In vitro drug-herb interaction potential of African medicinal plant products used by Type II diabetics

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By

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**ABSTRACT**

In Africa, use of medicinal plants for the treatment of diabetes is very common. However, efficacy on co-administering of medicinal plants with therapeutic drugs hasn’t been fully determined, especially for African medicinal plants. The current study focused on assessing the *in vitro* modulation effects of three popular African medicinal plants, namely: *Aloe ferox*, *Sutherlandia frutescens* and *Prunus africana* (including five commercial preparations containing these medicinal plants) on two of the most important anti-diabetic drug metabolising enzymes, Cytochrome P450 (CYP450) 2C9 and CYP3A4 and a key drug efflux transporter, P-glycoprotein (P-gp).

Vivid® microsome-based screening kits were used to assess inhibitory potency of plants preparations on CYP2C9 and CYP3A4 enzymes activities. The study showed that *P. africana* was a more potent inhibitor of CYP2C9 and CYP3A4 activity than the corresponding positive controls *Ginkgo biloba* and St. John’s wort, which are known to cause clinically significant drug-herb interactions. *S. frutescens* leaf extract demonstrated potent to moderate inhibition on both the tested CYP activities, while its commercial products (Promune® and Probetix®) possessed moderate to mild inhibitory effects on the activities of both CYPs. Potent inhibitory effect on CYP2C9 and CYP3A4 was seen with *Aloe Ferox®*. Prosit® and Aloes powder® showed potent to moderate inhibition on CYP2C9 activity and moderate to mild inhibition on CYP3A4 activity.

In addition to CYP450 activity, the present study also investigated the effects of the selected medicinal plant products on the activity of the main drug efflux protein, P-gp. A screening assay was specifically developed to assess the potential for herbal remedies to interact with P-gp mediated drug absorption. The assay is based on the principle of the reversal of drug resistance in modified Caco-2 cells specifically altered to express high
efflux protein activity. These cells display a multidrug resistance phenotype and the addition of a plant extract containing a P-gp inhibitor or substrate will inhibit or compete with any cytotoxic drug and consequently reverse the drug resistance. The suitability of the assay was confirmed using a known P-gp inhibitor.

The study observed that the anti-proliferation effect of vinblastine was significantly enhanced in vinblastine-resistant Caco-2 cells, which have high P-gp expression, when they were exposed to the selected African herbal preparations. This observation indicates that the studied plant preparations may alter P-gp functionality and therefore lead to interference with the absorption of co-administered drugs.

The outcomes of this study provide useful information on whether there are any potential drug-herb interactions between the commonly used African medicinal plants and oral anti-diabetic drugs, at the level of CYP and P-gp drug metabolism and could contribute to better therapeutic management of Type II diabetics. However these predicted interactions will need to be verified in a clinical setting.

**Key words:** Drug-herb interaction, African medicinal plants, type II diabetic care, *A. ferox*, *S. frutescens, P. africana*
# TABLE OF CONTENTS

DECLARATION .............................................................................................................................. vii

ACKNOWLEDGMENT ................................................................................................................... viii

LIST OF FIGURES ........................................................................................................................... ix

LIST OF TABLES ............................................................................................................................ x

ABBREVIATIONS .......................................................................................................................... xi

CHAPTER 1: INTRODUCTION TO PRESENT STUDY .............................................................. 1
  1.1: Type II diabetes mellitus ................................................................................................. 3
  1.2: Use of medicinal plants in the treatment of diabetes ...................................................... 5
  1.3: Role of African medicinal plants in Type II diabetic care ............................................. 6
    1.3.1: Aloe ferox .................................................................................................................... 7
    1.3.2: Prunus africana .......................................................................................................... 8
    1.3.3: Sutherlandia frutescens ............................................................................................ 8
  1.4: Interaction between herbal remedies and clinical drugs .............................................. 9
  1.5: The study aims and objectives ....................................................................................... 11
    1.5.1: Aims .......................................................................................................................... 11
    1.5.2: Objectives ............................................................................................................... 11
  1.6: The structure of the dissertation .................................................................................... 12

CHAPTER 2: DRUG-HERB INTERACTIONS: LITERATURE REVIEW ..................... 13
  2.1: Drug-herb interaction: Cytochrome P450s ................................................................. 14
    2.1.1: Overview of drug biotransformation ....................................................................... 14
    2.1.2: Human Cytochrome 450s ....................................................................................... 15
    2.1.3: Therapeutic drugs and the modulation of CYP450s activities ............................. 17
  2.2: Drug-herb interaction: P-glycoprotein ......................................................................... 22
    2.2.1: Overview drug efflux transporters ......................................................................... 22
    2.2.2: P-glycoprotein ........................................................................................................ 24
  2.3: Modulation of CYP450s and P-gp by medicinal herbs .................................................. 26
CHAPTER 3: MATERIALS, METHODS & METHOD DEVELOPMENT.................. 28

3.1: Materials ................................................................................................................. 28

3.2: Methods .................................................................................................................... 30

3.2.1: Stock solutions preparation .................................................................................. 30

3.2.1.1: Medicinal plant stock solutions preparation .................................................. 30

3.2.1.2: Conventional compounds stock solutions preparation .................................. 31

3.2.2: Vivid® CYP2C9 and CYP3A4 inhibitory assay .................................................... 31

3.2.3: P-gp assay using Caco-2 cell model ..................................................................... 34

3.2.3.1: Cell culture ......................................................................................................... 34

3.2.3.2: Development of a vinblastine resistant Caco-2 cell line .............................. 35

3.2.3.3: Toxicity assessment using MTT assay ............................................................... 38

3.2.3.4: Evaluation P-glycoprotein assay using vinblastine sensitivity .................... 40

CHAPTER 4: RESULTS & DISCUSSION........................................................................... 46

4.1: In vitro inhibition of CYP2C9 and CYP3A4 metabolism by African medicinal plants .................................................................................................................. 46

4.2: In vitro modulation of P-gp activity by African medicinal plants ....................... 51

4.2.1: Considerations for this P-gp assay ........................................................................ 55

4.2.2: The intrinsic cytotoxicity of medicinal herbs on cell lines ................................ 57

4.3: Clinical relevance of in vitro models ..................................................................... 58

4.3.1: Recombinant human hepatic supersome based CYPs assay model ............ 60

4.3.2: Cell line models .................................................................................................... 60

CHAPTER 5: CONCLUSION............................................................................................. 62

REFERENCES .................................................................................................................. 67

APPENDIX: A .................................................................................................................... 79
DECLARATION

I, Yuan Yuan Fang (Student number: 203004876), hereby declare that the work reported in this dissertation is my own, and that the dissertation has not been previously submitted in full or partial fulfilment of the requirements of another qualification.


Author’s signature: ______________________

Date: ______________________________
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LIST OF FIGURES

Figure 1.1: Metabolic complications associated with insulin resistance ....................... 4
Figure 1.2: African medicinal plants selected for the present study ............................ 7
Figure 2.1: Catalytic cycle of Cytochrome P450 ...................................................... 16
Figure 2.2: Distribution of CYPs in intestine and liver ............................................ 17
Figure 2.3: Cellular localization of ABC efflux transporters on intestinal epithelium...... 23
Figure 2.4: Schematic representation of P-gp efflux in absence and presence of P-gp inhibitor ............................................................ 24
Figure 3.1: The metabolism of Vivid® substrate into highly fluorescent products ....... 31
Figure 3.2: Growth rate of Caco-2WT cells in presence of VBS .............................. 36
Figure 3.3: The principle of P-gp assay using vinblastine sensitivity ......................... 41
Figure 3.4: Dose response curves of VBS in Caco-2WT and Caco-2VBS10 cells ....... 42
Figure 3.5: IC₅₀ values of VBS in Caco-2WT and Caco-2VBS10 cells at various passages ....................................................................................................................... 43
Figure 3.6: Dose response curve of VBS in presence of 20 µM verapamil in Caco-2VBS10 cells ...................................................................................................................... 44
Figure 3.7: IC₅₀ values of VBS in presence of 20 µM metformin in Caco-2VBS10 cells ...................................................................................................................... 44
Figure 4.1: IC₅₀ values of VBS in the presence of Aloe Ferox®, Prosit® and Aloes powder® in Caco-2VBS10 cells ...................................................................................................................... 52
Figure 4.2: IC₅₀ values of VBS in the presence of Promune®, Probetix® and S. frutescens leaf extracts in Caco-2VBS10 cells ...................................................................................................................... 53
Figure 4.3: IC₅₀ values of VBS in the presence of P. africana in Caco-2VBS10 cells... 55
Figure 4.4: IC₅₀ values of VBS in presence of investigated herbal extracts in Caco-2WT cells ...................................................................................................................... 57
Figure 4.5: Models have been used in the development of drug discovery ............... 59
Figure A.1: The Cytotoxicity of tested samples in Caco-2WT and Caco-2VBS cell lines ...................................................................................................................... 83
LIST OF TABLES

Table 2.1: Modulation effect of therapeutic drugs used in the management of type II diabetes on CYPs activity .............................................................................................................. 19
Table 2.2: Known P-gp substrates, inducers and inhibitors (drugs used in the management of type II diabetes)........................................................................................................ 25
Table 3.1: Descriptions of commercial medicinal herbal products ........................................ 29
Table 3.2: Concentrations of test samples used in CYPs assay ............................................. 32
Table 3.3: Assay parameters used in the experiment ............................................................ 33
Table 3.4: Concentrations of test samples used to determine cytotoxicity .......................... 40
Table 4.1: Inhibitory potency (IC\textsubscript{50}) of test samples on CYP2C9 and 3A4 activity .... 48
Table 4.2: % Inhibition of test samples on CYP2C9 and 3A4 activity at estimated gut concentrations .......................................................................................................................... 51
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td><em>A. ferox</em></td>
<td><em>Aloe ferox</em></td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme Inhibitors</td>
</tr>
<tr>
<td>ARBs</td>
<td>Angiotensin II Receptors Blockers</td>
</tr>
<tr>
<td>ARV</td>
<td>Antiretroviral</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the plasma concentration-time Curve</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast Cancer Resistance Protein</td>
</tr>
<tr>
<td>CAM</td>
<td>Complementary and Alternative Medicine</td>
</tr>
<tr>
<td>CCB</td>
<td>Calcium Channel Blocker</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra-acetic Acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HDL</td>
<td>High-Density Lipoprotein</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>Hydroxymethylglutaryl - Coenzyme A Reductase</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration inhibiting 50% of the effect</td>
</tr>
<tr>
<td>INR</td>
<td>International Normalised Ratio</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin Darby Canine Kidney</td>
</tr>
<tr>
<td>MDR</td>
<td>Multiple Drug Resistance</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MRP</td>
<td>Multiple Drug Resistance associated Proteins</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>NMMU</td>
<td>Nelson Mandela Metropolitan University</td>
</tr>
<tr>
<td>P. africana</td>
<td>Prunus africana</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate - Buffered Saline</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X Receptor</td>
</tr>
<tr>
<td>S. frutescens</td>
<td>Sutherlandia frutescens</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute Carrier</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine diphosphate Glucuronosyl Transferease</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine Diphosphate</td>
</tr>
<tr>
<td>VBS</td>
<td>Vinblastine Sulphate</td>
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CHAPTER 1:
INTRODUCTION TO PRESENT STUDY

