestinal tissue

Cell	J ($\times 10^{-2}$)		Net Flux (J_{net}) ($\times 10^{-2}$)		
	AP-BL	BL-AP	Betaine monohydrate	Negative control	Positive control
1	3.6782	6.5427	2.8645	0.52119	-1.78426
2	3.7832	5.4310	1.6478	0.33914	-1.57282
3	2.6088	4.7439	2.1351	0.66136	-1.42028
4	4.0899	4.3233	0.2334	0.48291	-1.55428
Mean	3.540025	5.260225	1.720200	0.50115	-1.58291
Stdev	0.644921	0.969248	1.110162	0.132475	0.150457

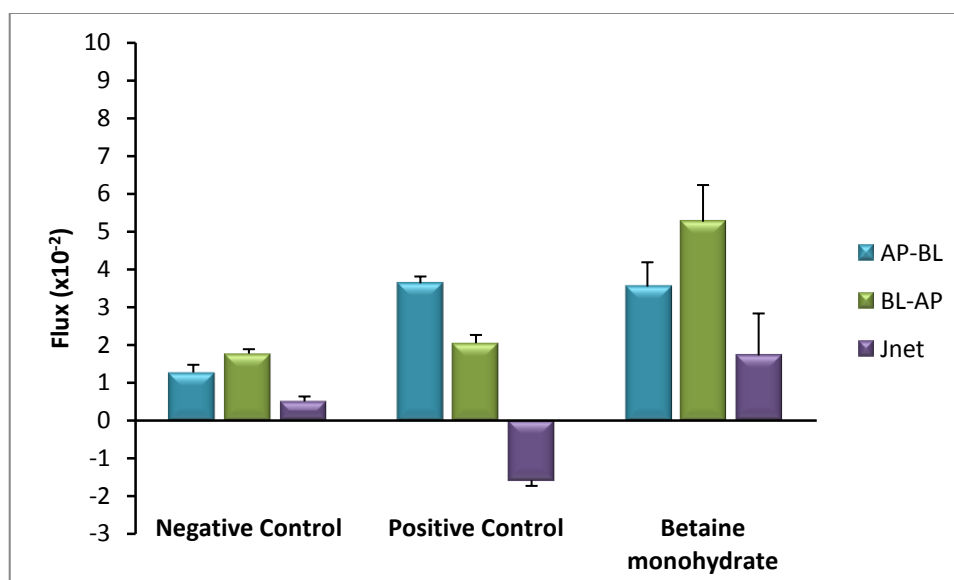


Figure 5.42: Mean J and J_{net} values for indinavir transport in the presence of betaine monohydrate across excised porcine intestinal tissue

As is evident from the data presented in Tables 5.29 and 5.30, the transport of indinavir in the BL-AP direction was clearly not influenced by the presence of betaine monohydrate across porcine intestinal tissue. Furthermore, the mean ER value for indinavir transport in the presence of betaine monohydrate also did not differ from the negative control group. However, the mean J_{net} value of the present group differed significantly from the value of the negative control group. When the values for indinavir transport were compared to the positive control, the mean ER and J_{net} values were statistically significant ($p < 0.05$) from the positive control group.

As different effects were observed for betaine monohydrate with respect to the ER and J_{net} values (used to evaluate the net drug transport) a conclusion could not be drawn regarding the effect of betaine monohydrate on indinavir transport. Tukey's comparison test classified betaine monohydrate in a homogeneous group with the negative control for both the ER and J_{net} values indicating no significant effect on indinavir transport (Tables 5.35 and 5.38).

5.4.5.3 Transport in the presence of β -carotene

The bi-directional transport of indinavir in the presence of 20 μM β -carotene across excised porcine intestinal tissue into the acceptor chamber expressed as a percentage relative to the initial concentration in the donor chamber (200 μM) over a period of 180 min is presented in Table 5.31 for each time interval.

Table 5.31: The bi-directional cumulative transport (%) of indinavir in the presence of β -carotene across excised porcine intestinal tissue

Time (min)	Cell 1		Cell 2		Cell 3		Cell 4	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20	0.32127	0.08190	0.11775	0.04256	0.27025	0.04802	0.28435	0.03253
40	0.31549	0.15748	0.15860	0.12217	0.35502	0.11200	0.41496	0.11221
60	0.36230	0.24949	0.21571	0.10631	0.36953	0.12110	0.45935	0.07295
80	0.44685	0.33697	0.29039	0.16800	0.52407	0.18026	0.50813	0.16054
100	0.65710	0.40476	0.39111	0.25990	0.66592	0.26996	0.68972	0.35771
120	0.90451	0.57063	0.57576	0.35952	0.75277	0.38646	0.74939	0.47329
140	1.11520	0.66199	0.66880	0.48264	0.95136	0.59327	0.96564	0.46551
160	1.37972	0.63364	0.86337	0.43735	1.05873	0.47027	1.11536	0.52605
180	1.80181	1.02739	1.13428	0.84784	1.29705	0.88889	1.33955	1.06026

The bi-directional cumulative transport (%) of indinavir across excised porcine intestinal tissue plotted as a function of time in the presence of the β -carotene is shown in Figure 5.43.

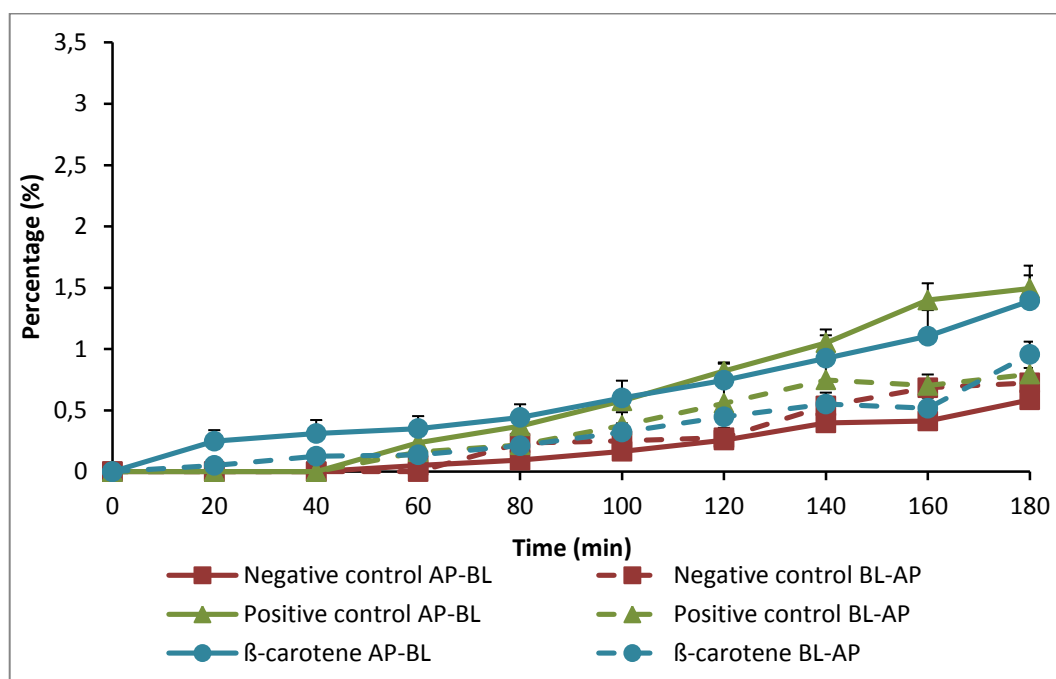


Figure 5.43: Bi-directional cumulative percentage transport of indinavir in the presence of β -carotene plotted as a function of time

The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values calculated from the cumulative transport of indinavir in the presence the β -carotene are presented in Table 5.32.

Table 5.32: P_{app} and ER values of indinavir transport in the presence of β -carotene across excised porcine intestinal tissue

Cell	P_{app} ($\times 10^{-7}$)		Efflux Ratio (ER)		
	AP-BL	BL-AP	β -carotene	Negative control	Positive control
1	8.3591	4.72771	0.565575	1.457467	0.521359
2	5.4363	3.70219	0.681013	1.235544	0.576364
3	6.0907	4.04264	0.663739	1.632445	0.617743
4	6.1509	4.58564	0.745528	1.334469	0.537216
Mean	6.50925	4.264545	0.663964	1.414981	0.563171
Stdev	1.274986	0.477182	0.074440	0.171051	0.043105

The mean apparent permeability coefficient (P_{app}) and the efflux ratio (ER) values for indinavir transport in the presence of β -carotene are illustrated in Figure 5.44.

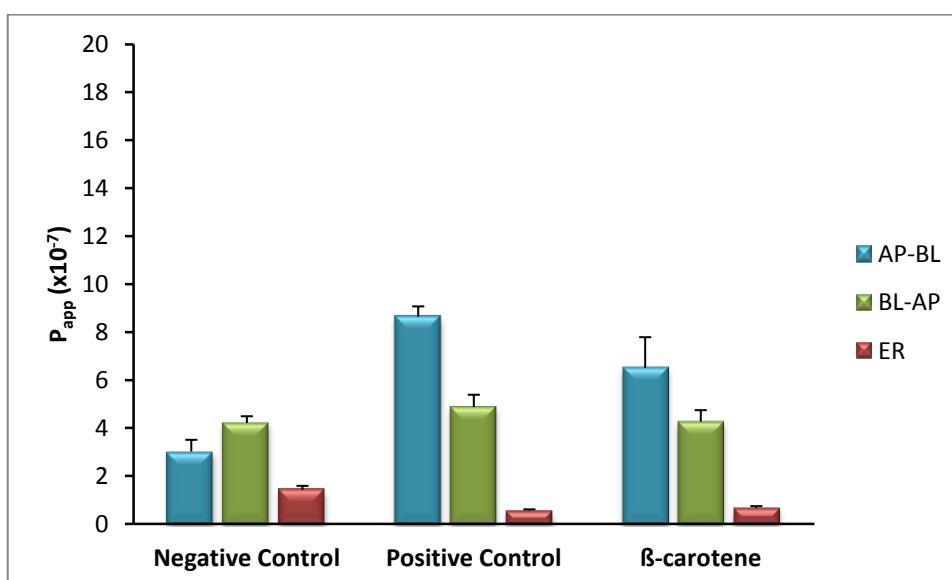


Figure 5.44: Mean P_{app} and ER values of indinavir transport in the presence of β -carotene across excised porcine intestinal tissue

The transepithelial flux (J) and net flux (J_{net}) values for indinavir transport in the presence of β -carotene are presented in Table 5.33 and compared in Figure 5.45.

Table 5.33: The J and J_{net} values of indinavir transport in the presence of β -carotene across excised porcine intestinal tissue

Cell	J ($\times 10^{-2}$)		Net Flux (J_{net}) ($\times 10^{-2}$)		
	AP-BL	BL-AP	β -carotene	Negative control	Positive control
1	3.51083	1.98564	-1.52519	0.52119	-1.78426
2	2.28324	1.55492	-0.72832	0.33914	-1.57282
3	2.55810	1.69791	-0.86019	0.66136	-1.42028
4	2.58336	1.92597	-0.65739	0.48291	-1.55428
Mean	2.733883	1.79111	-0.94277	0.50115	-1.58291
Stdev	0.535501	0.200417	0.397267	0.132475	0.150457

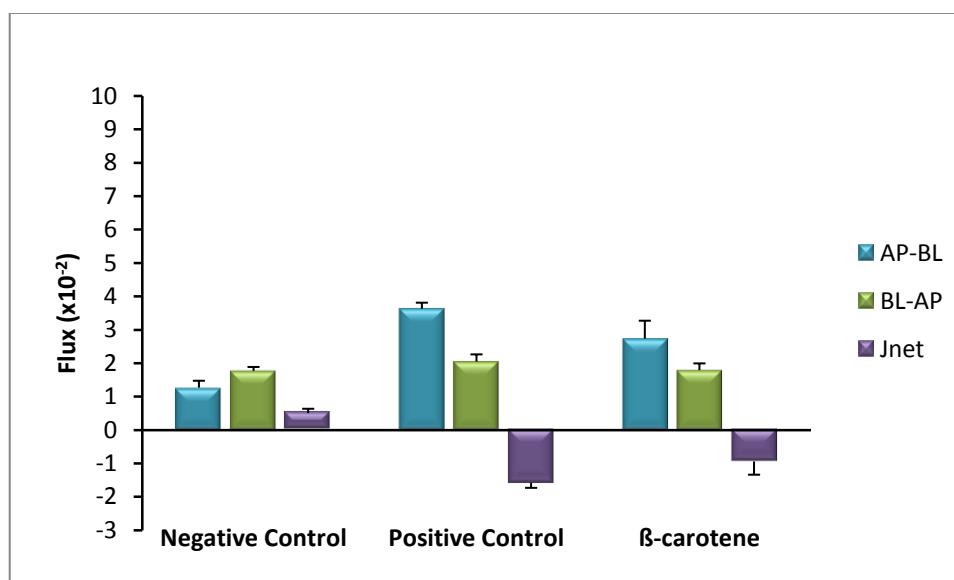


Figure 5.45: Mean J and J_{net} values for indinavir transport in the presence of β -carotene across excised porcine intestinal tissue

The transport of indinavir in the BL-AP direction was significantly ($p < 0.05$) decreased by the inclusion of β -carotene in the transport study across porcine intestinal tissue. From Tables 5.32 and 5.33 it is evident that the mean ER and J_{net} values of indinavir transport in the presence of β -carotene were significantly ($p < 0.05$) lower than the values of the negative control group. In fact, following the addition of β -carotene to the transport study, the J_{net} value was altered from being a positive value for the indinavir alone to a negative value in the presence of β -carotene. The negative J_{net} value is indicative of net absorption of indinavir transport in the AP-BL direction, which means that β -carotene inhibited P-gp mediated indinavir efflux.

When the transport profile of β -carotene is compared to the positive control group, it is clear that the transport data from these two groups is in accordance. Neither the mean ER nor J_{net} values differed significantly from the positive control group. These results are supported by Tukey's comparison test, where β -carotene was classified in a homogeneous group with the positive control group for the mean ER and J_{net} values (Tables 5.36 and 5.39). This indicates that β -carotene inhibited the P-gp related efflux of indinavir to the same extent as the verapamil in the positive control group.

5.5 Comparison of transport results

5.5.1 *Allium sativum* crude extract, L-alliin and diallyl disulphide

The P_{app} and ER values for indinavir transport obtained in the presence of *A. sativum* crude extract and two of its pure phytoconstituents (L-alliin and diallyl disulphide) are summarised in Figure 5.46.

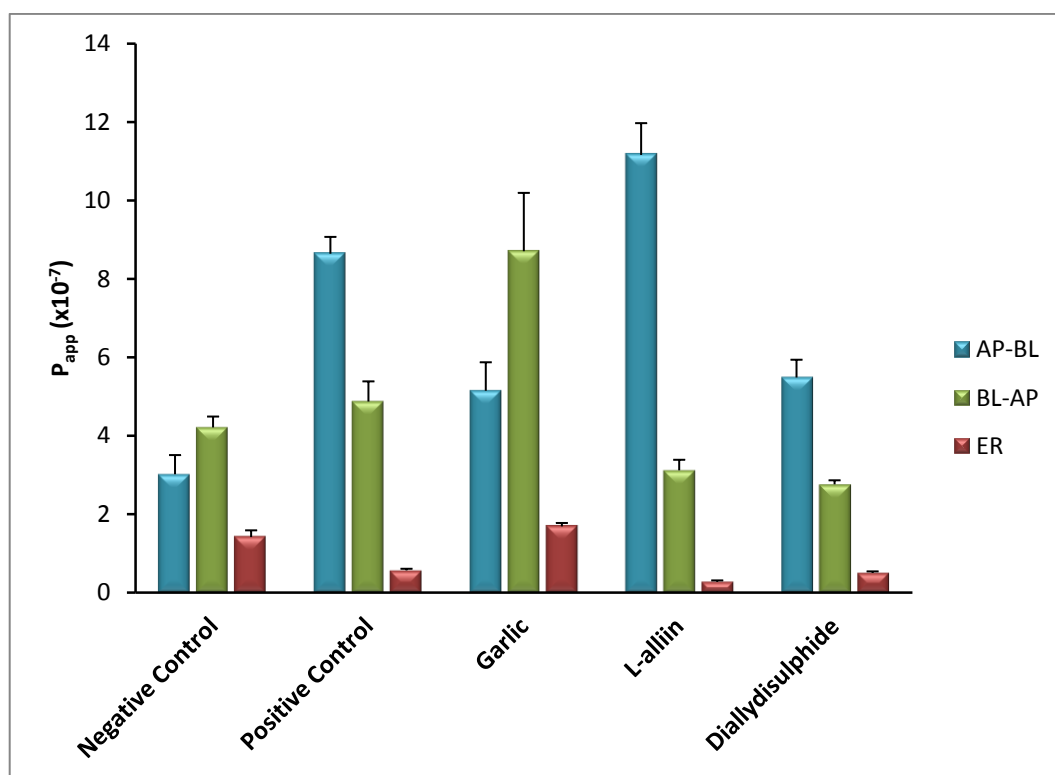


Figure 5.46: The mean P_{app} and ER values of indinavir transport in the presence of *Allium sativum* crude extract, L-alliin and diallyl disulphide

The mean J and J_{net} values for indinavir transport obtained for *A. sativum* crude extract and two of its pure phytoconstituents are summarised in Figure 5.46.

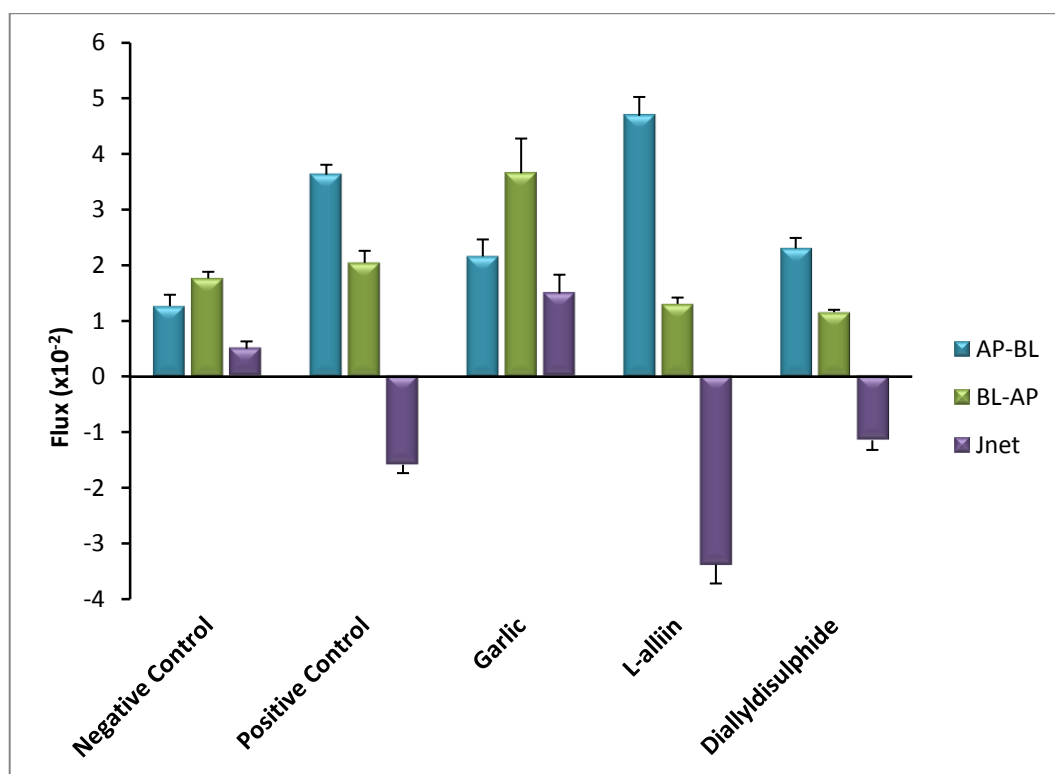


Figure 5.47: The mean J and J_{net} values of indinavir transport in the presence of *Allium sativum* crude extract, L-alliin and diallyl disulphide

The results indicate that the mean J_{net} value of indinavir transport in the presence of *A. sativum* crude extract was higher compared to that obtained in the negative control group, albeit not statistically significant. This indicates that indinavir transport across excised porcine intestinal tissue was not affected by the inclusion of *A. sativum* crude extract. On the other hand, it was demonstrated that with respect to the BL-AP direction, the pure compounds L-alliin and diallyl disulphide decreased indinavir transport significantly. The mean J_{net} values for both compounds differed significantly from that of the negative control group. Furthermore, it is clear that the J_{net} value changed from a positive value for indinavir alone to a negative value in the presence of both these compounds, resulting in a net influx (or absorptive transport) of indinavir. This indicates that P-gp related efflux of indinavir is inhibited by both L-alliin and diallyl disulphide. In this respect, L-alliin displayed a more robust effect than the positive control, verapamil.

The implication of the significant inhibition of P-gp mediated indinavir efflux by L-alliin and diallyl disulphide is that the co-administration of these compounds together with indinavir may increase indinavir plasma levels in patients. This pharmacokinetic interaction may result in a higher bioavailability of indinavir in HIV patients receiving ART, but at the same time contribute to a higher prevalence of adverse effects. However, the clinical significance of this

interaction will have to be established by *in vivo* studies before a final conclusion can be made.

The opposite effect that was demonstrated when L-alliin and diallyl disulphide were applied as single compounds compared to that of *A. sativum* crude extract, can possibly be explained by the fact that a higher concentration of these pure compounds was applied as compared to that which was present in the crude extract. Furthermore, it could be that the *A. sativum* crude extract displayed a combined effect caused by the presence of several phytoconstituents (see Figure 5.4 for chemical fingerprinting). It is known that the chemical composition of plants may vary due to factors such as the time of collection, the location where the plant is grown, climate and weather patterns (Gouws *et al.*, 2012:980). This could also explain the controversy in the literature concerning the different effects of *A. sativum* on P-gp efflux and complicate the analyses of findings from both *in vitro* and *in vivo* studies and the conclusions drawn from its data (Foster *et al.*, 2001:176).

5.5.2 *Citrus limonum* crude extract, hesperidin and eriocitrin

The P_{app} and ER values of indinavir transport obtained in the presence of *C. limonum* crude extract and two of its pure phytoconstituents (hesperidin and eriocitrin) are summarised in Figure 5.48.

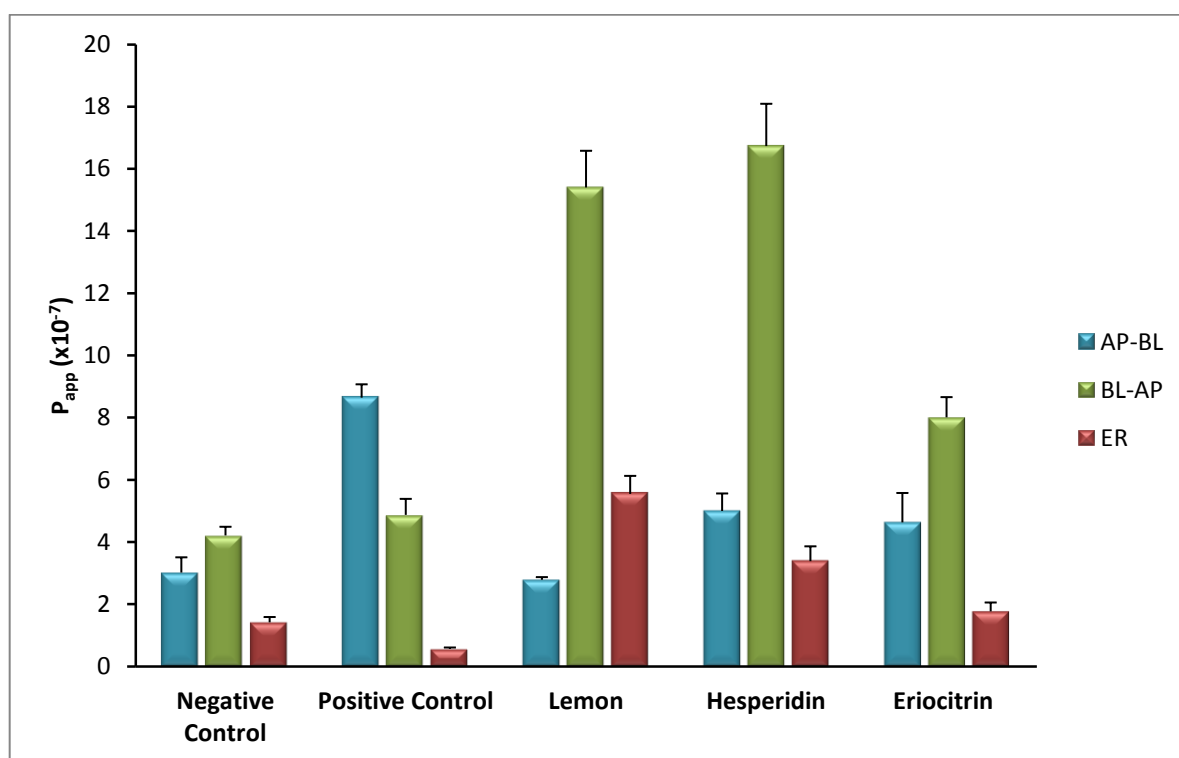


Figure 5.48: The P_{app} and ER values of indinavir transport in the presence of *Citrus limonum* crude extract, hesperidin and eriocitrin

The mean J and J_{net} values for indinavir transport obtained in the presence of *C. limonum* crude extract and the two pure phytoconstituents are summarised in Figure 5.49.

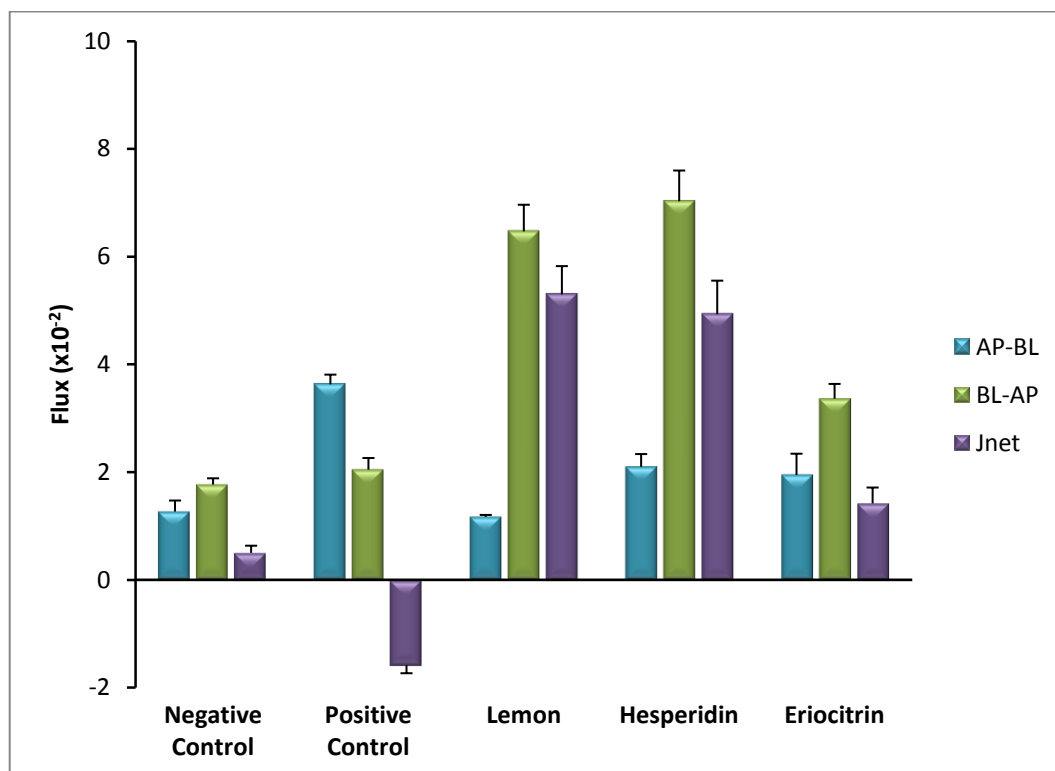


Figure 5.49: The J and J_{net} values of indinavir transport in the presence of *Citrus limonum* crude extract, hesperidin and eriocitrin

The results for the *C. limonum* crude extract and hesperidin indicate higher transport of indinavir in the BL-AP direction compared to the negative control group with an overall transport of indinavir in the secretory direction. The mean J_{net} values were significantly higher compared to the negative and positive control groups. Thus, the *C. limonum* crude extract and hesperidin therefore significantly promoted the efflux of indinavir. The transport profile of indinavir in the presence of eriocitrin tended to correspond with the results for *C. limonum* crude extract and hesperidin, although the J_{net} value did not differ significantly from the negative control group. This indicates that eriocitrin did not have a significant influence on the P-gp transporter.

It can be concluded that the co-administration of *C. limonum* crude extract and hesperidin with compounds that are substrates for efflux transporters such as P-gp will cause a higher efflux of these compounds back into the intestinal lumen which may potentially result in lower plasma levels of the substrate (in this case indinavir). The clinical significance of these effects will have to be determined *in vivo* before final conclusions can be made.

The present study implicates a role for *C. limonum* and hesperidin as P-gp promoters which are in direct contrast to the findings from previous studies (Bansal *et al.*, 2009:54). However, the promotion of P-gp related efflux by *C. limonum* and the pure compounds are consistent with a study done by Lim *et al.* (2006:48) that demonstrated a two-fold increase in the efflux of Rhodamine 123 with the addition of *C. limonum* extract. Further studies are needed to clarify this controversy.

5.5.3 *Beta vulgaris* crude extract, betaine monohydrate and β -carotene

The mean P_{app} and ER values for indinavir transport obtained in the presence of *B. vulgaris* crude extract and two of its pure phytoconstituents (β -carotene and betaine monohydrate) are summarised in Figure 5.50.