Africa has a rich variety of indigenous medicinal plants. Twenty five percent of the total number of higher plants in the world is found in Africa, south of the Sahara (van Wyk, 2008). Furthermore, due to cultural diversity and historical reasons, it is not surprising that medicinal herbs still play a significant role in Africa in treating or assisting the treatment of various illnesses including fatigue, malaria, diabetes and human immunodeficiency virus (HIV) infections. It is estimated that more than 80% of the southern African population make use of traditional medicines, often in combination with prescription drugs (Maduna, 2006).

When medicinal herbal therapies are co-administered with prescription drugs, there is an increasing risk of clinical treatment failures and adverse toxicity due to drug-herb interactions. Plant extracts contain multiple components, many of which have unknown biological activity. These constituents can potentially mimic, increase or decrease the effects of co-administered prescription drugs resulting in synergistic or antagonistic effects through simultaneous interaction on the same therapeutic target or by affecting the metabolic stability of these drugs (Sørensen, 2002). Although synergistic or additive effects may provide complementary therapeutic benefits, this could also lead to unfavourable toxicities and require an alternative dosing regimen during long term treatment especially for a chronic disease such as diabetes (Chavez et al., 2006). On the other hand, competitive or antagonistic interactions can decrease the therapeutic efficacy of prescription drugs ultimately leading to treatment failure. Constituents from herbal extracts may also alter the pharmacokinetics of therapeutic drugs by interfering with their absorption, metabolism and clearance (Chavez et al., 2006). Numerous studies have reported the involvement of herbal products in the inhibition and/or induction of liver and intestinal drug metabolizing enzymes such as the Cytochrome P450s and efflux pump
proteins such P-glycoprotein, leading to adverse clinical effects (Pal & Mitra, 2006; Zhou et al., 2004).

Currently there exists a serious lack of information about the efficacy and safety of traditional medicine in South Africa. Most of the information that has been published is anthropological research into the rituals that accompany African traditional healing. Despite this, the South African government has approved a bill (Traditional Health Practitioners Act [No. 35 of 2004]) to formalize the African traditional healing system. In all other countries, such as India and China where traditional medicine is recognized by their governments, much more scientific research had been done to validate their mechanism of action. Given the enormous economic burden of human diseases, South Africa can ill afford therapeutic failure due to the unwanted side effects of complementary medicines. Consequently, the investigation into potential drug interactions of popular traditional remedies is urgently warranted. *In vitro* screening of the most common traditional remedies used in South Africa will aid in identifying any potential drug-herb interactions and create a much needed platform for further clinical studies. In the present study three African medicinal plants (*Aloe ferox*, *Sutherlandia frutescens* and *Prunus africana*), traditionally used for their anti-diabetic properties, were selected to investigate potential drug-herb interaction adversities when co-administered with anti-diabetic prescription medications.

Since the present study has focused on the potential interaction between medicinal plant products and prescription drugs used in the treatment of diabetes, a brief description on the clinical management of diabetes, and its associated complications as well as the use of the selected traditional remedies in the treatment of this disease have been included as these aspects are also relevant to this study. A comprehensive literature survey specific to drug-herb interaction, the major focus of the present study, is detailed in the chapter 2.
1.1: Type II diabetes mellitus

Diabetes is a multi-factorial disease characterised by chronic hyperglycaemia due to defects in insulin secretion and insulin sensitivity. In type II diabetic patients, fasting hyperglycaemia is associated with increased hepatic gluconeogenesis, while in the insulin-stimulated state (fed state); blood glucose levels correlate with decreased utilisation in skeletal muscle and adipose tissue (Virally et al., 2007). During the initial developmental stages of the disease, defective insulin sensitivity is overcome and normal glucose tolerance maintained, by increased insulin secretion. However, as the disease progresses, a further increase in peripheral tissue insulin resistance and/or a decrease in insulin secretion occurs resulting in the development of overt diabetes (Buchanan, 2003).

Beside hyperglycemia, type II diabetes often associates with a cluster of cardiovascular risk factors, including dyslipidemia, hypertension, hypercoagulability, obesity, and other features characteristic in metabolic abnormalities (Scheen, 2005) (Figure 1.1). These abnormalities dramatically increase the risk for cardiovascular complications. Thus, in addition to therapeutic strategies aimed at maintaining glycemic control, the treatment of associated metabolic complications become equally important in the management of type II diabetes (Nathan et al., 2009).
Metabolic abnormalities associated with insulin resistance include defects such as dyslipidemia (low HDL, high LDL and high triglycerides levels), obesity, hypercoagulation, and atherosclerosis. HDL = High-Density Lipoprotein; LDL = Low-Density Lipoprotein. The figure was taken from Kudolo (2001).

In combination with lifestyle interventions such as regular exercise, weight loss and diet therapy, type II diabetic care often requires the use of multiple pharmacological therapies to attain adequate glucose control and to treat concurrent metabolic syndromes (Caprio et al., 1997; Nathan et al., 2009). The wide variety of pathogenic abnormalities associated with diabetes and its related complications, implies that numerous therapeutic targets can be exploited to treat this chronic disease. Currently there are a number of different mechanistic classes of oral anti-diabetic drugs used in treatment of diabetes, these include: (i) inhibitors of carbohydrate digestion which delay postprandial absorption of monosaccharides (α-glucosidase and α-amylase inhibitors), (ii) inhibition of hepatic glucose production via the inhibition of gluconeogenesis (biguanide), (iii) insulin secretagogues which increase the release of insulin from pancreatic β-cells (sulfonylureas and potassium-ATP channel stimulators), (iv) insulin sensitizing agents which increase the insulin responsiveness in peripheral tissues (Thiazolidinediones) and, (v) incretin
potentiators such as inhibitors of incretin degradation (Dipeptidyl Peptidase 4 inhibitors) and incretin mimetics which enhance β-cell function (Nathan et al., 2009; Virally et al., 2007). To reduce cardiovascular complications, diabetic patients are frequently co-prescribed with one or more cardiovascular treatments including (i) anti-hypertensive agents (angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs), thiazide diuretics and calcium channel blockers (CCBs)), (ii) lipid lowering agents (statins and fibrates) and (iii) anti-platelet agents (Triplitt, 2006).

1.2: Use of medicinal plants in the treatment of diabetes

In order to improve their quality of life, diabetic patients often combine conventional prescription drugs with complementary and alternative medicines (CAMs). Frequently used are nutritional supplements, herbal medicines, nutritional advices, spiritual healing and relaxation techniques (Ryan et al., 2001; Chang et al., 2007). A particular CAM selected by the patient largely depends on the individuals’ concept regarding the illness and its treatment, values, cultural influence and world view (Mahabir & Gulliford, 1997; Chang et al., 2007). A survey conducted by Chang et al. (2007) investigated the prevalence of using CAMs amongst people living with diabetes in nine countries from the period of 1990 to 2006. They found that in Australia, Canada and Arabia, the prevalence of using dietary supplements, herbal medicines and botanical products was between 23.6 to 31%. In Thailand, the United States, Mexico, Korea and India, a higher prevalence of CAMs use amongst diabetic patients was found, with a range between 47.8 and 72.8 %, and herbal medicines was recognized as one of the most frequently used CAMs (Chang et al., 2007). In Africa, more than 80% of the population use medicinal herbs as primary health care and thus a higher prevalence of medicinal herbs use amongst diabetic patients would be expected (Maduna, 2006).
1.3: Role of African medicinal plants in Type II diabetic care

The usage of medicinal plants in treating diabetes symptoms have been reported in several clinical studies in African regions (Eddouks et al., 2002; Diève et al., 2008; Erasto et al., 2005). Eddouks et al. (2002) reported 80% usage in the south-east region of Morocco among the type II diabetic patients - 25% used only medicinal plants, while 75% used in conjunction with therapeutic drugs. Diève et al. (2008) interviewed 220 Senegalese diabetics and found: (i) 65% of patients believed medicinal plants to be effective for the treatment of diabetes and (ii) 74% of patients used medicinal herbs combined with conventional treatments. In South Africa, the use of medicinal plants for the treatment of diabetes is very common; several ethnobotanical surveys have identified various indigenous medicinal plants used for the management and treatment of diabetes (Erasto et al., 2005; Thring & Weitz, 2006).

Knowledge of the widespread use of traditional herbal remedies has resulted in research into their role in disease management. Numerous in vitro and animal studies provide support for the anti-diabetic properties of several African medicinal plants (Chadwick et al., 2007; van de Venter et al., 2008; Karachi, 2009).

For the purposes of this study, the role of the anti-diabetic medicinal plants, Aloe ferox, Prunus africana and Sutherlandia frutescens (Figure 1.2) are briefly discussed in the following paragraphs.
1.3.1: Aloe ferox

*A. ferox* also known as “Cape Aloe”, is indigenous to South Africa. It is widely distributed from the Western to Eastern Cape provinces as well as in Southern KwaZulu-Natal and the extreme southeastern part of the Free State (Deutschländer et al., 2009). *A. ferox* has a long history of use in both African and Europe as laxative medicines (Van Wyk, 2008). Extracts from various parts of *A. ferox* have been used for arthritis, eczema, conjunctivitis, inflammation, and in the treatment of hypertension (Van Wyk, 2008; Deutschländer *et al.*, 2009), however the anti-diabetic properties are reported to be the most common application (Deutschländer *et al.*, 2009; Botes, 2009). A recent study by Botes, (2009) investigated the anti-diabetic properties of *A. ferox* and found that its leaf gel has anti-oxidant properties which may explain some of the health benefits in reducing symptoms associated with/or prevention of cardiovascular diseases, cancers, neurodegeneration, and diabetes. Furthermore, using streptozotocin induced diabetic
rats, treatment with A. ferox at 300 mg/kg/day resulted in a modest increase in the serum insulin levels and a slight improvement of the lipid profile (Botes, 2009).