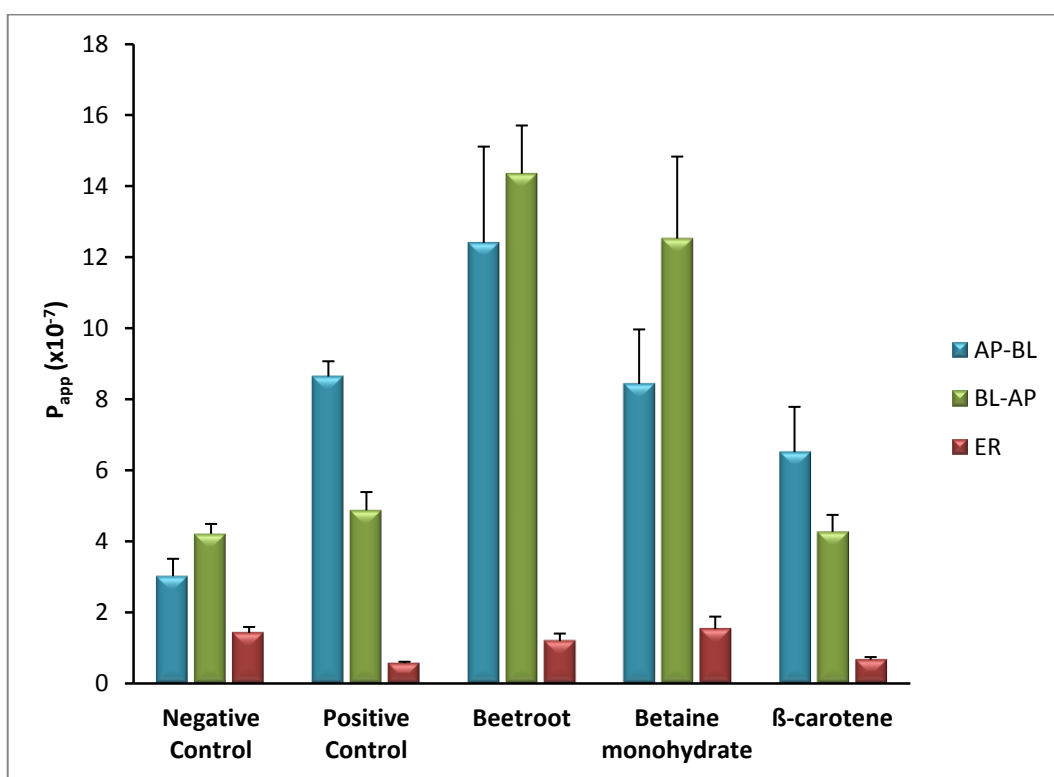


Figure 5.50: The P_{app} and ER values of indinavir transport in the presence of *Beta Vulgaris* crude extract, betaine monohydrate and β -carotene

The mean J and J_{net} values for indinavir transport obtained for *B. vulgaris* crude extract and the two pure phytoconstituents are summarised in Figure 5.51.

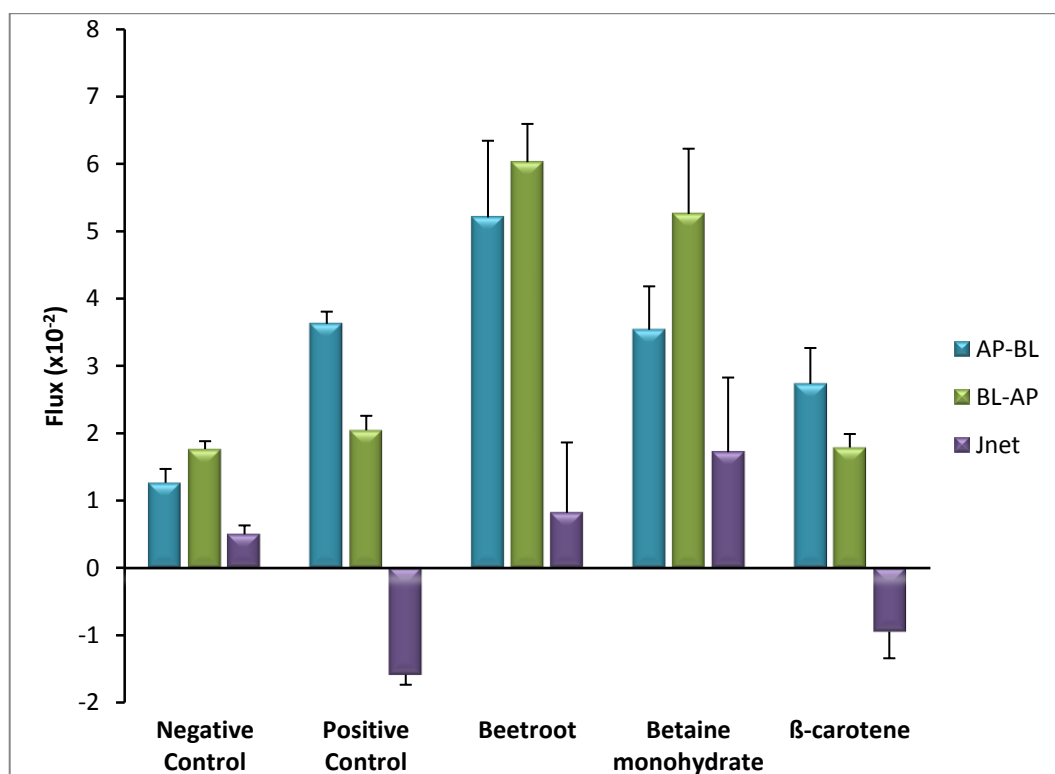


Figure 5.51: The mean J and J_{net} values of indinavir transport in the presence of *Beta vulgaris* crude extract, betaine monohydrate and β -carotene

The J_{net} values for indinavir in the presence of *B. vulgaris* crude extract and betaine monohydrate did not differ significantly when compared to the negative control group. The J_{net} value for indinavir transport in the presence of β -carotene however, differed with statistical significance from the value of the negative control group. It is apparent that β -carotene displays an effect similar to that of the positive control, indicating significant inhibition of P-gp related indinavir efflux. This may clinically manifest as an increased drug bioavailability when β -carotene is co-administered with drugs that are substrates for P-gp, potentially resulting in harmful adverse effects.

5.6 Conclusion

Statistically significant ($p < 0.05$) inhibition of indinavir efflux as indicated by reduced ER values was obtained for L-alliin, diallyl disulphide and β -carotene. Inhibition of indinavir efflux may lead to increased transport in the absorptive direction and therefore a potentially higher bioavailability. Statistically significant ($p < 0.05$) promotion of indinavir efflux was obtained for *C. limonum* crude extract and hesperidin, which may potentially lead to lower bioavailability of indinavir after oral administration. *B. vulgaris*, betaine monohydrate, *A. sativum* and eriocitrin displayed no statistically significant effects compared to the negative control group on indinavir transport across excised porcine intestinal tissue. Although these pharmacokinetic interactions may negatively affect the ART of HIV patients, the deliberate

and controlled inclusion of L-alliin, diallyl disulphide and β -carotene in dosage forms may result in a more effective delivery of PIs such as indinavir.

5.7 Statistical analysis

5.7.1 Efflux ratio (ER): Dunnett and Tukey tests

The ER values calculated from the indinavir transport in the presence of the different experimental compounds were subjected to Welch and Dunnett tests to determine whether statistically significant differences existed compared to the negative and positive control groups. The results of these tests are summarised in Table 5.34. The mean ER values of the experimental groups compared with the negative control group across porcine intestinal tissue are shown in Figure 5.52.

Table 5.34: p-values obtained for the different experimental groups by comparing the ER values of indinavir across porcine intestinal tissue with the control groups

Compound	n	Mean ER	SD	ANOVA p-value: Welch	p-value: Dunnett	
					Positive control	Negative control
<i>A. sativum</i>	4	1.6860	0.0884		< 0.00001*	0.7161
L-alliin	4	0.2803	0.0297		0.6622	< 0.0000*
Diallyl disulphide	4	0.5049	0.0346		1.0000	0.0010*
<i>C. limonum</i>	4	5.5508	0.5752		< 0.00001*	< 0.00001*
Hesperidin	4	3.3651	0.4765		< 0.00001*	< 0.00001*
Eriocitrin	4	1.7675	0.2878		< 0.00001*	0.4431
<i>B. vulgaris</i>	4	1.1847	0.2126		0.0317*	0.8452
B-carotene	4	0.6639	0.0746		0.9988	0.0077*
Betaine monohydrate	4	1.5224	0.3557		0.0004*	0.9982
Positive control	4	0.5629	0.0426	< 0.00001		
Negative control	4	1.4146	0.1701	< 0.00001		

*Statistically significant difference ($p < 0.05$)

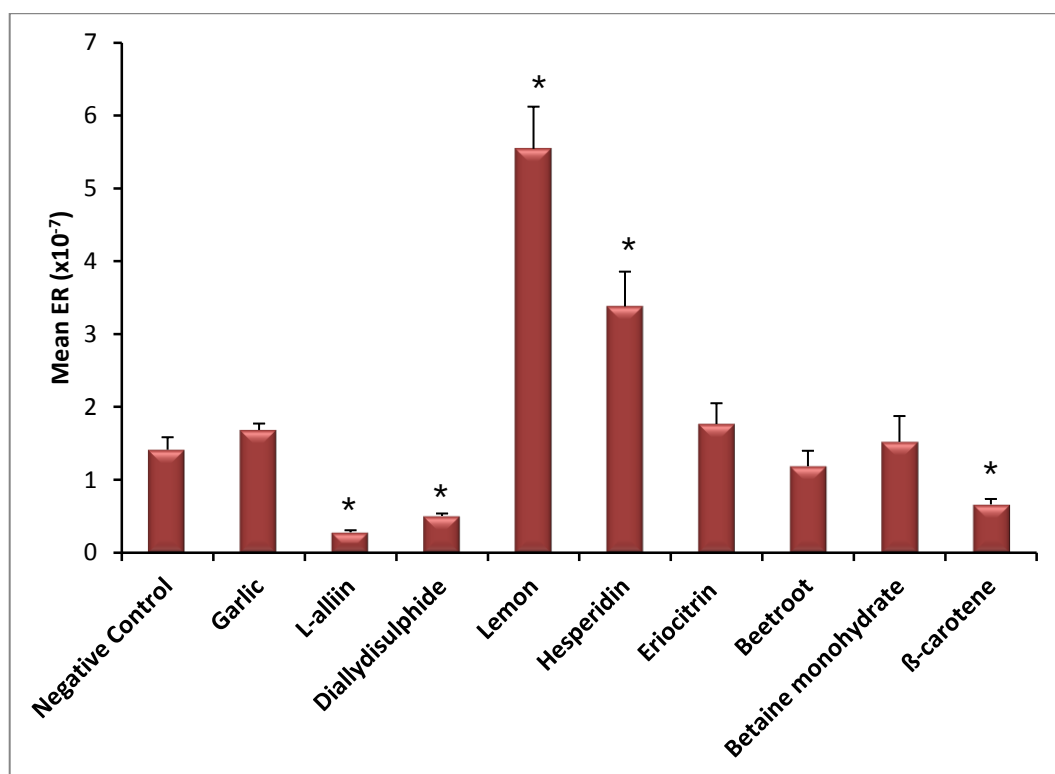


Figure 5.52: Mean ER values of the different experimental groups compared to the negative control group across porcine intestinal tissue. An asterisk (*) indicates statistically significant differences between the experimental group and the negative control

Tukey's comparison test was conducted to classify the experimental and control groups into homogeneous groups according to their mean ER values and these classifications are presented in Tables 5.35 and 5.36.

Table 5.35: Tukey's comparison test for the mean ER values compared to the negative control group

Compound	Mean ER	Group				
		1	2	3	4	5
L-alliin	0.280316		****			
Diallyl disulphide	0.504918		****	****		
β-carotene	0.663915		****	****		
<i>B. vulgaris</i>	1.184725	****		****		
Negative control	1.414635	****				
Betaine monohydrate	1.522426	****				
<i>A. sativum</i>	1.686021	****				
Eriocitrin	1.767451	****				
Hesperidin	3.385154				****	
<i>C. limonum</i>	5.550792					****

Table 5.36: Tukey's comparison test for the mean ER values compared to the positive control group

Compound	Mean ER	Group				
		1	2	3	4	5
L-alliin	0.280316	****				
Diallyl disulphide	0.504918	****	****			
Positive control	0.562909	****	****			
β-carotene	0.663915	****	****			
<i>B. vulgaris</i>	1.184725		****	****		
Betaine monohydrate	1.522426			****		
<i>A. sativum</i>	1.686021			****		
Eriocitrin	1.767451			****		
Hesperidin	3.385154				****	
<i>C. limonum</i>	5.550792					****

The results for the transport of indinavir across porcine intestinal tissue were used to calculate the ER values for each experimental group. These in turn were compared to the results from the negative control group. Statistically significant differences ($p < 0.05$) could be demonstrated for indinavir transport in the presence of L-alliin, diallyl disulphide, hesperidin, β-carotene and *C. limonum* crude extract compared to the negative control group. L-alliin, diallyl disulphide and β-carotene displayed statistically significant inhibition of indinavir efflux as is evident from reduced ER values. On the other hand, statistically significant promotion of indinavir efflux is indicated by increased ER values for *C. limonum* crude extract and hesperidin. Tukey's test also classified L-alliin, diallyl disulphide and β-carotene in a homogeneous group with the positive control group (Table 5.36).

The experimental groups that did not differ significantly from the negative control group include *B. vulgaris* crude extract ($p = 0.8452$), betaine monohydrate ($p = 0.9982$), *A. sativum* crude extract ($p = 0.7161$) and eriocitrin ($p = 0.4431$). Tukey's test also classified *B. vulgaris* crude extract, betaine monohydrate, *A. sativum* crude extract and eriocitrin in a homogeneous group with the negative control (Table 5.35).

5.7.2 Net flux (J_{net}): Dunnett and Tukey test

The J_{net} values calculated from the indinavir transport in the presence of the different experimental compounds were subjected to the Welch and Dunnett tests to determine whether statistically significant difference existed between the negative and positive control groups. The results of these tests are summarised in Table 5.37. The mean J_{net} values of the experimental groups compared to the negative control group across porcine intestinal tissue are shown in Figure 5.53.

Table 5.37: p-values obtained for the different experimental groups by comparing the J_{net} values of indinavir across porcine intestinal tissue with the control groups

Compound	n	Mean J_{net}	SD	ANOVA p-value: Welch	p-value: Dunnett	
					Positive control	Negative control
<i>A. sativum</i>	4	0.0149	0.0034		< 0.00001*	0.1406
L-alliin	4	-0.0338	0.0034		0.0014*	< 0.00001*
Diallyl disulphide	4	-0.0114	0.0017		0.8801	0.0035*
<i>C. limonum</i>	4	0.0530	0.0052		< 0.00001*	< 0.00001*
Hesperidin	4	0.0493	0.0063		< 0.00001*	< 0.00001*
Eriocitrin	4	0.0142	0.0030		< 0.00001*	0.1989
<i>B. vulgaris</i>	4	0.0082	0.0105		< 0.00001*	0.9771
B-carotene	4	-0.0094	0.0040		0.5634	0.0122*
Betaine monohydrate	4	0.0050	0.0013		< 0.00001*	0.0443*
Positive control	4	-0.0159	0.0015	< 0.00001		
Negative control	4	0.0050	0.0013	< 0.00001		

*Statistically significant difference ($p < 0.05$)

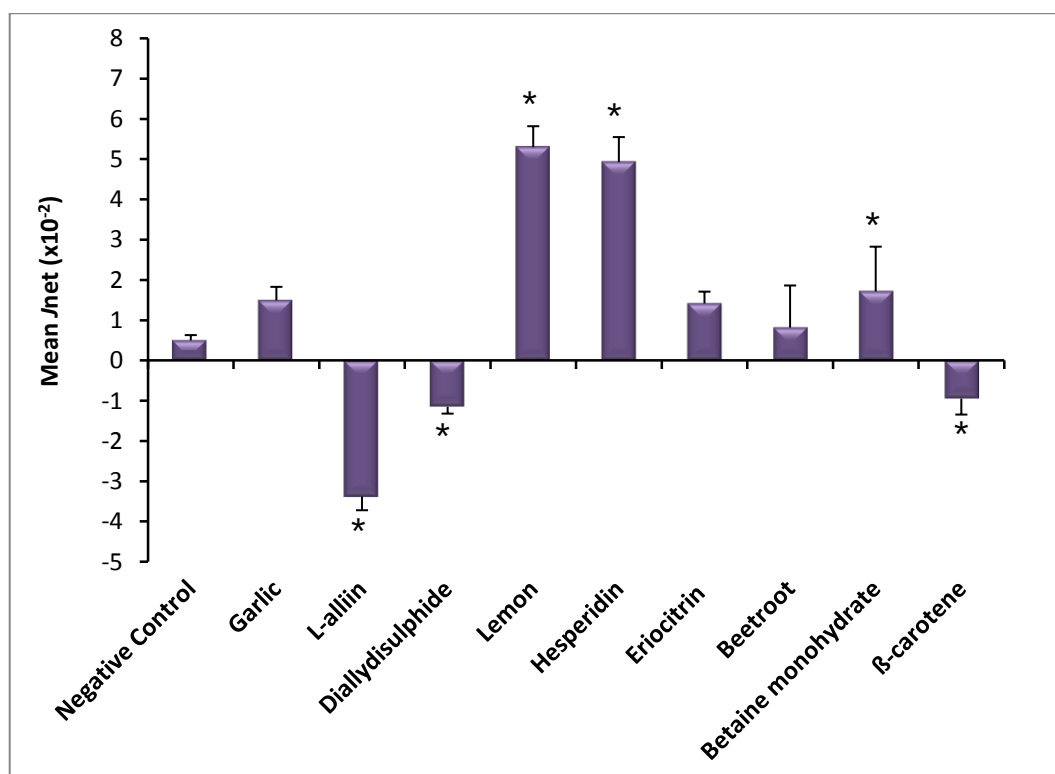


Figure 5.53: J_{net} values of the different experimental groups compared to the negative control group across porcine intestinal tissue. An asterisk (*) indicates statistically significant differences between the experimental group and the negative control

Tukey's comparison test was conducted to classify the experimental and control groups into homogeneous groups according to their mean J_{net} values and these classifications are presented in Tables 5.38 and 5.39.

Table 5.38: Tukey's comparison test for the J_{net} values compared to the negative control group

Compound	Mean J_{net}	Group			
		1	2	3	4
L-alliin	-0.033779				****
Diallyl disulphide	-0.011442		****		
β-carotene	-0.009428		****		
Negative control	0.005012	****			
<i>B. vulgaris</i>	0.008205	****			
Eriocitrin	0.014183	****			
<i>A. sativum</i>	0.014942	****			
Betaine monohydrate	0.017202	****			
Hesperidin	0.049282			****	
<i>C. limonum</i>	0.052986			****	

Table 5.39: Tukey's comparison test for the J_{net} values compared to the positive control group

Compound	Mean J_{net}	Group			
		1	2	3	4
L-alliin	-0.033779				****
Positive control	-0.015829		****		
Diallyl disulphide	-0.011442		****		
β -carotene	-0.009428		****		
<i>B. vulgaris</i>	0.008205	****			
Eriocitrin	0.014183	****			
<i>A. sativum</i>	0.014942	****			
Betaine monohydrate	0.017202	****			
Hesperidin	0.049282			****	
<i>C. limonum</i>	0.052986			****	

Concerning the p -values obtained for the comparison of the J_{net} values of the negative control group with the experimental compounds, L-alliin, diallyl disulphide, β -carotene, betaine monohydrate, hesperidin and *C. limonum* crude extract displayed statistical significance ($p < 0.05$). More specifically, L-alliin, diallyl disulphide and β -carotene displayed significant inhibition of indinavir efflux while *C. limonum* crude extract, hesperidin and betaine monohydrate on the other hand, displayed significant promotion of indinavir efflux.

The experimental groups that did not have a significant effect on the J_{net} values compared to that of the negative control group include *A. sativum* crude extract ($p = 0.1406$), eriocitrin ($p = 0.1989$) and *B. vulgaris* crude extract ($p = 0.9771$).

Tukey's test classified *A. sativum* crude extract, eriocitrin, *B. vulgaris* crude extract and betaine monohydrate in a homogeneous group with the negative control group (group 1). Diallyl disulphide and β -carotene were classified in a homogeneous group with the positive control (group 2), hesperidin and *C. limonum* were classified in group 3 and L-alliin in group 4 (the only group that exhibited a greater effect than the positive control group) (Tables 5.38 and 5.39).

5.7.3 Statistical analysis of pure compounds compared to crude plant extracts

5.7.3.1 *Allium sativum* crude extract, L-alliin and diallyl disulphide

A statistical analysis was done to compare the effects of L-alliin and diallyl disulphide with the effect of the *A. sativum* crude extract according to the mean ER and J_{net} values. These results are summarised in Figures 5.54 and 5.55.

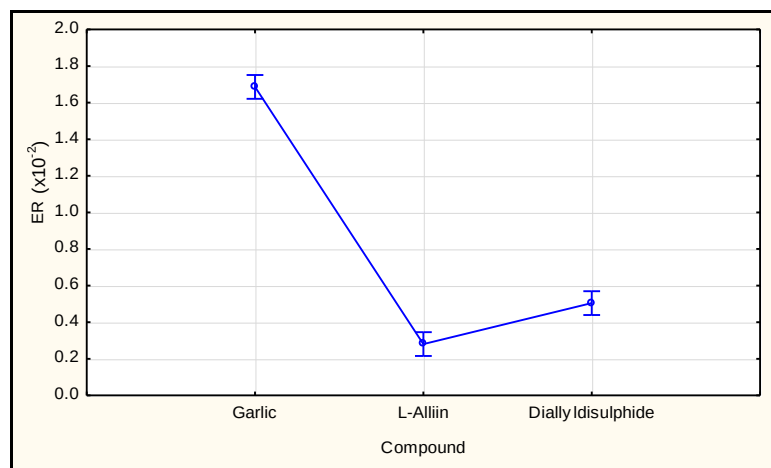


Figure 5.54: ER values of indinavir transport in the presence of L-alliin and diallyl disulphide using *Allium sativum* crude extract as the reference group

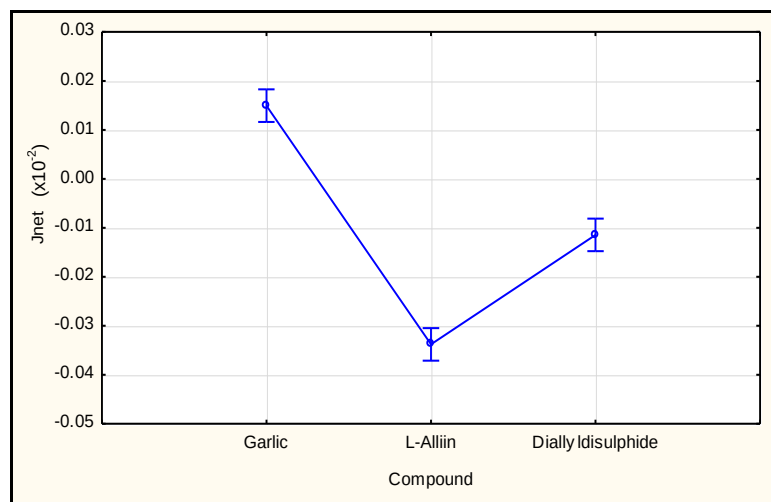


Figure 5.55: J_{net} values of indinavir transport in the presence of L-alliin and diallyl disulphide using *Allium sativum* crude extract as the reference group

As the ER and J_{net} values of indinavir transport in the presence of L-alliin and diallyl disulphide differed significantly with the values obtained for *A. sativum* crude extract, it is evident that the results for the two pure compounds present in *A. sativum* did not correlate with the effect of the crude extract on the transport of indinavir. Other compounds present in the crude extract may interact with the effect of L-alliin and diallyl disulphide on indinavir transport, which may be the reason why these two pure compounds did not indicate a similar effect on the indinavir transport than the *A. sativum* crude extract.

5.7.3.2 *Citrus limonum* crude extract, hesperidin and eriocitrin

A statistical analysis was done to compare the effects of hesperidin and eriocitrin to the effect of the *C. limonum* crude extract based on the mean ER and J_{net} values. These results are summarised in Figures 5.56 and 5.57.

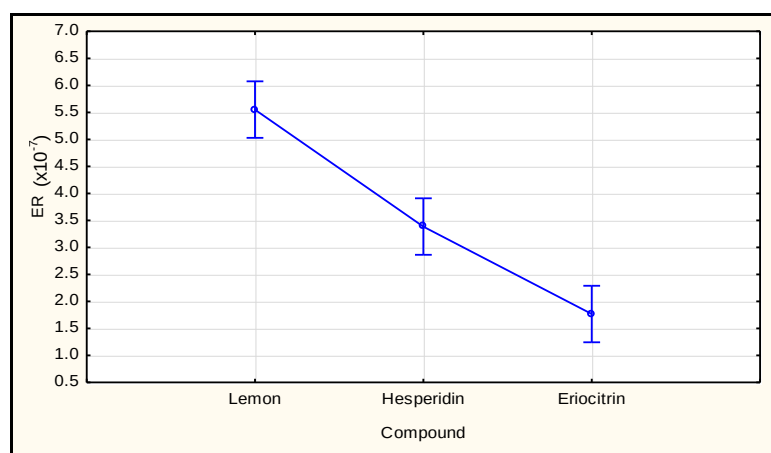


Figure 5.56: ER values of indinavir transport in the presence of hesperidin and eriocitrin using *Citrus limonum* extract as the reference group

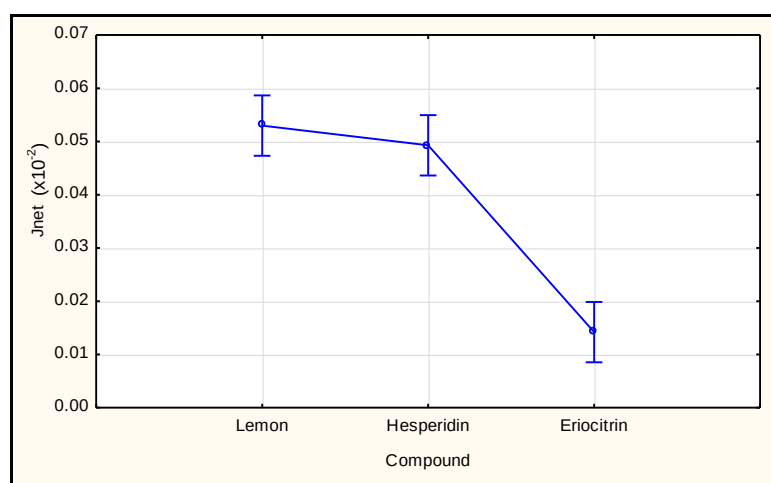


Figure 5.57: J_{net} values of indinavir transport in the presence of hesperidin and eriocitrin using *Citrus limonum* crude extract as the reference group

The ER values of hesperidin and eriocitrin indicated significant differences ($p < 0.05$) when compared to the ER values for *C. limonum* crude extract. Regarding the J_{net} values, hesperidin did not result in significant difference, while the J_{net} value of eriocitrin differed significantly ($p < 0.05$) from the *C. limonum* crude extract group. As hesperidin is a potent efflux modulator, it displays biological activity in relatively small concentrations (viz the concentration present in the *C. limonum* crude extract).

The fact that eriocitrin displayed a significant different J_{net} value compared to the *C. limonum* crude extract, can possibly be explained by the absence of eriocitrin in the crude extract which was identified by the chemical fingerprinting of the *C. limonum* crude extract (Figure 5.7).

5.7.3.3 *Beta vulgaris* crude extract, betaine monohydrate and β -carotene

A statistical analysis was done to compare the effects of β -carotene and betaine monohydrate to the effect of the *B. vulgaris* crude extract according to the mean ER and J_{net} values. These results are summarised in Figures 5.58 and 5.59.

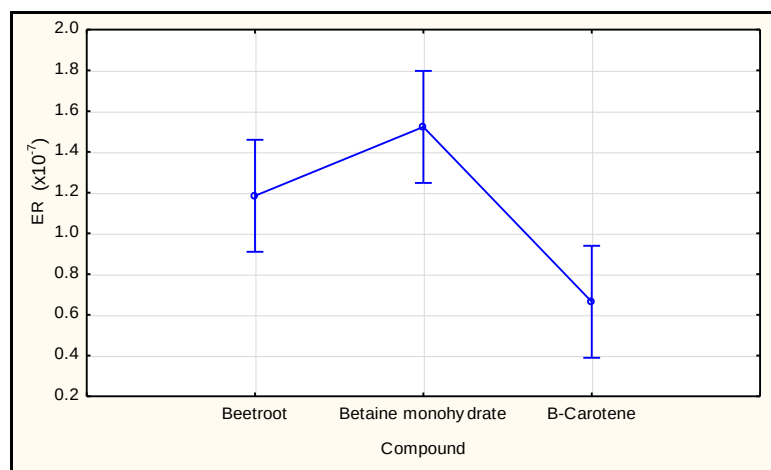


Figure 5.58: ER values of indinavir transport in the presence of β -carotene and betaine monohydrate using *Beta vulgaris* crude extract as the reference group

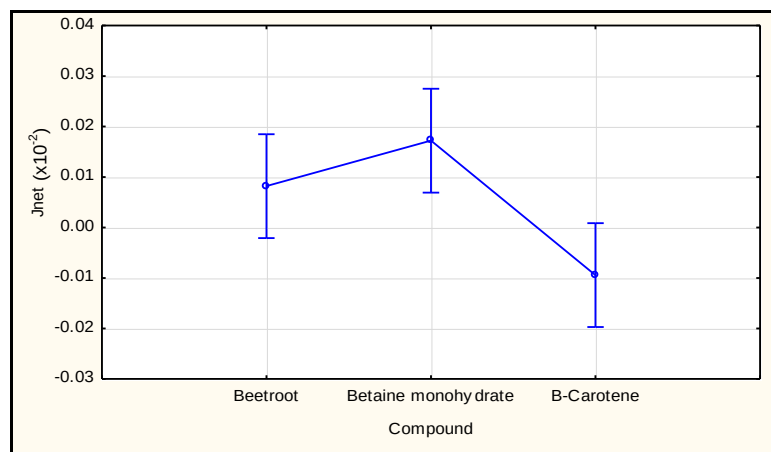


Figure 5.59: J_{net} values of indinavir transport in the presence of β -carotene and betaine monohydrate using *Beta vulgaris* crude extract as the reference group

The ER and J_{net} values of β -carotene differed significantly ($p < 0.05$) from that of the *B. vulgaris* crude extract. These results demonstrate that the transport of indinavir in the presence of a pure form of β -carotene did not display the same characteristics as its transport in the presence of *B. vulgaris* crude extract, while the effect of betaine monohydrate corresponds with that of the *B. vulgaris* crude extract.