1.3.2: Prunus africana

*P. africana* known as “African cherry”, is a medium to large canopy tree reaching 30-40 m in height. It is widely distributed throughout Africa and is most abundant in open areas along forest margins and in disturbed areas. The well known medicinal value of *P. africana* is its ability to reduce benign prostatic hyperplasia symptoms however it is also reported for the treatment of fever, gonorrhea, stomach pain, chest pain, intercostals pain, inflammatory disorders, kidney disease, urinary tract complications, wound healing, malaria and as an appetite stimulant or purgative (Stewart, 2003).

The use of *P. africana* in diabetic care has been documented in an ethnobotanical study and has been recommended by traditional medicinal healers and diabetic patients who have it used during their therapy (unpublished study by Mbaabu). Furthermore Karachi (2009) investigated its anti-diabetic properties using *in vitro* and animal models. The study reported that *P. africana* bark extract has strong anti-oxidant properties, and in the cell cultures it improved glucose utilization. Moreover, using pre-diabetic rats, a dramatic increase in the serum insulin levels was observed after treatment with *P. africana* extract for four weeks, suggesting the extract may function as an insulin secretagogue (Karachi, 2009).

1.3.3: Sutherlandia frutescens

*S. frutescens* also known as “Cancer Bush” is one of the most valuable South African indigenous medicinal plants (Figure 1.2); it has a long medicinal use in South Africa in different cultural groups (Van Wyk, 2008). In recent years, *S. frutescens* has been commercialized by several local pharmaceutical manufacturers and has drawn much scientific attention. The medicinal uses include fever reduction, cancer tonic, stress,
tuberculosis, colds, influenza, chicken pox, diabetes, chronic fatigue syndromes, inflammation, kidney and liver conditions, back ache, rheumatism, peptic ulcers, anxiety, osteoarthritis, menopausal symptoms, gastritis, poor appetite and HIV infection (Van Wyk, 2008).

*S. frutescens* has been used widely by diabetic patients, especially along the western coastal region of South Africa and it was marketed as potential hypoglycemic agent for the treatment of the type II diabetes (Van Wyk & Albrecht, 2008; Chadwick et al., 2007). Its hypoglycemic properties have been identified in several animal studies (Van Wyk & Albrecht, 2008). Chadwick et al. (2007) observed statistically significant increase in glucose uptake in both muscle and adipose tissues, a reduction in intestinal glucose uptake and no weight gain in pre-diabetic rats treated with *S. frutescens* extracts via their drinking water for eight weeks. These authors concluded that *S. frutescens* showed promise as a therapy for type II diabetics. More recently, MacKenzie et al. (2009) showed that a commercial *S. frutescens* extract prevented the onset of high fat diet-induced insulin resistance by attenuating plasma free fatty acid levels.

### 1.4: Interaction between herbal remedies and clinical drugs

Many naturally occurring plant components have been shown to interfere at different levels with the pharmacodynamic and pharmacokinetic properties of clinical drugs. Firstly, a plant component can be a substrate for one of the several proteins involved in the metabolism and/or efflux of therapeutic drugs. Such competitive inhibition could result in higher plasma concentrations of these drugs, thus exposing the patient to greater risk of serious side effects. Secondly, a plant constituent could enhance the expression of one or several drug metabolising enzymes and/or transport proteins. The increased metabolism and/or efflux of the drug would result in decreased plasma concentrations and may produce sub-therapeutic plasma drug concentrations leading to treatment failure.
and the development of drug resistance. Thirdly, the plant derived compound may act as an inhibitor of one of the drug metabolising enzymes and/or efflux proteins resulting in elevated plasma levels of the therapeutic drug (Delgoda & Westlake, 2004; Pal & Mitra 2006; Triplitt, 2006).

Traditional remedies are usually complex mixtures of different molecular entities that have the potential to interact with various drug metabolising pathways. Such interactions include the inhibition or transcriptional activation (induction) of drug metabolising enzymes and efflux proteins, the majority of which reside in enterocytes and hepatocytes (Sørensen 2002; Zhou et al., 2004). A typical drug metabolic pathway involves the initial oxidation of the parent molecule (phase I metabolism), which is followed by conjugation of the oxidised moiety with polar molecules such glucose, sulphate or glutathione (phase II metabolism) (Wilkinson, 2001). The major enzymes involved in human phase I metabolism are the different iso-forms of Cytochrome P450 (CYP450), namely; CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Zhou et al., 2007). The latter iso-form is the most abundant CYP isozyme and is believed to metabolise between 50 and 70% of the currently marketed drugs including HIV protease inhibitors, non-nucleoside reverse transcriptase inhibitors, macrolide antibiotics and azole antifungals (Pal & Mitra, 2006). The key phase II enzymes include uridine diphosphate (UDP)-dependent glucuronosyl transferase, sulfotransferase and glutathione-S-transferase (Wilkinson, 2001). In addition to drug metabolising enzymes, cells possess numerous transport proteins able to export compounds out of the cell, reducing the therapeutic efficacy of these drugs. The major transporters involved in the bioavailability of therapeutic drugs include P-glycoprotein (P-gp), multidrug resistance associated proteins (MRPs) and breast cancer resistance proteins (BCRP). Substrate specificity and tissue distribution of these drug transporters vary widely. MRP1 is ubiquitously expressed, while the expression P-gp appears to be restricted to tissues involved in absorption and secretion (Pal & Mitra, 2006; Pal et al., 2010). In the intestine, P-gp is primarily found within the brush border membrane on the apical surface of mature enterocytes and may thus significantly restrict the oral delivery of therapeutic agents (Brand et al., 2006).
1.5: The study aims and objectives

1.5.1: Aims

The primary aim of the present study was to determine the in vitro potential for pharmacokinetic drug-herb interaction between three selected medicinal plants, namely: *Aloe ferox, Sutherlandia frutescens, Prunus africana* (including a few commercial preparations containing these medicinal plants) and therapeutic agents used in type II diabetic management. This study also served to initiate a new research direction in the Department of Biochemistry at NMMU aimed at evaluating the safety of African herbal remedies, a much neglected aspect of African traditional medicines and an important requisite for the establishment of proper safety regulations and manufacturing standards for herbal products which is considered mandatory if traditional medicine is to be integrated into public health programs.

1.5.2: Objectives

To accomplish the study aim, the following objectives were derived:

- The CYP450 inhibitory effects of the selected African medicinal plants were investigated using Vivid® CYP2C9 red and CYP3A4 red recombinant enzyme screening kits
- The potential for selected medicinal plant preparations to modulate the activity of the major drug efflux protein; P-gp. To achieve this goal, a novel cell based assay was devised and its suitability as an alternative P-gp assay confirmed. A stable cell line expressing high levels of P-gp was induced by vinblastine treatment and used to assess the potential of selected African medicinal plants to interact with this drug efflux protein.
1.6: The structure of the dissertation

The reminder of this dissertation is structured into four chapters. A review of the current literature regarding drug-herb interaction is presented in chapter 2. In this chapter, the role of CYPs and P-gp in drug metabolism is presented with emphasis on the metabolism of anti-diabetic drugs and the known effects of medicinal herbs. In addition to first line anti-diabetic drugs, the metabolism of therapeutic drugs used in the management of associated complications is also discussed. Finally, a review on the impact of the well known medicinal herbs (Ginkgo biloba and St. John’s wort) on CYP and P-gp related drug metabolism and absorption is presented, to motivate their use as positive controls in the present study.

Chapter 3 describes the methodology used, including sections detailing the development of a new P-gp assay method. Due to the nature of assay method development, some subsections containing “Question-Answering” formatted discussions have been included to explain the developmental process. Further, in order to confirm the suitability of the new assay method results from a series of experiments designed to characterize the new model is also included in this chapter together with detailed discussion on these results.

Chapter 4 focuses on the potential for drug-herb interaction of the selected medicinal plant products (A. ferox, S. frutescens and P. africana) in terms of CYPs and P-gp interaction. In addition, a comparison of various in vitro models and discussions of in vivo relevance of these models are presented in order to predict the in vivo situation.

Chapter 5 is an integrated discussion and conclusion of the results of chapter 4. A discussion on the clinical relevance of the current study is included, and possible refinements to be sought in future research are discussed. Appendix A contains the results of cytotoxicity of test samples on cell lines obtained from chapter 3.
CHAPTER 2:

DRUG-HERB INTERACTIONS: LITERATURE REVIEW

Patients often take herbal products in combination with therapeutic drugs and most of them do not inform physicians on the use of herbal supplements. The reasons for this are that people believe that, since these preparations come from natural sources, medicinal herbs are safe to use. Furthermore, patients also assume that since these herbal remedies have been used for decades they must be safe and without harmful effects. Contrary to this common belief, however, an increasing number of case reports, animal and human studies have demonstrated that interactions between medicinal herbs and conventional drugs can lead to serious and sometimes fatal side effects (Hu et al., 2005; Colalto, 2010). Several interactions between medicinal herbs and prescription drugs have been identified and reported, for example, *Allium sativum* (Garlic) decreased peak plasma concentration of the protease inhibitor squinnavir by 54% in health volunteers. Co-administrating garlic extracts with warfarin causes increased clotting time and international normalised ratio (INR) in patients. *Ginkgo biloba* causes bleeding when used in conjunction with warfarin and aspirin. It raises blood pressure when used together with thiazide diuretics and causes coma when combined with trazodone in patients. *Panax ginseng* (Ginseng) has been reported to interact with the monoamine oxidase inhibitor phenelzine and patients experienced headache, tremors and maniac episodes. *Piper methysticum* (Kava) has been reported to have dopamine antagonistic activity. In several case reports, patients developed severe parkinsonism after taking Kava, and more frequent and longer “off” periods when using Kava and levodopa together. St. John’s wort decreases serum area under the plasma concentration-time curve (AUC) of amitriptyline, nortriptyline, midazolam, oral contraceptives, cyclosporine, digoxin, indinavir, simvastatin, theophylline and warfarin. The use of St. John’s wort has resulted in organ rejection in patients undergoing organ transplantation, as a consequence of decreased serum levels of cyclosporine. Co-administrating St. John’s
wort decreases anticoagulant activity in patients stabilised on warfarin (Delgoda & Westlake, 2004; Hu et al., 2005; Colalto, 2010 and references within).