CHAPTER 6

6 CONCLUSIONS AND FUTURE RECOMMENDATIONS

6.1 Final conclusions

In the current study, the efflux of indinavir in the negative control group (indinavir alone) was substantiated by demonstration of a higher transport of indinavir in the BL-AP direction than in the AP-BL direction across excised porcine intestinal tissue. With the inclusion of verapamil as the positive control group, a higher transport of indinavir was observed in the AP-BL direction than the BL-AP direction, confirming the role of verapamil as an inhibitor of efflux. Verapamil could therefore be used as a positive control to compare the effects of the various experimental compounds used in the current study. The results from both the positive and negative control studies confirmed the suitability of indinavir to serve as a reference compound to investigate the effect of the selected plant extracts and pure compounds on the intestinal transport of indinavir.

The mean ER and the J_{net} values for indinavir transport in the presence of *A. sativum* crude extract correspond with the values of the negative control group. This indicates that *A. sativum* crude extract did not have a significant effect on the efflux of indinavir and no inhibition or promotion of efflux was demonstrated. The mean ER value for indinavir transport in the presence of L-alliin was lower than the value for the positive control group, but did not indicate statistical significance. This is an indication that L-alliin displays the same degree of P-gp inhibition as the positive control. However, the mean J_{net} value was significantly lower than the same value for the positive control group, indicating that L-alliin had a more robust effect compared to the positive control. The mean ER and J_{net} values of diallyl disulphide did not differ significantly from the positive control group. It is clear that the J_{net} value was changed from a positive value for the negative control to a negative value with the inclusion of both L-alliin and diallyl disulphide resulting in a net influx of indinavir. This is an indication of significant inhibition of P-gp related indinavir efflux by both L-alliin and diallyl disulphide. In addition, the inhibition of efflux by L-alliin was more robust than that of verapamil for the concentrations investigated in this study. Several studies have demonstrated that raw *A. sativum* and constituents of *A. sativum* can inhibit the activities of efflux transporters such as P-gp, although the inhibition was low or moderate compared to the known P-gp inhibitor verapamil (Zhou *et al.*, 2004:63).

The results for *C. limonum* crude extract and hesperidin indicate a higher transport of indinavir in the BL-AP direction than the AP-BL direction resulting in an overall net efflux (i.e. transport in the secretory direction) of indinavir. The mean ER and J_{net} values were

significantly higher than the values for both the control groups. It is therefore evident that *C. limonum* crude extract and hesperidin promoted the efflux of indinavir. The transport profile for indinavir in the presence of eriocitrin corresponds with the results for *C. limonum* crude extract and hesperidin. However, the mean ER and J_{net} values did not indicate significant differences with the results for the negative control group and it can be concluded that eriocitrin did not have a significant influence on the efflux of indinavir. The results are in contrast with several studies performed earlier that demonstrated eriocitrin mediated inhibition of P-gp related efflux (Bansal *et al.*, 2009:54). However, the promotion of P-gp related efflux established in this study is consistent with a previous investigation done by Lim *et al.* (2006:48) indicating enhanced efflux of Rhodamine 123 with the addition of extracted *C. limonum* material.

The mean ER and J_{net} values of indinavir transport in the presence of *B. vulgaris* crude extract did not differ significantly from the negative control group, thus indicating no significant effect on P-gp related indinavir efflux. Different effects were observed for betaine monohydrate in the two parameters used and therefore non-conclusive effects were obtained on indinavir transport in the presence of this compound. The mean ER and J_{net} values for indinavir transport in the presence of β -carotene correspond with the values obtained in the presence of verapamil (positive control group), indicating inhibition of P-gp related efflux with the inclusion of β -carotene.

The effects of the selected crude plant extracts and phytoconstituents on the intestinal transport of indinavir are summarised in Table 6.1.

Table 6.1: A summary of the effects of the selected crude plant extracts and phytoconstituents on the intestinal transport of indinavir

Extract/Compound	Effect on indinavir efflux
<i>A. sativum</i> crude extract	Non-conclusive
L-alliin	Inhibition
Diallyl disulphide	Inhibition
<i>C. limonum</i> crude extract	Promotion
Hesperidin	Promotion
Eriocitrin	Non-conclusive
<i>B. vulgaris</i> crude extract	Non-conclusive
Betaine monohydrate	Non-conclusive
β -carotene	Inhibition

In conclusion, the results from the present study indicate that hesperidin and the crude extract of *C. limonum* significantly promote P-gp related indinavir efflux that may result in reduced indinavir bioavailability and potentially insufficient indinavir plasma levels. The pure phytoconstituents L-alliin, diallyl disulphide and β -carotene significantly affect indinavir transport by inhibiting its efflux across the porcine jejunum tissue. An increased plasma concentration and bioavailability of indinavir may occur with the co-administration of these phytoconstituents. An increase in indinavir plasma concentrations may potentially cause harmful reactions or adverse-effects. On the other hand, controlled and deliberate herb-drug interactions employing efflux inhibition could present the opportunity of enhanced permeability of indinavir resulting in the administration of lower drug doses in combination with herbal extracts or phytoconstituents.

6.2 Recommendations for future studies

The clinical significance of the interactions established in the present study will have to be verified during *in vivo* studies before any clinical conclusions can be made. The results can also be authenticated by comparing it with other *in vitro* transport models such as Caco-2 cells. It is further recommended that *in vitro* transport studies be performed with combined plant extracts to mimic the real-life situation in which patients consume 'cocktail' mixtures of herbal medicines. Future studies can include quantification of the pure phytoconstituents present in each plant extract and the measurement of drug transport in the presence of different concentrations of these compounds. Apart from P-gp, a number of different active efflux transporter systems (i.e. OATP transporters) also exist in the gastro-intestinal epithelium and it is recommended that the effects of the experimental compounds on specific transporters should be investigated.

References

- ABC Local. 2012. Lemon butter. <http://www.abc.net.au/nightlife/stories/2236818.htm> Date of access: 13 Jun. 2012.
- Ajazuddin & Saraf, S. 2010. Evaluation of physicochemical and phytochemical properties of Safoof-E-Sana, a Unani polyherbal formulation. *Pharmacognosy research*, 2(5):318-322.
- Aller, S.G., Yu, J., Ward, A., Weng, Y., Chittaboina, S., Zhou, R., Harell, P.M., Trinh, Y.T., Zhang, Q., Urbatsch, I.L. & Chang, G. 2009. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science*, 323(5922): 1718-1722.
- Amagase, H., Petesch, B.L., Matsuura, H., Kasuga, S. & Itakura, Y. 2001. Intake of garlic and its bioactive components. *The journal of nutrition*, 131:955S-962S.
- Ambudkar, S. V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I. & Gottesman, M.M. 1999. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annual review of pharmacology and toxicology*, 39:361-398.
- Ambudkar, S.V., Kim, I. & Sauna, Z.E. 2006. The power of the pump: mechanisms of action of P-glycoprotein (ABCB1). *European journal of pharmaceutical sciences*, 27:392-400.
- Amon, J.J. 2008. Dangerous medicines: unproven AIDS cures and counterfeit antiretroviral drugs. *Globalization and health*, 4(5):1-10.
- Apredica: Caco-2 permeability assay services. Caco-2 permeability assay. <http://www.apredica.com/caco2.php> Date of access: 12 Oct 2012.
- Artursson, P., Ungell, A. & Löfroth, J. 1993. Selective paracellular permeability in two models of intestinal absorption: cultured monolayers of human intestinal epithelial cells and rat intestinal segments. *Pharmaceutical research*, 10(8):1123-1129.
- Babb, D.A., Pemba, L., Seatlanyane, P., Charalambous, S., Churchyard, G.J. & Grant, A.D. 2007. Use of traditional medicine by HIV-infected individuals in South Africa in the era of antiretroviral therapy. *Psychology, health and medicine*, 12(3):314-320.
- Balayssac, D., Aurthier, N., Cayre, A. & Coudore, F. 2005. Does inhibition of P-glycoprotein lead to drug-drug interactions? *Toxicology letters*, 156:319-329.
- Balimane, P.V. & Chong, S. 2005. Cell culture-based models for intestinal permeability: a critique. *Drug discovery today*, 10(5):335-343.

- Balimane, P.V., Chong, S. & Morrison, R.A. 2000. Current methodologies used for evaluation of intestinal permeability and absorption. *Journal of pharmacological and toxicological methods*, 44:301-312.
- Balimane, P.V., Han, Y. & Chong, S. 2006. Current industrial practices of assessing permeability and P-glycoprotein interaction. *The American association of pharmaceutical sciences*, 8(1):E1-E13.
- Bansal, T., Jaggi, M., Khar, R.K. & Talegaonkar, S. 2009. Emerging significance of flavonoids as P-glycoprotein inhibitors in cancer chemotherapy. *Journal of pharmacy and pharmaceutical sciences*, 12(1):46-78.
- Belsinger, S. 2004. The goodness of garlic. <http://www.herbcompanion.com/cooking/the-goodness-of-garlic.aspx> Date of access: 9 Oct 2012.
- Benet, L.Z. & Cummins, C.L. 2001. The drug efflux metabolism alliance: biochemical aspects. *Advanced drug delivery reviews*, 50(1):S3-S11.
- Berggren, S. 2006. Drug transport and metabolism in rat and human intestine. Uppsala: Uppsala University. (Dissertation – Ph.D.).
- Bohets, H., Annaert, P., Mannens, G., van Beijsterveld, T. L., Anciaux, K., Verboven, P., Meuldermans, W. & Lavrijsen, K. 2001. Strategies for absorption screening in drug discovery and development. *Current topics in medicinal chemistry*, 1:367-383.
- Borrelli, F., Capasso, R. & Izzo, A. A. 2007. Garlic (*Allium sativum* L.): adverse effects and drug interactions in humans. *Molecular nutrition and food research*, 51:1386-1397.
- Brown, L., Heyneke, O., Brown, D., Van Wyk, J.P.H. & Hamman, J.H. 2008. Impact of traditional medicinal plant extracts on antiretroviral drug absorption. *Journal of ethnopharmacology*, 119:588-592.
- Chan, L.M.S., Lowes, S. & Hirst, B.H. 2004. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. *European journal of pharmaceutical sciences*, 21:25-51.
- Chavez, M.L., Jordan, M.A. & Chavez, P.I. 2006. Evidence-based drug-herbal interactions. *Life sciences*, 78:2146-2157.
- Corzo-Martínez, M., Corzo, N. & Villamiel, M. 2007. Biological properties of onions and garlic. *Trends in food science and technology*, 18:609-625.

- Dausch, J.G. & Nixon, D.W. 1990. Garlic: a review of its relationship to malignant disease. *Preventive medicine*, 19:346-361.
- Deferme, S., Annaert, P. & Augustijns, P. 2008. In vitro screening models to assess intestinal drug absorption and metabolism. *Biotechnology: pharmaceutical aspects*, 7(2):182-215.
- Del Río, J.A., Fuster, M.D., Gómez, P., Porras, I., García-Lidón, A. & Ortuño, A. 2004. *Citrus limon*: a source of flavonoids of pharmaceutical interest. *Food chemistry*, 84:457-461.
- De Zwart, F.J., Slow, S., Payne, R.J., Lever, M., George, P.M., Gerrard, J.A. & Chambers, S.T. 2003. Glycine betaine and glycine betaine analogues in common foods. *Food chemistry*, 83:197-204.
- Dias, M.G., Camões, M.F.G.F.C. & Oliveira, L. 2009. Carotenoids in traditional Portuguese fruits and vegetables. *Food chemistry*, 113:808-815.
- Endres, C.J., Hsiao, P., Chung, F.S. & Unadkat, J.D. 2006. The role of transporters in drug interactions. *European journal of pharmaceutical sciences*, 27:501-517.
- Evans, A.M. 2000. Influence of dietary components on the gastrointestinal metabolism and transport of drugs. *Therapeutic drug monitoring*, 22:131-136.
- Figiel, A. 2010. Drying kinetics and quality of beetroots dehydrated by combination of connective and vacuum-microwave method. *Journal of food engineering*, 98:461-470.
- Foster, B.C., Foster, M.S., Vandenhoeck, S., Krantis, A., Budzinski, J.W., Arnason, J.T., Gallicano, K.D. & Choudri, S. 2001. An *in vitro* evaluation of human cytochrome P450 3A4 and P-glycoprotein inhibition by garlic. *Journal of pharmaceutical sciences*, 4(2):176-184.
- Fugh-Berman, A. 2000. Herb-drug interactions. *The lancet*, 355:134-138.
- Garg, A., Garg, S., Zaneveld, L.J.D. & Singla, A.K. 2001. Chemistry and pharmacology of the citrus bioflavonoid hesperidin. *Phytotherapy research*, 15:655-669.
- Gil-Izquierdo, A., Riquelme, M.T., Porras, I. & Ferreres, F. 2004. Effect of the rootstock and interstock grafted in lemon tree (*Citrus limon* (L.) Burm.) on the flavonoid content of lemon juice. *Journal of agriculture and food chemistry*, 52:324-331.
- González-Molina, E., Moreno, D.A. & García-Viguera, C. 2009. Comparison of 'Verna' lemon juice quality for new ingredients and food products. *Scientia horticulturae*, 120:353-359.

- Gore-Felton, C., Vosvick, M., Power, R., Koopman, C., Ashton, E., Bachmann, M.H., Israelski, D. & Spiegel, D. 2003. Alternative therapies: a common practice among men and women living with HIV. *Journal of the association of nurses in AIDS care*, 14(3):17-27.
- Gouws, C., Steyn, D., Du Plessis, L., Steenekamp, J. & Hamman, J.H. 2012. Combination therapy of Western drugs and herbal medicines: recent advances in understanding interactions involving metabolism and efflux. *Expert opinion on drug metabolism and toxicology*, 8(8):973-984.
- Grass, G.M. 1997. Simulation models to predict oral drug absorption from in vitro data. *Advanced drug delivery reviews*, 23:199-219.
- Grass, G.M. & Sweetana, S.A. 1988. *In vitro* measurement of gastrointestinal tissue permeability using a new diffusion cell. *Pharmaceutical research*, 5(6):372-376.
- Greenbeck, J.F. Reproducibility of the shimadzu DTG-60.
<http://www2.shimadzu.com/apps/appnotes/reproducibilitydtg.pdf> Date of access: 23 Apr. 2012.
- Hansen, T.S. & Nilsen, O.G. 2009. *Echinacea purpurea* and P-glycoprotein drug transport in Caco-2 cells. *Phytotherapy research*, 23:86-91.
- Hayeshi, R., Masimirembwa C., Mukanganyama, S. & Ungell, A.B. 2006. The potential inhibitory effect of antiparasitic drugs and natural products on P-glycoprotein mediated efflux. *European journal of pharmaceutical sciences*, 29:70-81.
- Hidalgo, I.J. 2001. Assessing the absorption of new pharmaceuticals. *Current topics in medicinal chemistry*, 1(5):385-401.
- Hochman, J.H., Yamazaki, M., Ohe, T. & Lin, J.H. 2002. Evaluation of drug interactions with P-glycoprotein in drug discovery: *in vitro* assessment of the potential for drug-drug interactions with P-glycoprotein. *Current drug metabolism*, 3(3):257-273.
- Hu, Z., Yang, X., Chi Lul Ho, P., Yung Chan, S., Wan Sia Heing, C., Chan, E., Duan, W., Ling Koh, H. & Zhou, S. 2005. Herb-drug interactions. *Drugs*, 65(9):1240-1282.
- Huisman, M.T., Smit, J.W. & Schinkel, A.H. 2000. Significance of P-glycoprotein for the pharmacology and clinical use of HIV protease inhibitors. *AIDS*, 14:237-242.
- ICH **see** International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Ingersoll, R.E. 2005. Herbaceuticals: an overview for counsellors. *Journal of counseling and development*, 83:434-43.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). 1996. Validation of analytical procedures: text and methodology Q2(R1).

http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf Date of access: 24 Oct. 2012.

Jastrebova, J., Witthöft, C., Grahn, A., Svensson, U. & Jägerstad, M. 2003. HPLC determination of folates in raw and processed beetroots. *Food chemistry*, 80:579-588.

JP XIV. General tests.

http://jpub.nihs.go.jp/jp14e/14data/General_Test/Loss_on_Drying_Test.pdf Date of access: 03 Jul. 2012.

Kamath, A.V., Yao, M., Zhang, Y. & Chong, S. 2005. Effect of fruit juices on the oral bioavailability of Fexofenadine in rats. *Journal of pharmaceutical sciences*, 49(2):233-238.

Katragadda, S., Budda, B., Anand, B.S. & Mitra, A.K. 2005. Role of efflux pumps and metabolizing enzymes in drug delivery. *Expert opinion on drug delivery*, 2(4):683-705.

Kerns, E.H. & Di, L. 2008. Drug-like properties: concepts, structure design and methods. s.l.: Academic press.

Le Ferrec, E., Chesne, C., Artusson, P., Brayden, D., Fabre, G., Gires, P., Guillou, F., Rousset, M., Rubas, W. & Scarino, M. 2001. *In vitro* models of the intestinal barrier: the report and recommendations of ECVAM workshop 46. *Alternatives to laboratory animals*, 29:649-668.

Lim, S. & Lim, L. 2006. Effects of citrus fruit juices on cytotoxicity and drug transport pathways of Caco-2 cell monolayers. *International journal of pharmaceutics*, 307:42-50.

Lüker, J., El Tamer, M.K., Schwab, W., Verstappen, F.W.A., Van Der Plas, L.H.W., Bouwmeester, H.J. & Verhoeven, H.A. 2002. Monoterpene biosynthesis in lemon (*Citrus limon*). *European journal of biochemistry*, 269:3160-3171.

McFadden, R. & Peterson, N. 2011. Interactions between drugs and four common medicinal herbs. *Nursing standard*, 25(19):65-68.

- Mills, E., Cooper, C. & Kanfer, I. 2005. Traditional African medicine in the treatment of HIV. *Lancet*, 5:465-467.
- Minocha, M., Mandava, N.K., Kwatra, D., Pal, D., Folk, W.R., Earla, R. & Mitra, A.K. 2011. Effect of short term and chronic administration of *Sutherlandia frutescens* on pharmacokinetics of nevirapine in rats. *International journal of pharmaceutics*, 413:44-50.
- Mitsunaga, Y., Takanaga, H., Matsuo, H., Naito, M., Tsuruo, T., Ohtani, H & Sawada, Y. 2000. Effect of bioflavonoids on vincristine transport across blood-brain barrier. *European journal of pharmacology*, 395:193-201.
- Müller, A.C. & Kanfer, I. 2011. Potential pharmacokinetic interactions between antiretrovirals and medicinal plants used as complimentary and African traditional medicines. *Biopharmaceutics and drug disposition*, 32:458-470.
- Nejdfor, P., Ekelund M., Jeppsson, B. & Weström, B.R. 2000. Mucosal *in vitro* permeability in the intestinal tract of the pig, the rat and man: species- and region-related differences. *Scandinavian journal of gastroenterology*, 5:501-507.
- Norris, D.A., Puri, N. & Sinko, P.J. 1998. The effect of physical barriers and properties on the oral absorption of particulates. *Advanced drug delivery reviews*, 34:135-154.
- Okwu, D.E. & Emenike, I.N. 2006. Evaluation of the phytonutrients and vitamins content of citrus fruit. *International journal of molecular medicine and advanced sciences*, 2(1):1-6.
- Omar, S.H. & Al-Wabel, N.A. 2010. Organosulfur compounds and possible mechanism of garlic in cancer. *Saudi pharmaceutical journal*, 18:51-58.
- Pal, D. & Mitra, A.K. 2006. MDR- and CYP3A4-mediated drug-herbal interactions. *Life sciences*, 78:2131-2145.
- Palumbo, P., Picchini, U., Beck, B., Van Gelder, J., Delbar, N. & Degaetano, A. 2008. A general approach to the apparent permeability index. *Journal of pharmacokinetics and pharmacodynamics*, 35:235-248.
- Panchagnula, R., Bansal, T., Varma, M.V.S. & Lal Kaul, C. 2004. Reversed-phase liquid chromatography with ultraviolet detection for simultaneous quantification of indinavir and propranolol from *ex vivo* rat intestinal permeability studies. *Journal of chromatography*, 806:277-282.

Petry, J. J. M. D. 1995. Garlic and postoperative bleeding. *Plastic and reconstructive surgery*, 96(2):483.

Robertson, H. *Beta vulgaris* (chaud, beetroot, sugarbeet, mangel-wurzel).

http://www.biodiversityexplorer.org/plants/amaranthaceae/beta_vulgaris.htm Date of access: 13 Jun. 2012.

Sendl, A. 1995. *Allium sativum* and *Allium ursinum*: chemistry, analysis, history, botany. *Phytomedicine*, 4:323-339.

Sharom, F.J. 2006. Shedding light on drug transport: structure and function of the P-glycoprotein multidrug transporter (ABCB1). *Biochemistry and cell biology*, 84(6):979-992.

Sharom, F.J. 2008. ABC multidrug transporters: structure, function and role in chemoresistance. *Pharmacogenomics*, 9(1):105-127.

Solvo® Technology. Intestine. <http://www.solvo.jp/Solvo%20Solutions/intestine.html> Date of access: 9 Okt 2012.

StatSoft®. 2007. Statistica, version 8.0. Tulsa. [data analysis software system].

Suana, Z.E. & Ambudkar, S.V. 2000. Evidence for a requirement for ATP hydrolysis at two distinct steps during a single turnover of the catalytic cycle of human P-glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America*, 97(6):2515-2520.

Sweetana, S.A. & Grass, G.M. 1993. Apparatus for *in vitro* determination of substances across membranes, biological tissues, or cell cultures. (Patent: US 5183760).

Swindle, M.M. & Smith, A.C. 1998. Comparative anatomy and physiology of the pig. *Scandinavian journal of laboratory animal science. Supplement*, 25:11-21.

Tabachnick, B.G. & Fidell, L.S. 2001. *Using Multivariate Statistics*, 4th Ed., Allyn & Bacon, Boston. 966 p.

Tamai, J. 2012. Oral drug delivery utilizing intestinal OATP transporters. *Advanced drug delivery reviews*, 64:508-514.

Tarirai, C., Viljoen, A.M. & Hamman, J.H. 2010. Herb-drug pharmacokinetic interactions reviewed. *Expert opinion on drug metabolism and toxicology*, 6(12):1515-1538.

- Tarirai, C., Viljoen, A.M. & Hamman, J.H. 2012. Effects of dietary fruits, vegetables and a herbal tea on the *in vivo* transport of cimetidine: comparing the Caco-2 model with porcine jejunum tissue. *Pharmaceutical biology*, 50(2):254-263.
- Ulbricht, C., Chao, W., Costa, D., Rusie-Seamon, E., Weissner, W. & Woods, J. 2008. Clinical evidence of herb-drug interactions: A systemic review by the natural standard research collaboration. *Current drug metabolism*, 9:1063-1120.
- University of Tokyo. Division of Life Sciences. 2010. Intercellular Communication. http://csls-text.c.u-tokyo.ac.jp/active/11_04.html Date of access: 8 Oct. 2010.
- Ussing, H.H. & Zerahn, K. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta physiologica scandinavica*, 23:110-127.
- Vaes, L.P.J. & Chyka, P.A. 2000. Interactions of warfarin with garlic, ginger, ginko, or ginseng: nature of the evidence. *The annals of pharmacotherapy*, 34:1478-1482.
- Váli, L., Stefanovits-Bányai, É., Szentmihályi, K., Fébel, H., Sárdi, É., Lugasi, A., Kocsis, I. & Blázovics, A. 2007. Liver protecting effects of table beet (*Beta vulgaris* var. *ruba*) during ischemia-perfusion. *Nutrition*, 23:172-178.
- Van Asperen, J., Van Tellingen, O. & Beijnen, J.H. 1998. The pharmacological role of P-glycoprotein in the intestinal epithelium. *Pharmacological research*, 37(6):429-435.
- Varma, M.V.S., Ashokraj, Y., Dey, C.S. & Panchagnula, R. 2003. P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. *Pharmacological research*, 48:347-359.
- Varma, M.V.S., Perumal, O.P. & Panchagnula, R. 2006. Functional role of P-glycoprotein in limiting peroral drug absorption: optimizing drug delivery. *Current opinion in chemical biology*, 10:367-373.
- Vermani, K., Garg, S. 2002. Herbal medicines for sexually transmitted diseases and AIDS. *Journal of ethnopharmacology*, 80:49-66.
- Wilson, T.H. & Wiseman, G. 1953. The use of everted small intestine for the study of the transference of substances from the mucosal to serosal surface. *Journal of physiology*, 123:116-125.
- Zhou, S., Lim, L.Y. & Chowbay, B. 2004. Herbal modulation of P-glycoprotein. *Drug metabolism reviews*, 36(1): 57-104.

Zhou, S., Zhou, Z., Li, C., Chen, X., Yu, X., Xue, C.C. & Herington, A. 2007. Identification of drugs that interact with herbs in drug development. *Drug discovery today*, 12:664-673.

Annexure A

Table A.1: Peak area values of indinavir alone (negative control group) across excised porcine intestinal tissue

Time (min)	AP-BL					BL-AP				
	Cell 1	Cell 2	Cell 3	Cell 4	Mean	Cell 1	Cell 2	Cell 3	Cell 4	Mean
20	0.000	0.000	0.000	0.000	0.00000	0.000	0.000	0.000	0.000	0.00000
40	0.000	0.000	0.000	0.000	0.00000	0.000	0.000	0.000	0.000	0.00000
60	0.038	1.016	1.032	0.878	0.74100	0.000	0.000	0.000	0.000	0.00000
80	1.448	1.463	1.629	0.809	1.33725	2.072	1.462	1.315	2.419	1.81700
100	2.609	2.368	2.076	2.417	2.36750	2.285	1.664	1.825	1.880	1.91350
120	3.057	3.91	3.223	4.464	3.66350	2.138	1.569	1.732	2.965	2.10100
140	5.742	6.268	4.332	6.262	5.65100	3.868	3.745	4.352	4.521	4.12150
160	3.956	6.644	4.381	8.301	5.82050	4.869	4.971	5.228	5.806	5.21850
180	9.001	9.309	7.230	7.714	8.31350	5.107	6.177	5.004	5.720	5.50200

Table A.2: Peak area values of indinavir transport in the presence of verapamil (positive control group) across excised porcine intestinal tissue

Time (min)	AP-BL					BL-AP				
	Cell 1	Cell 2	Cell 3	Cell 4	Mean	Cell 1	Cell 2	Cell 3	Cell 4	Mean
20	0.000	0.000	0.000	0.000	0.00000	0.000	0.000	0.000	0.000	0.00000
40	0.000	0.000	0.000	0.000	0.00000	0.000	0.000	0.000	0.000	0.00000
60	1.362	1.657	2.371	1.779	1.79225	1.685	2.721	2.691	1.476	2.14325
80	2.845	2.814	3.106	2.299	2.76600	2.928	1.548	4.041	2.861	2.84450
100	3.909	4.050	4.879	4.322	4.29000	3.691	5.349	6.741	3.967	4.93700
120	5.675	5.870	6.845	5.928	6.07950	5.47	6.597	10.135	6.897	7.27475
140	7.518	7.336	8.976	7.291	7.78025	8.256	10.406	12.79	7.488	9.73500
160	10.765	10.545	11.249	8.900	10.36475	9.737	9.095	9.958	7.431	9.05525
180	11.59	11.777	10.113	10.563	11.01075	9.982	11.296	10.003	9.951	10.30800

Table A.3: Peak area values of indinavir transport in the presence of *Allium Sativum crude* extract across excised porcine intestinal tissue

Time (min)	AP-BL					BL-AP				
	Cell 1	Cell 2	Cell 3	Cell 4	Mean	Cell 1	Cell 2	Cell 3	Cell 4	Mean
20	0.000	0.000	0.000	0.000	0.00000	0.000	0.000	0.000	0.000	0.00000
40	0.000	0.000	0.000	0.000	0.00000	1.644	1.373	1.594	1.645	1.56400
60	2.714	3.197	4.186	4.845	3.73550	3.027	1.772	2.208	4.168	2.79375
80	4.211	4.560	6.373	5.097	5.06025	4.114	4.677	3.105	6.221	4.52925
100	6.765	6.771	10.302	7.531	7.84225	7.523	7.015	4.423	7.566	6.63175
120	8.085	5.038	13.137	10.043	9.07575	8.626	11.865	7.661	13.854	10.5015
140	11.813	15.72	17.198	12.816	14.38675	13.743	14.268	10.785	15.018	13.4535
160	13.685	16.368	20.372	15.341	16.44150	17.954	19.432	13.406	20.841	17.90825
180	18.651	20.924	24.415	20.211	21.05025	25.523	25.814	17.712	27.131	24.04500

Table A.4: Peak area values of indinavir transport in the presence L-alliin across excised porcine intestinal tissue

Time (min)	AP-BL					BL-AP				
	Cell 1	Cell 2	Cell 3	Cell 4	Mean	Cell 1	Cell 2	Cell 3	Cell 4	Mean
20	0.000	0.000	0.000	0.000	0.00000	3.673	4.278	4.222	1.816	3.49725
40	0.000	0.000	0.000	0.000	0.00000	4.079	4.7	4.807	2.35	3.98400
60	0.319	2.345	0.561	3.095	1.58000	3.76	6.674	9.951	2.096	5.62025
80	1.012	2.686	2.663	5.295	2.91400	4.752	7.567	8.63	5.044	6.49825
100	6.863	6.138	5.621	6.207	6.20725	5.784	8.428	11.592	4.568	7.59300
120	9.871	12.583	8.066	9.823	10.08575	9.604	9.952	10.3	3.736	8.39800
140	11.957	11.744	11.746	14.63	12.51925	4.345	3.905	12.053	5.774	6.51925
160	16.272	13.554	15.54	15.089	15.11375	10.485	8.471	6.58	4.649	7.54625
180	20.189	16.055	16.148	17.999	17.59775	5.538	10.277	9.695	8.938	8.61200