Both Cytochrome P450s and drug efflux transporters are important in explaining many reported drug-herbal interactions (Zhou et al., 2007; Schwab et al., 2003). In 2007, Zhou et al. reviewed previous literature on clinical drugs that interact with herbal medicines in humans. Of the 34 therapeutic drugs that they listed, 82.4% were substrates of various CYPs (especially CYP3A4 and CYP2C9); 29.4 % were reported as substrates for P-glycoprotein (P-gp) and 23.5% were identified as substrates for both CYP3A4 and P-gp (Zhou et al., 2007). Furthermore, Pelkonen et al., (2008) reported that CYPs are involved in about 96% of metabolism-based drug interaction. Therefore, it is important to assess the potential modulation on CYPs and P-gp from medicinal herbs using various in vitro and in vivo models.

2.1: Drug-herb interaction: Cytochrome P450s

2.1.1: Overview of drug biotransformation

Biotransformation is a key process to metabolize drugs into more polar and inactive metabolites that are readily eliminated from the body. It occurs between the absorption of drug into the systemic circulation and its renal elimination (Correia, 2009). Thus, it can affect overall therapeutic and toxic profile of a drug. Biotransformation is mainly localized in the liver; however drug metabolism may also occur in some extra-hepatic sites including gastrointestinal tract, kidney, lung and blood (SØrensen, 2002).

Biotransformation is divided into two major categories, namely phase I (oxidation, reduction and hydrolysis) and phase II (conjugation). Phase I metabolism primarily serves to increase hydrophilicity of a parent drug by introduction or exposure of a polar functional group (-OH, -NH₂ –SH or -CO₂H). Phase II metabolism conjugates the parent drug or phase I metabolite with endogenous substrates such as glucuronic acid, sulphuric
acid, acetic acid or an amino acid to further increase the polarity of the compound (Ansede & Thakker, 2004; Correia, 2009). Phase II metabolism often, but not always, occurs after metabolism by the CYP450 system. Glucuronidation, which is catalyzed by uridine diphosphate glucuronosyl transferase (UGTs), is the most important reaction in phase II metabolism (Wilkinson, 2001). It has been reported to be the underlying cause for one important drug-drug interaction between gemfibrozil (fibric acid derivatives) and several statins (Shitara et al., 2004).

CYP450 enzyme system is the main catalyser of oxidative metabolism, which is the most important biotransformation in drug metabolism (Ansede & Thakker, 2004). As a result, CYPs have been shown to be involved in numerous interactions between drugs, nutrients, medicinal herbs and other drugs. Thus, to assess the drug-herb interaction potential of medicinal herbs, assessing their ability of altering CYPs activity has become an important first step (Delgoda & Westlake, 2004).

2.1.2: Human Cytochrome 450s

Human CYP450s is a super family of haem-thiolate proteins and is expressed predominantly in the liver (Delgoda & Westlake, 2004). It metabolizes a variety of xenobiotics including therapeutic drugs, carcinogens, steroids and eicosanoids into more hydrophilic metabolites, using nicotinamide adenine dinucleotide phosphate (NADPH) as energy source (Zhou et al., 2005). The catalytic mechanism for drug metabolism involves the binding of the substrate (drug) to oxidized P450 [Fe\(^{3+}\)] to form the “P450 [Fe\(^{3+}\)]-drug” complex, followed by activating a molecular oxygen and subsequently transferring the activated molecular oxygen atom to the “P450 [Fe\(^{3+}\)]-drug” complex, resulting in the creation of a more polar product (Figure 2.1) (Delgoda & Westlake, 2004; Correia, 2009).
RH = Parent Drug; ROH = Oxidized metabolite; e^- = electrons. The figure was taken from Correia (2009)

Fifty seven CYP450 iso-forms have been identified in the human and 47 contributing to the metabolism of xenobiotics (Cavallari & Lam, 2005). Of these, CYP 1A2, 2A6, 2B6, 2C9, 2D6, 2E1 and 3A4 are considered to be the most important iso-forms involved in drug metabolism (Correia, 2009). In addition, individual CYP450 iso-enzymes metabolise specified substrates based on the chemical and structural features of these compounds. For example, CYP3A4 is responsible for metabolising the most lipophilic substrates and it is known to metabolise over 50% of prescription drugs (Elliott & Kenakin, 2001). Most positively charged substrates (usually associated with basic nitrogen) are metabolised by CYP2D6, while weakly anionic molecules are metabolised by CYP2C9 which is responsible for the metabolism of more than 15% of clinical drugs. Poly-aromatic hydrocarbons are metabolised by CYP1A2, and small relatively soluble organics by CYP2E1 (Delgoda & Westlake, 2004). Taken as a whole, human CYP450s can metabolize almost all organic xenobiotics and consequently the hepatic CYP450-mediated drug metabolism is considered the main pathway to eliminate xenobiotics.
In addition to the liver, CYPs have been found in small and large intestines, kidney, lung and nervous tissue. Of these extra hepatic tissues, intestinal CYP is reported to be the most important contributor in drug biotransformation (Lin & Lu, 2001). Paine et al. (2006) characterized the human CYP450 iso-enzymes profile in small intestine and found CYP3A4 is the most abundant CYPs in small intestine, accounting for 80% of total CYPs (Figure 2.2) (Paine et al., 2006). Since such a high expression level of CYP3A4 was found in the intestine, and given the fact that most therapeutic drugs, food substances and herbal remedies are taken through oral route, intestinal CYP3A4 may contribute significantly to drug biotransformation, even if the liver is unaffected (Wilkinson, 2001). CYP2C9 represents the second largest expressed CYPs in the small intestine and comprises 14% of total CYPs (Figure 2.2) (Paine et al., 2006).

![Figure 2.2: Distribution of CYPs in intestine and liver](image)

Schematic representation of the percentage distribution of individual CYP450 isoform expressed as a total of the immunoquantified CYP450 content in liver and intestine. The figure was taken from Paine et al. (2006).

### 2.1.3: Therapeutic drugs and the modulation of CYP450s activities

Several important therapeutic agents used in the management type II diabetes have been identified as CYPs substrates (Table 2.1). Some of these have potential to induce or
inhibit the activity of CYPs. The importance of CYPs in the metabolism of these drugs leads to increased pharmacokinetic interaction potential with other therapeutic substances. Numerous clinically significant drug interactions associated with these agents have been reported (Triplitt, 2006; Scheen, 2005).

Most sulfonylurea drugs are mainly metabolized by CYP2C9; therefore, co-administering them with other CYP2C9 inducers or inhibitors can affect their metabolism (Triplitt, 2006). For instance, a decrease in the anti-hyperglycemia effect of sulfonylureas was reported when they were combined with HIV protease inhibitor (ritonavir and nelfinavir) or rifampicin (CYP2C9 inducers). Lipid lowering agents have been prescribed extensively as a first-line therapy to reduce cardiovascular risk in diabetic patients. Most of them are metabolized by CYPs, therefore they have great potential to interact with co-administered agents via interference with drug metabolism. In addition, it has been recommended that caution be taken when combining statins (such as atorvastatin, lovastatin and simvastatin), which are mainly metabolized by CYP3A4 with drugs that inhibit CYP3A4. Furthermore, troglitazone (thiazolidinediones) was removed from the market due to severe hepatotoxicity. Troglitazone induces CYP3A4, and it has been associated with significant drug interactions, especially when used with CYP3A4 substrates (Scheen, 2005 and references within).