Table A.5: Peak area values of indinavir transport in the presence diallyl disulphide across excised porcine intestinal tissue

Time (min)	AP-BL					BL-AP				
	Cell 1	Cell 2	Cell 3	Cell 4	Mean	Cell 1	Cell 2	Cell 3	Cell 4	Mean
20	0.000	0.000	0.000	0.000	0.00000	0.000	0.000	0.000	0.000	0.000000
40	0.000	0.000	0.000	0.000	0.00000	0.000	0.000	0.000	0.000	0.000000
60	0.000	2.185	0.541	1.800	1.13150	0.000	0.000	0.000	0.000	0.000000
80	0.938	4.738	0.984	3.972	2.65800	2.106	1.809	1.439	2.021	1.843750
100	4.407	7.389	5.774	7.232	6.20050	2.630	3.172	2.910	3.378	3.022500
120	7.324	6.684	3.464	7.853	6.33125	2.625	4.301	2.867	3.073	3.216500
140	7.763	9.195	5.421	8.655	7.75850	4.115	4.596	4.868	5.810	4.847250
160	10.55	11.017	7.509	13.514	10.64750	5.598	5.586	5.684	6.067	5.733750
180	11.096	13.082	19.061	14.559	14.44950	7.069	7.530	7.297	6.350	7.061500

Table A.6: Peak area values of indinavir transport in the presence *Citrus Limonum* crude extract across excised porcine intestinal tissue

Time (min)	AP-BL					BL-AP				
	Cell 1	Cell 2	Cell 3	Cell 4	Mean	Cell 1	Cell 2	Cell 3	Cell 4	Mean
20	0.000	0.000	0.000	0.000	0.00000	0.000	0.000	0.000	0.000	0.000000
40	0.000	0.000	0.000	0.000	0.00000	2.645	2.189	2.887	2.465	2.546500
60	1.109	1.168	0.474	0.000	0.68775	4.970	6.029	5.854	4.261	5.278500
80	2.071	1.769	1.645	2.351	1.95900	11.008	13.544	12.052	12.911	12.37875
100	3.622	3.04	3.114	3.312	3.27200	17.013	19.359	19.224	17.228	18.20600
120	4.732	3.583	2.446	4.552	3.82825	28.868	27.679	25.594	22.795	26.23400
140	5.772	4.326	4.541	4.879	4.87950	29.670	43.49	35.606	29.831	34.64925
160	7.321	6.469	7.369	7.330	7.12225	36.511	46.242	47.661	36.321	41.68375
180	9.379	10.249	10.249	9.951	9.95700	45.406	50.364	46.725	55.998	49.62325

Table A.7: Peak area values of indinavir transport in the presence hesperidin across excised porcine intestinal tissue

Time (min)	AP-BL					BL-AP				
	Cell 1	Cell 2	Cell 3	Cell 4	Mean	Cell 1	Cell 2	Cell 3	Cell 4	Mean
20	0.000	0.000	0.000	0.000	0.00000	2.079	2.906	2.458	2.671	2.52850
40	0.000	0.000	0.000	0.000	0.00000	4.769	5.055	4.081	4.386	4.57275
60	0.000	0.000	0.000	0.000	0.00000	12.147	9.939	9.939	8.273	10.07450
80	1.071	1.121	1.648	1.491	1.33275	13.288	13.818	14.304	11.298	13.17700
100	2.032	2.049	2.9	3.851	2.70800	21.523	20.347	15.76	17.372	18.75050
120	4.98	4.195	4.313	5.945	4.85825	25.509	20.875	22.006	22.961	22.83775
140	6.864	6.466	7.115	8.245	7.17250	28.467	24.949	25.012	27.599	26.50675
160	6.06	8.096	10.14	10.367	8.66575	35.755	31.568	30.671	31.404	32.34950
180	12.64	10.411	12.682	12.876	12.15225	39.204	32.541	33.987	35.998	35.43250

Table A.8: Peak area values of indinavir transport in the presence eriocitrin across excised porcine intestinal tissue

Time (min)	AP-BL					BL-AP				
	Cell 1	Cell 2	Cell 3	Cell 4	Mean	Cell 1	Cell 2	Cell 3	Cell 4	Mean
20	0.000	0.000	0.000	0.000	0.00000	0.000	0.000	0.000	0.000	0.00000
40	2.053	1.109	1.861	1.231	1.56350	0.000	0.000	0.000	0.000	0.00000
60	3.213	2.139	3.218	2.845	2.85375	2.276	2.564	1.172	2.102	2.02850
80	3.972	3.795	4.103	4.596	4.11650	4.181	3.049	5.031	7.126	4.84675
100	5.902	5.098	4.041	6.338	5.34475	6.961	5.693	12.280	10.285	8.80475
120	6.789	6.843	7.383	10.075	7.77250	14.315	9.084	9.392	15.72	12.12775
140	9.259	11.134	9.272	17.224	11.72225	17.755	14.666	17.340	19.563	17.33100
160	11.635	11.825	12.497	16.415	13.09300	24.838	20.038	20.283	27.478	23.15925
180	13.467	16.243	16.287	19.71	16.42675	27.096	29.758	25.785	27.94	27.64475

Table A.9: Peak area values of indinavir transport in the presence *Beta vulgaris* across excised porcine intestinal tissue

Time (min)	AP-BL					BL-AP				
	Cell 1	Cell 2	Cell 3	Cell 4	Mean	Cell 1	Cell 2	Cell 3	Cell 4	Mean
20	0.000	0.000	0.000	0.000	0.00000	0.000	0.000	0.000	0.000	0.00000
40	1.222	2.388	1.398	1.163	1.54275	4.297	4.057	4.797	4.275	4.35650
60	3.32	5.808	3.226	2.291	3.66125	8.904	10.096	9.469	8.787	9.31400
80	7.491	11.425	7.076	6.551	8.13575	15.19	17.038	12.473	14.522	14.80575
100	10.984	17.14	10.793	9.942	12.21475	24.174	24.445	22.786	22.398	23.45075
120	15.407	22.768	14.663	13.651	16.62225	31.092	30.664	24.504	28.784	28.76100
140	19.764	29.204	20.687	17.051	21.67650	36.964	36.984	31.869	31.862	34.41975
160	26.728	36.803	24.795	22.663	27.74725	44.196	42.721	36.872	40.043	40.95800
180	30.675	42.911	29.706	27.573	32.71625	51.178	49.188	39.987	47.722	47.01875

Table A.10: Peak area values of indinavir transport in the presence diallyl disulphide across excised porcine intestinal tissue

Time (min)	AP-BL					BL-AP				
	Cell 1	Cell 2	Cell 3	Cell 4	Mean	Cell 1	Cell 2	Cell 3	Cell 4	Mean
20	1.181	1.275	1.673	1.000	1.28225	3.108	3.520	1.442	1.110	2.29500
40	1.25	1.324	1.509	1.659	1.43550	2.351	1.937	1.894	1.452	1.90850
60	2.465	2.654	2.208	2.911	2.55950	4.910	3.649	3.009	2.912	3.62000
80	3.698	3.754	2.952	4.491	3.72375	8.470	6.858	5.576	5.337	6.56025
100	5.608	5.477	4.072	7.100	5.56425	12.855	9.397	7.649	8.330	9.55775
120	8.033	8.874	6.455	9.929	8.32275	17.299	15.309	12.439	12.068	14.27875
140	11.376	11.806	8.448	13.180	11.20250	23.270	19.033	16.259	14.086	18.16200
160	14.463	15.659	10.993	15.878	14.24825	26.694	22.533	19.848	18.343	21.85450
180	18.702	18.469	13.381	20.202	17.68850	32.870	27.544	23.244	20.749	26.10175

Table A.11: Peak area values of indinavir transport in the presence β -carotene across excised porcine intestinal tissue

Time (min)	AP-BL					BL-AP				
	Cell 1	Cell 2	Cell 3	Cell 4	Mean	Cell 1	Cell 2	Cell 3	Cell 4	Mean
20	3.394	1.244	2.855	3.004	2.62425	1.634	0.849	0.958	0.649	1.02250
40	3.236	1.640	3.669	4.298	3.21075	3.095	2.413	2.207	2.220	2.48375
60	3.735	2.232	3.799	4.730	3.62400	4.889	2.052	2.353	1.392	2.67150
80	4.614	3.004	5.428	5.233	4.56975	6.583	3.293	3.529	3.163	4.14200
100	6.810	4.046	6.880	7.137	6.21825	7.887	5.091	5.285	7.046	6.32725
120	9.361	5.967	7.756	7.713	7.69925	11.159	7.027	7.559	9.241	8.74650
140	11.514	6.895	9.829	9.981	9.55475	12.888	9.428	11.620	9.023	10.73975
160	14.247	8.924	10.904	11.490	11.3933	12.273	8.456	9.050	10.237	10.00400
180	18.628	11.728	13.391	13.820	14.3925	20.146	16.673	17.475	20.86	18.78850

Annexure B

*The Academy of Pharmaceutical Sciences of the
Pharmaceutical Society of South Africa*

*Die Akademies vir Farmaseutiese Wetenskappe van die
Aptekersvereniging van Suid-Afrika*



hereby commends

Karin Roos

*as the Runner-up of the
Young Scientist award for
2012*

A handwritten signature in black ink, appearing to read 'M. M. M.', written over a horizontal line.

Chairman/Voorsitter

A handwritten signature in black ink, appearing to read 'S. J. J.', written over a horizontal line.

Secretary/Sekretaris

Effect of plant extracts and phytoconstituents on the intestinal transport of the anti-retroviral drug, indinavir

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Purpose:

Herbal medicines are widely used by HIV infected patients and combined use with anti-retroviral drugs could potentially cause pharmacokinetic and/or pharmacodynamic interactions. In this study the effect of crude extracts and pure phytoconstituents of garlic (*Allium sativum*), lemon (*Citrus limonum*) and beetroot (*Beta vulgaris*) on the *in vitro* transport of indinavir, an anti-retroviral drug across excised intestinal tissue will be determined.

Methods:

The transport of indinavir was determined in the apical to basolateral (AP-BL) direction and the basolateral to apical (BL-AP) direction across excised pig jejunum segments using the Sweetana-Grass diffusion apparatus. In the negative control group, the transport of indinavir alone (200 μ M) was determined. In the positive control group verapamil (100 μ M), a known P-gp inhibitor, was added. The transport of indinavir in the presence of the plant extracts (5 mg/ml) and pure phytoconstituents (20 μ M) was then measured. Samples collected at pre-determined time intervals were analyzed by a validated HPLC method and transport was expressed as the apparent permeability coefficient (P_{app}) values from which the efflux ratio (ER) and the transepithelial flux (J) values were calculated.

Results:

The mean ER value for indinavir in the negative control group was 1.41 ± 0.170 and in the positive control group it was 0.56 ± 0.0426 . Statistically significant ($p < 0.05$) inhibition of indinavir efflux as indicated by reduced ER values was obtained for L-alliin (ER = 0.280 ± 0.030), diallyl disulphide (ER = 0.505 ± 0.034) and β -carotene (ER = 0.664 ± 0.075). Inhibition of indinavir efflux will lead to increased transport. Statistically significant ($p < 0.05$) promotion of indinavir efflux was obtained for lemon crude extract (ER = 5.551 ± 0.575) and hesperidin (ER = 3.385 ± 0.477). Beetroot, betaine monohydrate, garlic and eriocitrin showed no statistically significant effect.

Conclusion:

The results from this study showed that L-alliin, diallyl disulphide and β -carotene may significantly increase indinavir plasma levels after oral administration. Lemon crude extract and hesperidin may significantly reduce indinavir plasma levels. These pharmacokinetic interactions between certain compounds may negatively affect HIV patients' antiretroviral treatment, but controlled inclusion into dosage forms may cause more effective delivery of indinavir.

Effect of plant extracts and phytoconstituents on the intestinal transport of the anti-retroviral drug indinavir

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Background

There is a global rise in the use of natural products in combination with allopathic medicines, while most patients do not inform their health care providers of the use of these natural products (Ingersoll, 2005:434). Both pharmacodynamic and pharmacokinetic interactions between herbs and conventional drugs must be identified for the wellbeing of the patient (Ulbricht *et al.*, 2008:1063). Increasing evidence from *in vitro* and *in vivo* studies indicated that changed drug pharmacokinetics by co-administered herbs may be attributed to modulation of efflux drug transporters such as P-glycoprotein (P-gp). This states the importance of early identification of potential modulators of P-gp from herbal medicines (Zhou *et al.*, 2004:57-59). Herbal medicines including garlic, lemon and beetroot are widely used by HIV patients, especially after the pronouncement by the previous health ministers that promoted the use of these botanicals in HIV patients (Amon, 2008:4).

Aim

The aim of this study is to determine whether crude extracts and pure phytoconstituents of garlic (*Allium sativum*), lemon (*Citrus limonum*) and beetroot (*Beta vulgaris*) have an effect on the bi-directional transport of indinavir across excised porcine intestinal tissue.

Results and discussion

The J_{net} values for indinavir in the presence of the crude plant extracts from garlic, lemon and beetroot as well as selected phytoconstituents in comparison with the control groups are shown in Figure 1.