As mentioned previously, type II diabetic patients have often used the medicinal herbs together with their conventional therapeutic agents. Therefore it is important to recognize any modulation effects on CYPs from medicinal herbs. However, not all the CYPs are inducible, and for diabetes, the only clinically relevant CYPs appear to be CYP2C9 and CYP3A4 (Triplitt, 2006) (Table 2.1). For this reason, the current study focused only on assessing CYP2C9 and CYP3A4 activity.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug class</th>
<th>CYPs Involved</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>HMG-CoA reductase inhibitor (lipid-lowering agent)</td>
<td>CYP3A4 (S)</td>
<td>Extensively metabolized by CYP3A4; with 20-30% hepatic first-pass extraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4 (ID)</td>
<td>Effect of CYP3A4 inducers is unknown (Cautious)</td>
</tr>
<tr>
<td>Enalapril</td>
<td>ARBs (anti-hypertensive agent)</td>
<td>CYP3A4 (S)</td>
<td>Metabolized by CYP3A4</td>
</tr>
<tr>
<td>Captopril</td>
<td>ACEs (anti-hypertensive agent)</td>
<td>CYP2D6 (S)</td>
<td>Mainly metabolized by CYP2D6</td>
</tr>
<tr>
<td>Cervastatin*</td>
<td>HMG-CoA reductase inhibitor (lipid-lowering agent)</td>
<td>CYP2C8/3A4 (S)</td>
<td>A high affinity for CYP2C8; also metabolized by CYP3A4; with 50-60% hepatic first-pass extraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4 (ID)</td>
<td>Effect of CYP3A4 inducers is unknown</td>
</tr>
<tr>
<td>Chlorpropamide</td>
<td>Sulfonyleureas (anti-diabetic agent)</td>
<td>CYP3C9 (S)</td>
<td>Primarily metabolized (40-60%) by CYP2C9</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>CCBs (anti-hypertensive agent)</td>
<td>CYP3A4 (S)</td>
<td>Extensively metabolized (&gt;60%) by CYP3A4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4 (IH)</td>
<td>A weaker inhibitor of CYP3A4</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>HMG-CoA reductase inhibitor (lipid-lowering agent)</td>
<td>CYP2C9 (S)</td>
<td>Predominantly metabolized (50-80%) by CYP2C9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4/2C8 (S)</td>
<td>Metabolized by CYP3A4/2C8 (minor)</td>
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<tr>
<td></td>
<td></td>
<td>CYP2C9 (IH)</td>
<td>A potent inhibitor of CYP2C9</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>Fibrates (lip-lowering agent)</td>
<td>CYP2C8/9 (IH)</td>
<td>Potent inhibition of CYP2C8 activity, with minor inhibition of CYP2C9 activity</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>Sulfonyleureas (anti-diabetic agent)</td>
<td>CYP3C9 (S)</td>
<td>Mainly utilize CYP2C9 for metabolism</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4 (IH)</td>
<td>Inhibition of CYP3A4-mediated metabolism of cyclosporine</td>
</tr>
<tr>
<td>Gliclazide</td>
<td>Sulfonyleureas (anti-diabetic agent)</td>
<td>CYP2C9 (S)</td>
<td>Metabolized (30-40%) by CYP2C9</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>Sulfonyleureas (anti-diabetic agent)</td>
<td>CYP2C9 (S)</td>
<td>Metabolized (&gt;80%) by CYP2C9</td>
</tr>
<tr>
<td>Glipizide</td>
<td>Sulfonyleureas (anti-diabetic agent)</td>
<td>CYP2C9 (S)</td>
<td>Metabolized (30-40%) by CYP2C9</td>
</tr>
<tr>
<td>Irbesartan</td>
<td>ARBs (anti-hypertensive agent)</td>
<td>CYP2C9 (S)</td>
<td>Metabolized by CYP2C9</td>
</tr>
<tr>
<td>Drug</td>
<td>Drug class</td>
<td>CYPs Involved</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------</td>
<td>---------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Losartan</td>
<td>ARBs (anti-hypertensive agent)</td>
<td>CYP2C9/3A4 (S)</td>
<td>Extensively metabolized by CYP2C9/3A4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4</td>
<td>Significant interact with CYP3A4 inducers or inhibitors</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>HMG-CoA inhibitor (lipid-lowering</td>
<td>CYP3A4 (S)</td>
<td>Predominantly metabolized by CYP3A4 with 40-70% hepatic first-pass extraction</td>
</tr>
<tr>
<td>Nateglinide</td>
<td>Meglitinide derivatives (anti-diabetic agent)</td>
<td>CYP2C9 (S)</td>
<td>Extensively metabolized (50-70%) by CYP2C9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4 (S)</td>
<td>CYP3A4 contributes to 30% of its clearance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP2C9 (IH)</td>
<td>Effect of CYP2C9 inhibitor is unknown (<em>In vitro</em>, inhibit the metabolism of tolbutamide, a CYP2C9 substrate)</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>CCBs (anti-hypertensive agent)</td>
<td>CYP2C8/9/19;</td>
<td>Inhibits CYP2C8/9/19 and CYP3A4/D6 activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4/D6 (IH)</td>
<td></td>
</tr>
<tr>
<td>Phenprocoumon</td>
<td>Anti-coagulants</td>
<td>CYP2C9 (S)</td>
<td>Metabolized (50-70%) by CYP2C9</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>Thiazolidinediones (anti-diabetic</td>
<td>CYP2C8/3A4 (S)</td>
<td>Metabolized (39%) by CYP2C8; 17% by CYP3A4</td>
</tr>
<tr>
<td></td>
<td>agent)</td>
<td>CYP2C9 (S)</td>
<td>Metabolized (40-60%) by CYP2C9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4 (ID)</td>
<td>Effect of CYP3A4 inducers is unknown (Cautious)</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>Meglitinide derivatives (anti-diabetic agent)</td>
<td>CYP2C8/3A4 (S)</td>
<td>Dual CYP2C8/3A4 biotransformation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4</td>
<td>May competitively inhibit CYP3A4 conversion of other drugs which is a CYP3A4 substrate</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>Thiazolidinediones (anti-diabetic</td>
<td>CYP2C8/9 (S)</td>
<td>Primarily metabolized by CYP2C8; metabolized (5-10%) by CYP2C9</td>
</tr>
<tr>
<td></td>
<td>agent)</td>
<td>CYP3A4 (ID/ <em>in vitro</em>)</td>
<td><em>In vitro</em>, it is a CYP3A4 inducer ; however, it does not markedly alter CYP3A4 mediated drug metabolism <em>in vivo</em></td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>HMG-CoA inhibitor (lipid-lowering</td>
<td>CYP2C9 (S)</td>
<td>Metabolized by (60-80%) by CYP2C9</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>agent)</td>
<td>CYP3A4 (S)</td>
<td>Extensively metabolized by CYP3A4; with 50-80% hepatic first-pass extraction</td>
</tr>
<tr>
<td>Drug</td>
<td>Drug class</td>
<td>CYPS involved</td>
<td>Description</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------------</td>
<td>---------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>Sulfonylureas (anti-diabetic agent)</td>
<td>CYP2C9 (S)</td>
<td>Metabolized (78-93%) by CYP2C9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP2C9 (IH)</td>
<td>Inhibit CYP2C9 activity</td>
</tr>
<tr>
<td>Troglitazone*</td>
<td>Thiazolidinediones (anti-diabetic agent)</td>
<td>CYP2C9 (S)</td>
<td>Metabolized (5-15%) by CYP2C9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4 (ID)</td>
<td>Decrease serum concentration of drugs metabolized by CYP3A4</td>
</tr>
<tr>
<td>Valsartan</td>
<td>ACE inhibitor</td>
<td>CYP2C9 (S)</td>
<td>Metabolized (9%) by CYP2C9</td>
</tr>
<tr>
<td>Verapamil</td>
<td>CCBs (anti-hypertensive agent)</td>
<td>CYP3A4 (S)</td>
<td>Metabolized by CYP3A4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4 (IH)</td>
<td>Inhibits CYP3A4 activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP2C9 (S)</td>
<td>Metabolized (15-25%) by CYP2C9</td>
</tr>
<tr>
<td>Warfarin</td>
<td>Anti-coagulant agent</td>
<td>CYP2C9 (S)</td>
<td>Metabolized by (60-80%) by CYP2C9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A4 (ID)</td>
<td>Induce CYP3A4 activity</td>
</tr>
</tbody>
</table>

Note:
*: Have been withdrawn from the market due to serious hepatotoxicity

ACE = Angiotensin-Converting Enzyme Inhibitors; ARBs = Angiotensin II Receptors Blockers; CCBs = Calcium Channel Blockers; CYP = Cytochrome P450; Fibrates = Fibric acid derivatives; HMG-CoA = Hydroxymethylglutaryl-Co enzyme A Reductase; ID = Inducer; IH = Inhibitor; S = Substrate

(Compiled for data presented by Williams & Feely, 2002; Buse, 2003; Dorne et al., 2003; Niemi et al., 2005; Scheen, 2005; Triplitt, 2006; Scheen, 2007a; Scheen, 2007b; Zhou et al., 2009 and references within)
2.2: Drug-herb interaction: P-glycoprotein

2.2.1: Overview drug efflux transporters

Transporters are membrane-bounded proteins regulating the influx and efflux of drugs across the plasma membrane. They are classified into two families, namely solute carrier (SLC) and ATP-binding cassette (ABC) transporter. They are involved in drug absorption and disposition with broad substrate specificities and the ability to transport structurally unrelated compounds (Kusuhara & Yuichi, 2008). The remainder of this subsection focuses on the ABC transporters, which are involved in efflux of drugs from intestinal enterocytes either to basolateral surface (enhancing absorption) or back into intestinal luminal (reducing bioavailability).

The ABC transporters include multidrug resistance (MDR) proteins - P-glycoprotein (P-gp/MDR1), multidrug resistance associated proteins (MRPs), and breast cancer resistance protein (BCRP) (Kusuhara & Yuichi, 2008). These transporters are localized either in the apical surface or basolateral surface of intestinal enterocytes (Figure 2.3). P-gp is the most extensively studied transporter, and is localized in the apical membrane of enterocytes and plays a very important role in limiting the absorption of many orally administrated xenobiotics (Pal & Mitra, 2006). The substrate specificity of P-gp is very broad, and a large number of compounds have been identified as P-gp substrates. In general, these substrates are hydrophobic and either positively charged or neutral compounds (Kusuhara & Yuichi, 2008). Unlike P-gp, MRPs mainly transport hydrophobic anionic conjugates and neutral compounds (Pal et al., 2010). So far, eight different MRPs have been identified (Pal et al., 2010). Of these, MRP1, MRP2, and MRP3 have been reported to be involved in transporting numerous therapeutic agents such as antineoplastic drugs, fluoroquinolones and protease inhibitors (Pal & Mitra, 2006). For BCRP, the exact spectrum of transported substrates has not been established. They exhibit substrate specificity for various anticancer drugs such as mitoxantrone and topotecan, and drugs such as pitavastatin,
sulfasalazine and cimetidine, fluoroquinolones and glucuronide and sulfate conjugates, and dietary carcinogens (Kusuhara & Yuichi, 2008 and references within).

**Figure 2.3: Cellular localization of ABC efflux transporters on intestinal epithelium**

BCR, MDR1 (P-gp) and MRP2 are localized in the apical surface. MRP1, MRP3, MRP5 and MRP6 are present in the basolateral surface of intestinal enterocytes. Little known is about MRP4 which could be localized in both the apical and basolateral surface (Brand et al., 2006).

A = ATP binding sites; ABC = ATP-binding cassette; BCRP = breast cancer resistance protein; MDR = multidrug resistance proteins; MRP = multidrug resistance associated proteins; P-gp = P-glycoprotein. The figure was taken from Pal et al. (2010).

Among these efflux transporters, P-gp has been noted be especially important in modulating drug disposition. It has become well known that P-gp expression in healthy tissues plays a significant role in drug interactions (Eberl et al., 2007). Evidences from *in vitro*, *in vivo* and clinical studies have indicated that P-gp is involved in many reported drug-herbal interactions (Zhou *et al.*, 2004). Although the role of P-gp in drug-herbal interaction is increasingly recognized, there is very little information on whether P-gp activity can be altered by African medicinal herbs, which are routinely taken by a number of type II diabetic patients in combination with their prescription drugs.
2.2.2: P-glycoprotein

P-glycoprotein (P-gp), a 170 kDa transmembrane protein, is encoded by human MDR1 gene. It was first identified in certain tumour cells associated with the multidrug resistance phenomenon (Kim, 2002). Later in addition to tumour cells, P-gp was also found in a number of normal human tissues including adrenal gland, blood-brain barrier, small intestine, colon, pancreas, liver and kidney (Brand et al., 2006; Shirasaka et al., 2006; Eberl et al., 2007). In the intestine, P-gp is normally expressed in the apical membrane of enterocytes of the lower gastro-intestinal tract (jejunum, duodenum, ileum and colon) (Kim, 2002). It functions as a biochemical barrier for the entry of drug substances by actively pumping them out of the cell and back into the intestinal lumen (Figure 2.4); thus, the amount of orally administrated drugs available for systemic absorption is reduced (Varma et al., 2003).

Figure 2.4: Schematic representation of P-gp efflux in absence and presence of P-gp inhibitor.

P-gp works against the drug absorption process. It actively effluxes incoming drugs out of the enterocytes back into intestinal lumen in an ATP dependent manner. Thus, lower drug concentrations are available for systemic absorption. Changes in P-gp activity will alter systemic absorption of a drug. For instance, inhibited P-gp activity by any of its inhibitors leads to an increased concentration of a drug systemically (Hellum & Nilsen, 2008). **P-gp** = P-glycoprotein; **ATP** = Adenosine triphosphate

P-glycoprotein = P-gp
Because of the importance of P-gp in drug absorption and disposition, alterations of its functionality have been associated with a number of clinically significant drug interactions. Like CYPs, P-gp is susceptible to induction and inhibition by many drug substances. Many drug interactions mediated by P-gp inhibition have been reported. For instance, co-administering certain macrolides antibiotics (a P-gp inhibitor) with digoxin (a well-known P-gp substrate), resulted in increasing serum digoxin level in a clinical study (Eberl et al., 2007). In terms of P-gp induction, impaired digoxin absorption resulted from drug interaction with rifampicin (a P-gp inducer), associated with increased P-gp level (Kim, 2002).

A number of therapeutic agents used in Type II diabetic management are known P-gp substrates, inhibitors and/or inducers (Table: 2.2).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitors</th>
<th>Inducers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atenolol (Anti-hypertensives)</td>
<td>Amiodarone (Cardiac therapy)</td>
<td>Nifedipine (Anti-hypertensives)</td>
</tr>
<tr>
<td>Atorvastatin (Lipid-lowering agent)</td>
<td>Atorvastatin (Lipid-lowering agent)</td>
<td>Verapamil (Anti-hypertensives)</td>
</tr>
<tr>
<td>Carvedilol (Anti-hypertensives)</td>
<td>Carvedilol (Anti-hypertensives)</td>
<td></td>
</tr>
<tr>
<td>Cerivastatin (Lipid-lowering agent) *</td>
<td>Digoxin (Cardiac therapy)</td>
<td></td>
</tr>
<tr>
<td>Digitoxin (Cardiac therapy)</td>
<td>Lovastatin (Lipid-lowering agent)</td>
<td></td>
</tr>
<tr>
<td>Digoxin (Cardiac therapy)</td>
<td>Nifedipine (Anti-hypertensives)</td>
<td></td>
</tr>
<tr>
<td>Lovastatin (Lipid-lowering agent)</td>
<td>Quinidine (Cardiac therapy)</td>
<td></td>
</tr>
<tr>
<td>Losartan (Anti-hypertensives)</td>
<td>Reserpine (Anti-hypertensives)</td>
<td></td>
</tr>
<tr>
<td>Quinidine (Cardiac therapy)</td>
<td>Simvastatin (Lipid-lowering agent)</td>
<td></td>
</tr>
<tr>
<td>Quinine (Cardiac therapy)</td>
<td>Spironolactone (Anti-hypertensives)</td>
<td></td>
</tr>
<tr>
<td>Verapamil (Anti-hypertensives)</td>
<td>Verapamil (Anti-hypertensives)</td>
<td></td>
</tr>
</tbody>
</table>

Note:
*: have been removed from the market due to severe hepatotoxicity;
(Pal & Mitra, 2006; Takano et al., 2006; Brand et al., 2006 and references within)
2.3: Modulation of CYP450s and P-gp by medicinal herbs

Evidence from *in vitro*, *in vivo* and clinical studies have indicated that many medicinal herbal constituents can modulate activities of CYPs and P-gp, and consequently modify the absorption and metabolism many therapeutic agents used in the treatment of Type II diabetes (Chave *et al.*, 2006; Delgoda & Westlake, 2004; Zhou *et al.*, 2004) (Table 2.1 & 2.2). While the mechanisms of drug-herb interactions of many western medicinal plants are well studied, there is limited information regarding the interactions between African medicinal plants and therapeutic drugs.

For comparative purpose, this study investigated inhibitory potency of two well studied medicinal herbs (*G. biloba*, an inhibitor of CYP2C9 and St. John’s wort, an inhibitor of both CYP3A4 and P-gp) on CYPs and P-gp activities as a reference in current study. The following reviews present the modulation effect of *G. biloba* on CYP2C9 actives and St. John’s wort on both CYP3A4 and P-g activities from previous literatures and their potential role in drug-herb interactions.

Constituents of *in vitro* studies have indicated that *G. biloba* inhibited several CYPs including: CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Zhou *et al.*, 2002). Furthermore, a few publications have report that *G. biloba* extracts induce CYP2C9 activity, in both animal models and in clinical studies. Sugiyama *et al.* (2004) found that treatment of aged rats with 0.1% of *G. biloba* extract significantly attenuated the hypoglycemic effect of tolbutamide (Sugiyama *et al.*, 2004). Also, in healthy individuals, Uchida *et al.* (2006) reported oral administration of *G. biloba* extract at a dose of 360 mg/day for 28 days significantly lowered the hypoglycemic effect of tolbutamide. This study concluded that the significant decrease of the area under the concentration versus time curve (AUC$_{0\rightarrow\infty}$) of tolbutamide was possibly a result of induction of CYP2C9 in the healthy subjects (Uchida *et al.*, 2006). In 2007, Umegaki *et al.* identified bilobalide in *G. biloba* extract as a major substance that induces hepatic CYP2C9 expression or activity (Umegaki *et al.*, 2007).
Ironically, St. John’s wort is better known for its potential drug interaction than its medicinal properties. An earlier in vitro study using recombinant human CYP enzyme assays, reported that St. John’s wort (active components identified as hyperforin and quercetin) significantly inhibited CYP3A4 activity (Zou et al., 2002). Further, numerous case reports and clinical studies have reported its ability to interact with therapeutic drugs such as cyclosporine, indinavir, nevirapine, irinotecan, imatinib mesylate, alprazolam, midazolam, quazepam, digoxin, simvastatin, amitriptyline, methadone, omeprazole, oral contraceptives, theophylline, verapamil, fexofenadine and warfarin (Zhou et al., 2004; Chavez et al., 2006 and references within). The underlying bases for these reported drug-herb interactions are suggested to be due to increased CYP3A4 and intestinal P-gp activity, and consequently a decreased therapeutic level and increased clearance of these agents.

Further, previous study has found that compositions of St. John’s wort particularly hyperforin are potent ligands for the nuclear xenobiotic pregnane X receptor (PXR) which regulates CYP3A4 gene expression level (Watkins et al., 2003). In vivo and human studies reported that St. John’s wort induce CYP3A4, upon chronic exposure, but not in short term administering (Pal & Mitra, 2006). Studies have also demonstrated that St. John’s wort induces the orphan nuclear receptor P-gp which regulates MDR1 and many other gene drug transporters (Chavez et al., 2006 and references within).
CHAPTER 3: MATERIALS, METHODS & METHOD DEVELOPMENT

This chapter describes the research methodology used. The first two sections describe stock solutions preparation and the assay procedures for determining the inhibitory effect on the Cytochrome P450 2C9 and 3A4 activity by the selected medicinal plant extracts using Vivid® CYP450 screening kits. This is followed by a description of the development of an appropriate P-glycoprotein expressing cell model. The assay procedure for assessing cytotoxicity as well as modulation effect on P-gp activity by test medicinal plants is described. Finally, results from a series of experiments designed to verify the correlation between P-gp activity and vinblastine sensitivity are included together with detailed discussion on these results.

3.1: Materials

Caco-2 wild type (WT) cell line (at passage number 11) was obtained from Highveld Biological (Pty) Ltd (South Africa). Dulbecco’s modified Eagle’s medium (DMEM) with high glucose 4.5 g/l (25 mmol/l) and Foetal Bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Trypsin, trypan blue solution (0.4 %), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), vinblastine sulphate (VBS), verapamil, ketoconazole and metformin were purchased from Sigma-Aldrich (St. Louis, USA). Dimethyl sulfoxide (DMSO) and acetonitrile were purchased from MERCK (Germany). Vivid® CYP4502C9 red and CYP3A4 red screening kits were purchased from Invitrogen Corporation.

Fresh Sutherlandia frutescens leaves were collected in the Eastern Cape (South Africa) and identified by Prof. Campbell (Botany Department, Nelson Mandela Metropolitan University). A plant specimen has been deposited in the NMMU herbarium (PEU 14 800). The leaves were air dried away from direct sunlight. Prunus africana bark extracts (freeze dried powder) was collected and prepared in Kenya by Dr. Mbaabu (University of Nairobi).
Commercial medicinal herbal products were purchased from local pharmacies (Port Elizabeth, South Africa). A detailed description for each commercial product is listed in the Table 3.1.