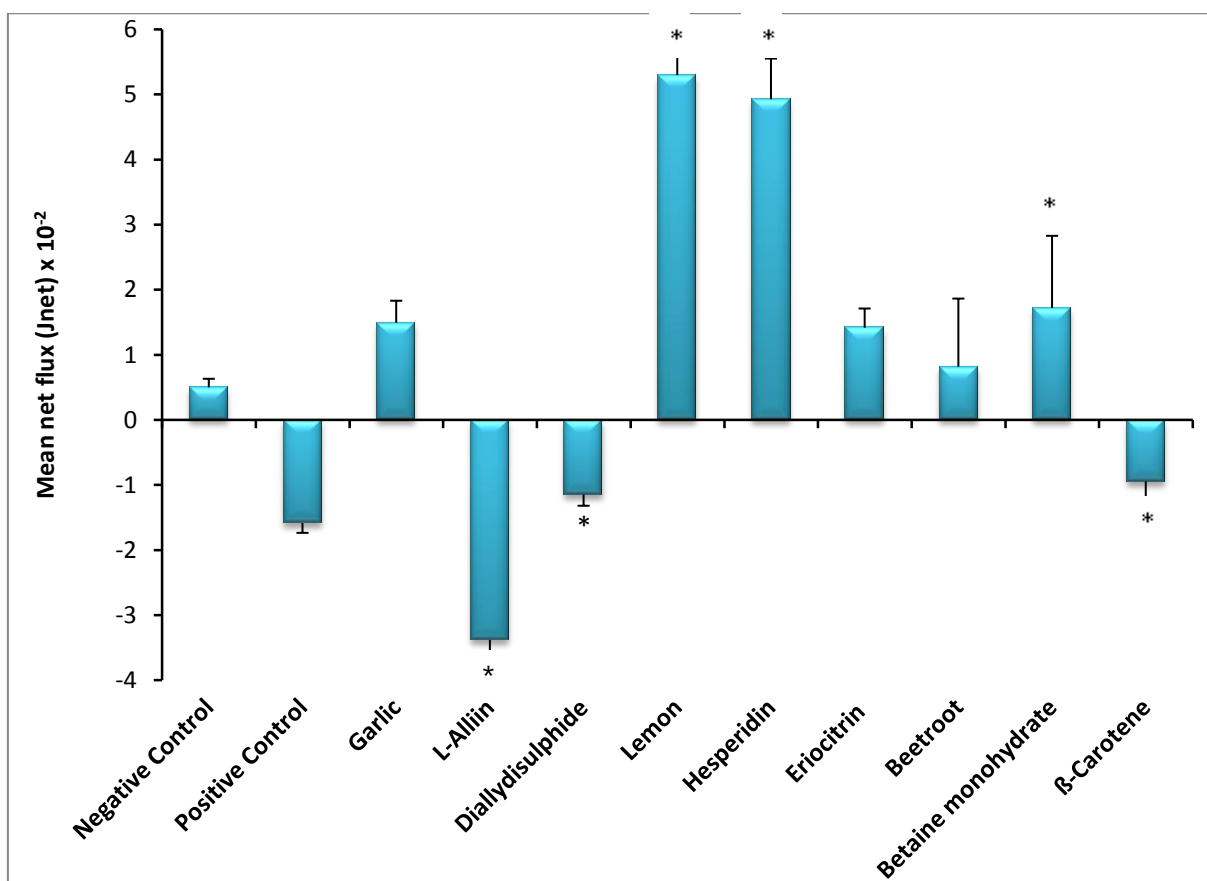


Figure 1: J_{net} values for indinavir in the presence of crude plant extracts and phytoconstituents in comparison with the control groups. * Statistically significantly different from negative control group.

Effect of garlic, L-alliin and diallyl disulphide on indinavir transport

The mean J_{net} value for indinavir in the presence of garlic crude extract was 0.015 ± 0.003 compared to 0.005 ± 0.001 for the negative control group. The efflux of indinavir was therefore not modulated significantly by the inclusion of garlic crude extract. The mean J_{net} value for indinavir in the presence of L-alliin was -0.034 ± 0.003 and in the presence of diallyl disulphide it was -0.011 ± 0.002 , which differs significantly ($p < 0.05$) from the negative control group. Both L-alliin and diallyl disulphide significantly inhibited P-gp related efflux of indinavir and may therefore improve the bioavailability of indinavir. In addition, L-alliin inhibited indinavir efflux to a significantly larger extent than the positive control group (verapamil).

Effect of lemon crude extract, hesperidin and eriocitrin on indinavir transport

The mean J_{net} value for indinavir in the presence of lemon was 0.05 ± 0.005 which was significantly higher compared to that of the negative control. This indicates the promotion of efflux of indinavir by lemon crude extract. The mean J_{net} value for indinavir in the presence of hesperidin was 0.049 ± 0.006 and in the presence of eriocitrin it was 0.014 ± 0.003 . The J_{net}

value was significantly ($p < 0.05$) higher compared to the negative control group in the presence of hesperidin, but not for eriocitrin.

Effect of beetroot crude extract, betaine monohydrate and β -carotene on indinavir transport

The mean J_{net} value for indinavir in the presence of beetroot crude extract was 0.008 ± 0.010 with no statistically significant difference compared to the negative control group. The J_{net} value for indinavir in the presence of betaine monohydrate was 0.017 ± 0.011 , which differed statistically significantly ($p < 0.05$) from the negative control group. The J_{net} value for β -carotene was -0.009 ± 0.004 , which was significantly lower ($p < 0.05$) than the value for the negative control group indicating significant inhibition of P-gp related indinavir efflux by this phytoconstituent of beetroot.

Conclusion and future recommendations

The results from this study showed that L-alliin, diallyl disulphide and β -carotene have an inhibition effect on indinavir efflux, which may lead to increased indinavir plasma levels after oral administration. Lemon crude extract and hesperidin promote indinavir efflux, which may reduce indinavir plasma levels. These pharmacokinetic interactions between certain phytoconstituents and plant extracts may negatively affect HIV patients' antiretroviral treatment. On the other hand, controlled inclusion of L-alliin, diallyl disulphide and β -carotene into dosage forms may cause more effective delivery of indinavir.

References

- Amon, J.J. 2008. Dangerous medicines: unproven AIDS cures and counterfeit antiretroviral drugs. *Globalization and health*, 4(5):1-10, Feb.
- Ingersoll, RE. 2005. Herbaceuticals: an overview for counsellors. *Journal of counseling and development*, 83:434-43.
- Ulbricht, C., Chao, W., Costa, D., Rusie-Seamon, E., Weissner, W. & Woods, J. 2008. Clinical evidence of herb-drug interactions: A systemic review by the natural standard research collaboration. *Current drug metabolism*, 9:1063-1120.
- Zhou, S., LIM, L.Y. & Chowbay, B. 2004. Herbal modulation of P-glycoprotein. *Drug metabolism reviews*. 36(1): 57-104.

Annexure C

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Rahway NJ 07065-0900 USA



Certificate of Analysis

Indinavir Sulfate

LOT No. 0000042180

TEST

Characteristics
Water
Assay: Indinavir Sulfate
Total Impurities
Heavy Metals
Residue on Ignition
Specific Rotation
Identity: Indinavir Sulfate (IR & HPLC)

RESULTS

Conforms
0.3 wt%
100.9% (wt/wt)
0.1 %
Conforms
0.0 % (wt/wt)
126 Degrees
Conforms

Retest Date:

August 2012

Charise Griffin
Research Operations

Certificate of Analysis

Product Name: (±)-L-ALLIIN
>= 90 % HPLC
Product Number: 74264
Product Brand: Sigma
Molecular Formula: $C_6H_{11}NO_3S$
Molecular Mass: 177.22
CAS Number: 17795-26-5

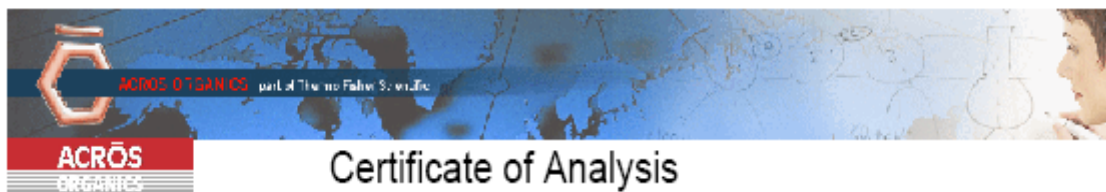
TEST	SPECIFICATION	LOT BCBD9440V RESULTS
APPEARANCE (COLOR)	WHITE TO FAINTLY BEIGE	WHITE
APPEARANCE (FORM)	POWDER TO FINE CRYSTALS	FINE CRYSTALS
PURITY (HPLC AREA %)	≥ 90.0 %	98.7 %
PROTON NMR SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

QC RELEASE DATE 21/JAN/11



Edeltraud Schwärzler, Manager
Quality Control
Buchs, Switzerland

Sigma-Aldrich warrants, that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product for its particular use. See reverse side of Invoice for additional terms and conditions of sale. The values given on the 'Certificate of Analysis' are the results determined at the time of analysis.



Certificate of Analysis

Product: 29332
Diallyldisulfide, 70%, remainder mainly diallyl sulfide

General Product Data
Version: 0
CAS No: 2179-57-9
Country of origin: UNITED KINGDOM
Molecular weight: 146.28
Molecular formula: C₆H₁₀S₂
Linear formula: (CH₂=CHCH₂S)₂
Flash point (°C): 51

Product Specifications

Appearance: CLEAR YELLOW LIQUID
Infrared spectrometry: AUTHENTIC
Separat. techn. GC: >=65 %
Refractive Index: 1.5350 to 1.5450 (20°C, 589 nm)

Lot Specific Data : A0232495

Appearance: CLEAR YELLOW LIQUID
Infrared spectrometry: AUTHENTIC
Separat. techn. GC: 85.7 %
Refractive Index: 1.5391 (20°C, 589 nm)

Issued: 01-10-12

L. Van den Broek, QA Manager

Acros Organics

ENA23, zone 1, nr 1350, Janssen Pharmaceuticaalaa 5a, B-2440 Geel, Belgium
Tel +32 14/57 52.11 - Fax +32 14/59 34.34 Internet: <http://www.acros.com>
1 Reagent Lane, Fair Lawn, NJ 07410, USA
Fax 201-796-1329

Certificate of Analysis

Product Name:

Resveratrol - $\geq 90\%$

Product Number:

H5254

Lot Number:

090M1879V

Brand:

SIGMA

CAS Number:

520-26-3

MDL Number:

MFCD00075663

Formula:

C₂₈H₃₄O₁₅

Formula Weight:

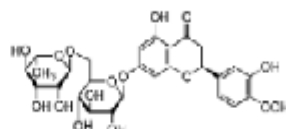
610.56 g/mol

Storage Temperature:

Store at 2 - 8 °C

Quality Release Date:

12 OCT 2010



Test	Specification	Result
Appearance (Color)	Yellow to Brown	Light Beige
Appearance (Form)	Powder	Powder
Solubility (Color)	Yellow to Yellow-Brown	Yellow-Brown
Solubility (Turbidity)	Clear to Hazy	Hazy
50 mg/mL, Pyridine		
Infrared spectrum	Conforms to Structure	Conforms
% Purity (HPLC)	≥ 80	92

Rodney Burbach, Manager
Analytical Services
St. Louis, Missouri US

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Certificate of Analysis

Product Name: ERIOCITRIN
>= 98.0 % HPLC
Product Number: 45714
Product Brand: Sigma
Molecular Formula: $C_{27}H_{32}O_{16}$
Molecular Mass: 596.53
CAS Number: 13463-28-0

TEST	SPECIFICATION	LOT BCBD9458V RESULTS
APPEARANCE (COLOR)	WHITE TO FAINTLY BEIGE	ALMOST WHITE
APPEARANCE (FORM)	POWDER	POWDER
PURITY (HPLC AREA %)	≥ 98.0 %	98.6 %
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

QC RELEASE DATE 21/JAN/11



Edeltraud Schwärzler, Manager
Quality Control
Buchs, Switzerland

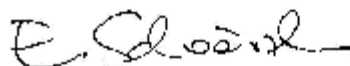
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Certificate of Analysis

Product Name: BETAINE MONOHYDRATE
>= 99.0 % NT
Product Number: 14300
Product Brand: Sigma
Molecular Formula: $C_5H_{11}NO_2 \cdot H_2O$
Molecular Mass: 135.16
CAS Number: 590-47-6

TEST	SPECIFICATION	LOT BCBD0080V RESULTS
APPEARANCE (COLOR)	WHITE/COLORLESS	WHITE
APPEARANCE (FORM)	POWDER TO FINE CRYSTALS WITH LUMPS	FINE CRYSTALS
TITRATION (NT) HClO4 0.1M	99.0 - 101.0 %	99.6 %
PURITY (TLC AREA %)	≥ 99.0 %	100.0 %
SOLUBILITY (COLOR)	COLORLESS	COLORLESS
SOLUBILITY (TURBIDITY)	CLEAR	CLEAR (<3.5 NTU)
SOLUBILITY (METHOD)	—	1G IN 10 ML H2O
WATER	10 - 16 %	13.5 %
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

QC RELEASE DATE 20/AUG/10
RECOMMENDED RETEST DATE AUG/12



Edeltraud Schwärzler, Manager
Quality Control
Buchs, Switzerland

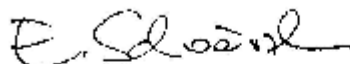
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Certificate of Analysis

Product Name: β -CAROTENE
BioChemika, purum
Product Number: 22040
Product Brand: Fluka
Molecular Formula: C₄₀H₅₆
Molecular Mass: 536.87
CAS Number: 7235-40-7

TEST	SPECIFICATION	LOT BCB08870V RESULTS
APPEARANCE (COLOR)	RED TO DEEP RED	DEEP RED
APPEARANCE (FORM)	POWDER	POWDER
MELTING POINT	176 - 184 °C	178 °C
SOLUBILITY (COLOR)	ORANGE TO RED TO DEEP RED	DEEP RED
SOLUBILITY (TURBIDITY)	CLEAR (< 3.5 NTU)	CLEAR
SOLUBILITY (METHOD)	—	0.01G IN 10 ML CHCL ₃
LOSS ON DRYING	≤ 0.2 %	0.07 %
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS
ASSAY (UV)	≥ 97.0 %	98.2 %
REM. ON UV SPECTROSCOPY	A 455/340 > 1.50	A455/340-1.72 (PH. EUR.4)
PROOF OF IDENTITY	A455/483 = 1.14 - 1.18	A 455/483-1.15 (PH. EUR. 4)
METAL TRACE ANALYSIS (ICP)	≤ 10 PPM	< 2 PPM

QC RELEASE DATE 22/DEC/10
RECOMMENDED RETEST DATE OCT/12



Edeltraud Schwärzler, Manager
Quality Control
Buchs, Switzerland

Sigma-Aldrich guarantees the 'Sales-Specification' values only, additional lot specific tests may be included for further information. The current 'Sales-Specifications' sheet is available on request. For further inquiries, please contact our Technical Service. Sigma-Aldrich warrants, that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice for additional terms and conditions of sale. The values given on the 'Certificate of Analysis' are the results determined at the time of analysis.



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NOORDWES-UNIVERSITEIT

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Web: <http://www.nwu.ac.za>

Ethics Committee

Tel +27 18 299 4850
Fax +27 18 293 5329
Email Ethics@nwu.ac.za

ETHICS APPROVAL OF PROJECT

2010/05/30

The North-West University Ethics Committee (NWU-EC) hereby approves your project as indicated below. This implies that the NWU-EC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

PROJECT TITLE: COMPARISON OF THE SWEETANA-GRASS AND CACO-2 CELL MODELS AS METHODS TO STUDY THE IN VITRO EFFLUX OF RHODAMINE 123

Ethics number:

N	W	U	-	0	0	1	8	-	0	9	-	A	5
Institution				Project Number				Year		Status			

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Approval date: 13 May 2008

Expiry date: 12 May 2014

Special conditions of the approval (if any): None

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- The project leader (principle investigator) must report in the prescribed format to the NWU-EC:
 - annually (or as otherwise requested) on the progress of the project,
 - without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.
- The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-EC. Would there be deviation from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-EC and new approval received before or on the expiry date.
- In the interest of ethical responsibility the NWU-EC retains the right to:
 - request access to any information or data at any time during the course or after completion of the project;
 - withdraw or postpone approval if:
 - any unethical principles or practices of the project are revealed or suspected,
 - it becomes apparent that any relevant information was withheld from the NWU-EC or that information has been false or misrepresented,
 - the required annual report and reporting of adverse events was not done timely and accurately,
 - new institutional rules, national legislation or international conventions deem it necessary.

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Prof MMJ Lowes
(chair NWU Ethics Committee)