<table>
<thead>
<tr>
<th>Species name</th>
<th>Commercial name</th>
<th>Compositions (Per Capsule / Tablet)</th>
<th>Daily dose</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. ferox</em></td>
<td>Aloe Ferox®</td>
<td><em>A. ferox</em> 150 mg</td>
<td>1-2 tablets</td>
<td>Spiraforce Enterprises</td>
</tr>
<tr>
<td><em>A. ferox</em></td>
<td>Prosit®</td>
<td><em>A. ferox</em> 242.537 mg, Zingiber officinale 9.702 mg, Capsicum frutescens 4.851 mg, Glycyrrhiza glabra 4.851 mg, Allium sativum 4.851 mg</td>
<td>1-2 capsules daily</td>
<td>African Cures cc</td>
</tr>
<tr>
<td>Aloe species</td>
<td>Aloes powder® a</td>
<td>Aloe species in powder form</td>
<td>Not indicated</td>
<td>Barrs Pharmaceutical industries</td>
</tr>
<tr>
<td><em>S. frutescens</em></td>
<td>Promune®</td>
<td><em>S. frutescens</em> extract 90 mg</td>
<td>2 capsules daily</td>
<td>Ferlot manufacturing &amp; packaging (Pty) Ltd.</td>
</tr>
<tr>
<td><em>S. frutescens</em></td>
<td>Probetix®</td>
<td><em>S. frutescens</em> extract 90 mg, Alpha-Lipoic acid 75 mg</td>
<td>2 capsules daily</td>
<td>Ferlot manufacturing &amp; packaging (Pty) Ltd.</td>
</tr>
<tr>
<td>St. John’s Wort</td>
<td>St. John’s Wort®</td>
<td>St. John’s Wort (Equivalent to hypericin 490 µg) 165 mg</td>
<td>1 capsule twice daily</td>
<td>Aspen Pharmacare</td>
</tr>
<tr>
<td><em>G. biloba</em></td>
<td>Ginkgo Biloba®</td>
<td><em>G. biloba</em> leaf extract (Equivalent to leaf 6000 mg)</td>
<td>1 capsule daily</td>
<td>Clicks</td>
</tr>
</tbody>
</table>

**Note:**
All the information was obtained from corresponding package inserts.

*a:* Aloes powder® does not disclose the species used but it is most likely *A. ferox* because this represent the species that is commercially harvested in South Africa.
3.2: Methods

3.2.1: Stock solutions preparation

3.2.1.1: Medicinal plant stock solutions preparation

De-ionized water was used as extraction solvent to prepare medicinal plant stock solutions. To minimize light exposure, the test tubes containing extracts were wrapped with the aluminium foil. All the stock solutions were transferred into eppendorf tubes (1.5 ml) and stored at 4 ºC.

Commercial medicinal herbal products were either tablets or capsules (Table 3.1). The tablets were ground into fine powder using a mortar and pestle. To start the extraction, the pre-weighed capsule contents or ground tablet powder were dispersed into pre-warmed de-ionized water (37 ºC) with vigorous shaking to produce herbal suspensions. The herbal suspensions were incubated in a water bath at 37 ºC for 30 minutes, followed by 15 minutes sonication. Incubation and sonication were repeated three times. After extraction, the obtained extracts were centrifuged at 382 xg for 6 minutes, and the resulting supernatant carefully transferred using a pipette into a pre-weighed test tube prior to freeze drying.

Dried S. frutescens leaves were used to prepare the non-commercial S. frutescens extract for this study. The infusion method, a traditional extraction method, was used to obtain the plant extract. To do so, shade dry leaves were shredded into fine powder using a blender. Then, 1 g of fine leave powder was weighed and left in boiled de-ionized water (30 ml) for 2 hours. Finally, the extract was filtered through Whatman No.1 filter paper. The filtrate was collected and transferred into a pre-weighed centrifuge tube prior to freeze drying.
3.2.1.2: Conventional compounds stock solutions preparation

Vinblastine, verapamil and metformin were easily dissolved in DMSO. The final concentration was 0.737 mM for the vinblastine stock and 20 mM for both the metformin and verapamil stocks. Ketoconazole was dissolved in acetonitrile with a final concentration 10 mM.

3.2.2: Vivid® CYP2C9 and CYP3A4 inhibitory assay

The inhibitory effect of medicinal plant extracts on CYP2C9 and CYP3A4 iso-enzymes were determined using Vivid® CYP450 (2C9 red and 3A4 red) screening kits. The kit enables rapid analysis of the CYP450 interactions in a multi-well plate format. It contains CYP450 BACULOSOMES® reagent and Vivid® substrate. The CYP450 BACULOSOMES® reagent is a mixture of microsomes derived from baculovirus infected-insect cells expressing a single human CYP450 iso-enzyme and nicotinamide adenine dinucleotide phosphate (NADPH)-P450 reductase. The kit also contained a regeneration system (333 mM glucose-6-phosphate and 30 U/ml glucose-6-phosphate dehydrogenase) used to convert NADP⁺ into NADPH. The energy released from this conversion initiates the metabolism of the Vivid® substrate (dibenzylfluorescein/ benzyloxyresorufin) by its specific CYP450 (2C9 and 3A4) iso-enzymes into highly fluorescent products (Figure 3.1).

![Figure 3.1: The metabolism of Vivid® substrate into highly fluorescent products](image)

CYPs are able to metabolise the substrate at two potential sites R1 and R2. Oxidation at either site releases the highly fluorescent metabolites. The figure was taken from Vivid® CYP450 Screening Kit Protocol, Invitrogen Corporation.
Assay procedures of the Vivid® CYP2C9 and CYP3A4 enzyme assay were followed as per manufacturer’s specifications (Invitrogen Corporation; Carlsbad, CA, USA). In brief, the stock solutions of medicinal plants were serially diluted with de-ionized water to various concentrations (Table 3.2).

The assay was conducted in a black 96-well plate (NUNC®, Denmark). In each well, 50 µl of master premix solution (consisting of CYP450 BACULOSOMES® reagent and regeneration system) was added to 40 µl of plant extracts or controls. The mixture was incubated at room temperature for 20 minutes. The reaction was initiated by adding 10 µl of a mixture of Vivid® substrate and NADP+. In CYP3A4 inhibitory assay, St. John’s wort and ketoconazole were selected as positive controls (Zou et al., 2002; Hellum & Nibsen, 2008). In CYP2C9 inhibitory assay, the positive control was G. biloba (Yale & Glurich, 2005).

Table 3.2: Concentrations of test samples used in CYPs assay

<table>
<thead>
<tr>
<th>Tested Samples</th>
<th>[Stock] (mg/ml)</th>
<th>CYP 3A4 assay</th>
<th>CYP 2C9 assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aloe Ferox®</td>
<td>3.13</td>
<td>5, 10, 25, 46, 100, 250, 457</td>
<td>5, 10, 30, 50, 80, 120, 200, 457</td>
</tr>
<tr>
<td>Prost®</td>
<td>8.89</td>
<td>2, 10, 25, 46, 100, 250, 457</td>
<td>5, 10, 30, 50, 80, 120, 200, 457</td>
</tr>
<tr>
<td>Aloes Powder®</td>
<td>11.84</td>
<td>5, 10, 25, 50, 150, 250, 500</td>
<td>5, 10, 25, 50, 100, 150, 250, 500</td>
</tr>
<tr>
<td>Promune®</td>
<td>5.03</td>
<td>4, 34, 100, 250, 338, 500, 1000</td>
<td>15, 100, 150, 200, 300, 500, 800</td>
</tr>
<tr>
<td>Probetix®</td>
<td>5.26</td>
<td>4, 34, 100, 250, 338, 500, 1000</td>
<td>25, 80, 150, 300, 500, 800</td>
</tr>
<tr>
<td>S. frutescens leaves</td>
<td>15.88</td>
<td>10, 30, 50, 80, 100, 150, 250</td>
<td>10, 30, 50, 80, 100, 150, 300, 600</td>
</tr>
<tr>
<td>P. africana</td>
<td>18.59</td>
<td>10, 15, 20, 30, 50</td>
<td>5, 15, 25, 30, 50</td>
</tr>
<tr>
<td>St. John’s wort®a</td>
<td>13.59</td>
<td>4, 10, 25, 50, 75, 100, 200</td>
<td>ND</td>
</tr>
<tr>
<td>Ginkgo Biloba®b</td>
<td>23.88</td>
<td>ND</td>
<td>5, 15, 30, 40, 50, 100, 250</td>
</tr>
<tr>
<td>Ketoconazole®</td>
<td>10 mM</td>
<td>10⁶, 10⁵, 10⁴, 10³, 10², 10¹, 1, 10</td>
<td>ND</td>
</tr>
</tbody>
</table>

Note:
a: Positive controls used in CYP3A4 inhibitory assay; b: Positive control used in CYP2C9 inhibitory assay; ND = Not determined
The fluorescence measurements were taken at every 30 seconds using a Fluoroskan Ascent® Microplate Fluorometer (Thermo Labsystem, Finland) over 1 hour with an excitation wavelength 544 nm and an emission wavelength 590 nm. The experimental parameters used in the study are listed in Table 3.3.

The results were analysed according to the manufacturer’s protocol. The reaction rate was determined by calculating the rate of the change in fluorescence per unit time using the initial linear range of the progress curve. The percent enzyme inhibition in the presence of test compounds or positive controls were calculated using the following formula:

\[
\% \text{ Enzyme Inhibition} = (1 - \frac{\text{the rate in presence of test compounds or positive control}}{\text{the rate in absence of test compounds}}) \times 100\%
\]

As a measure of inhibition potency on CYP2C9 and CYP3A4 activity, IC\(_{50}\) values were determined for each test sample. The IC\(_{50}\) value is the concentration that is required to decrease the activity of the enzyme by 50%. The IC 50 values were obtained using Prism software (Version 4, Graphpad Prism software, San Diego, USA). Significance of the results was evaluated using Student’s T-test, and p-values smaller than 0.05 were considered significant.

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>[Vivid® CYP Baculosomes](^a) (nM)</th>
<th>[Vivid® CYP Substrates](^a) (µM)</th>
<th>The liner range of the changes fluorescence per unit time (minutes)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C9 Red</td>
<td>10</td>
<td>2</td>
<td>2 - 10</td>
</tr>
<tr>
<td>CYP3A4 Red</td>
<td>5</td>
<td>3</td>
<td>4 - 30</td>
</tr>
</tbody>
</table>

Note:
\(a\): Determined by the manufacturer protocol; \(b\): Determined by visual inspection.
3.2.3: P-gp assay using Caco-2 cell model

3.2.3.1: Cell culture

Caco-2WT (wild type) cells (at passage number 11) were cultured in a medium containing DMEM and 10 % FBS and maintained at 37 °C in a humidified environment with 5 % CO₂ in the air. The medium was renewed every 72 hours. Once the cells reached approximately 70 % confluence, they were sub-cultured by trypsinization. Briefly, after removing the medium, cells were washed once with 10 ml of warm PBS-EDTA (without Ca²⁺ and Mg²⁺) followed by adding 1 ml of trypsin (0.1 %)-EDTA (0.02%) solution and incubation at 37 °C for 10 to 15 minutes. Once the cells had detached from the bottom of a culture dish, 3 ml of complete medium was added to inactivate trypsin. To separate the cells, the cell suspension was gently pipetted a number of times to avoid cell aggregates and then 1 ml of cell suspension was transferred into a new culture dish containing 10 ml of complete medium. For storage, after trypsinization, a mixture containing 80 % DMEM, 10 % FBS and 10 % DMSO was added to the culture dish and mixed well. Then, 1 ml of cell suspension was transferred into a cryotube and stored in liquid nitrogen. Caco-2 cells used in the study were tested for mycoplasma contamination and found to be free.

- Why choose Caco-2 cell line as a cell model for studying P-gp activity?

Only a few suitable cell lines are available for studying the functionality of P-gp, these include P-gp transfected Madin Darby Canine Kidney (MDCK) and LLC-PK1 (a porcine kidney epithelial cell line) cells (Ungell, 2004). However, the Caco-2 cell line appears to be the most favourable cell model used to study P-gp functionality (Hidalgo & Li, 1996; Maubon et al., 2007). Unlike other non-human or non-intestinal cell lines, Caco-2, a human colorectal adenocarcinoma, exhibits morphological characteristics such as brush border enzymes and drug metabolising enzymes, which are found in human intestinal enterocytes. The Caco-2 cell line is well characterised with respect to the expression of various transporters such as oligopeptide transporters and efflux proteins (P-gp and other multi-
drug resistance associated efflux proteins) (Maubon et al., 2007). Most importantly, P-gp expressed by Caco-2 has remarkable biochemical and functional similarities to P-gp expressed by intestinal enterocytes (Hidalgo & Li, 1996). In addition, Caco-2 cells are easy to culture and handle, and have good experimental reproducibility and robustness (Ungell, 2004). Thus, Caco-2 cells were considered a suitable cell model for studying the effects of medicinal plant extracts on P-gp activity.

3.2.3.2: Development of a vinblastine resistant Caco-2 cell line

Caco-2VBS10 (vinblastine treated) clone was created from wild-type Caco-2 cells. Briefly, Caco-2WT cells at passage number 23 were cultured in a medium containing 10 nM vinblastine as described by Shirasaka et al. (2006). Cells were routinely sub-cultured at about 80 % confluence in an identical manner as for Caco-2WT.

To demonstrate the changes in cell growth, cells were seeded at a density of $3.4 \times 10^5$ cells /ml in a culture plate (100 mm). After 3 days, the number of living cells were counted using a Brightline Haemocytometer and by staining cells with 0.4 % trypan blue. The study observed that Caco-2WT cell growth rate was initially inhibited to 8% of the control group (in absence of vinblastine) and recovered gradually. After 8 passage cycles, cells reached normal growth rate despite the continuous presence of 10 nM vinblastine (Figure 3.2). This observation is in line with previous studies by Shirasaka et al. (2006) and Siissalo et al. (2007). Caco-2VBS10 cells were only used for experimental purposes once their growth rate had completely normalized.
Figure 3.2: Growth rate of Caco-2WT cells in presence of VBS

In the control group, Caco-2WT cells grow in a medium without VBS. The gray bars represent cells growing in the medium containing VBS (10 nM). The numbers of living cells were counted at 3 day intervals at each passage. Data represents a single experiment. VBS = Vinblastine sulphate

- Why develop Caco-2VBS10 cell line?

Though Caco-2 cells are widely used as a cell model to study P-gp activity, previous studies, surprisingly, reported that this cell model has failed to identify reference P-gp substrates. Generally, the highly variable and rather low expression of P-gp in Caco-2 cells seems to be a limiting factor in accurately identifying P-gp substrates or studying P-gp related interactions (Shirasaka et al., 2006; Hellinger et al., 2010).

P-gp expression in Caco-2 cells is highly influenced by time in culture. Specifically, P-gp expression is significantly lower in higher passage number than at earlier starting passage number (Anderle et al., 1998; Shirasaka et al., 2006). P-gp expression is also affected by culturing conditions. For instance, though no changes of P-gp expression over time in culture were observed when Caco-2 cells were cultured on polycarbonate filters, a significant decrease was detected when the cells were cultured directly on plastic (Anderle et al., 1998). In addition, Bethrens & Kissel (2003) observed that a significant difference in carrier-mediated transporter expression in Caco-2 cells can result from even a small
change in culture conditions such as culture time, seeding density and membrane support. This in turn may affect the sensitivity and specificity of the P-gp efflux screening assay. Further, Maubon et al. (2007) reported that mRNA expression of P-gp varies between two Caco-2 cell clones by at least two fold. For P-gp, mRNA expression correlates with protein abundance and functionality in Caco-2 cells (Siissalo et al., 2007). These findings suggest that the expression of P-gp in Caco-2 cells is highly dependent on tissue culture environment and consequently prone to variability. It also implies the importance of characterizing transport level in any Caco-2 cell system (Maubon et al., 2007) prior to assess P-gp interactions.

To improve P-gp expression and functionality in Caco-2 cell system, many studies have demonstrated that culturing Caco-2 cells in the presence of vinblastine (10 nM) leads to a significantly increased P-gp expression level as compared to control groups without vinblastine (Anderle et al., 1998; Shirasaka et al., 2006). Importantly, high P-gp expression level induced by vinblastine remained consistent across a broad range of cell passage numbers. This finding demonstrated that the vinblastine treated Caco-2 cell system can be used over wide passage ranges (Siissalo et al., 2007). Indeed, vinblastine treated Caco-2 cell system successfully identified 11 reference P-gp substrates, out of which the wild type Caco-2 cell line only identified four (Hellinger et al., 2010). These findings support the use of vinblastine treated Caco-2 as is an accurate in vitro system for screening potential P-gp substrates/inhibitors.

- **What is the mechanism of action by which vinblastine treatment increases P-gp expression level in Caco-2 cell line?**

Vinblastine, an anti-cancer drug, belongs to vinka alkoloid group. It inhibits cell division by inhibiting microtubulin formation and thus cell cycle arrest. In addition, it is a well known P-gp substrate and inducer (Hellinger et al., 2010).
The exact mechanism by which vinblastine treatment results in elevated P-gp level in Caco-2 is still unclear. Two mechanisms have been suggested in the literature namely; induction and selection. For the induction mechanism, it has been reported that vinblastine increases the P-gp mRNA expression level, via activating the human Pregnane X Receptor (PXR) which is involved in the regulation of P-gp gene expression; however, vinblastine is reported to be a weak activator of the receptor itself (Hellinger et al., 2010). Furthermore, a study by Mitin et al. (2004) reported that Caco-2 cells lack PXR. Another possibility is that vinblastine directly affects the mRNA stability and consequently the level of mRNA, thereby enhancing P-gp protein levels in Caco-2 cells (Shirasaka et al., 2006).

The explanation for the selection mechanism is that the individual cell levels of P-gp is not uniform, therefore vinblastine accumulates to a greater extent in cells with low P-gp expression due to limited P-gp efflux activity. Since vinblastine inhibits cell proliferation, these cells are more susceptible to the growth inhibition effects of vinblastine. On other hand, in the cells with high P-gp expression, vinblastine is less likely to accumulate, therefore cell proliferation is unhindered and these cells will become predominant during successive passage (Shirasaka et al., 2006). From the data in the present study, this selection hypothesis is in accord with the observation that the cell growth rate was inhibited substantially at initial vinblastine-treatment and recovered slowly; eventually cells with high P-gp expression become dominant (Figure 3.2). This hypothesis is further supported by a finding of Hellinger et al. (2010); they reported that vinblastine treated Caco-2 cells maintained high level of P-gp proteins or P-gp functionality even after a prolonged absence of vinblastine (Hellinger et al., 2010). However, this is not in the scope of the present study and further experiments are needed to clarify this issue.

3.2.3.3: Toxicity assessment using MTT assay

In order to determine the highest sub-lethal concentrations of test samples, the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used. The MTT assay, a common cytotoxicity screening assay, measures the number of viable cells based
on mitochondrial activity (Mosmann, 1983). Briefly, 100 μl Caco-2WT or Caco-2VBS10 cells were seeded into 96-well plates at a density of 20,000 cells/ml. The plates were then incubated at 37 ºC for 24 hours to allow attachment and initiate growth. Thereafter the cells were incubated with test samples at various concentrations (Table 3.4) for 72 hours at 37 ºC. The final volume of incubation medium in each well was 200 μl. At the end of this incubation period, the medium was removed and 100 μl of fresh culture medium containing MTT (5 mg/ml) was then added to each well. The plates were incubated for 1 hour at 37 ºC. During this period, the mitochondria of living cells reduced the soluble yellow MTT dyes into insoluble blue formazan, which accumulated in these living cells.

The reaction was stopped by removing incubation medium. To solubilise the dark blue formazan crystals, 100 μl of DMSO was added to each well and plates were left for 15 minutes at room temperature. In order to quantify the intensity of the colour, the absorbance was read on a microtiter-plate reader using a wavelength of 570 nm. The amount of formazan crystals is directly proportional to the number of living cells (Mosmann, 1983). The absorbance intensity of these test groups was compared with control groups containing culture medium only. The significance was determined using a paired Student’s T-test.

The MTT assay was also used to assess whether selected medicinal plants enhance the anti-proliferatory potency of vinblastine in Caco-2VBS10 relative to Caco-2WT cells. Cells were incubated with various concentrations of vinblastine for 72 hours, either alone or in presence of sub-lethal concentration of tested samples determined previously. The concentrations ranges of vinblastine were 1 nM - 400 nM in Caco-2VBS10 and 0.5 nM - 100 nM in Caco-2WT. The percentage cell survival was calculated as following:

\[% \text{ cell survival} = \frac{\text{the mean absorbance of treated wells}}{\text{the mean absorbance of untreated well}} \times 100\]
In order to determine the IC$_{50}$ values of vinblastine, a dose-response curve was plotted and fitted by using Prism GraphPad 4. For each dose response curve, at least 6 concentrations were used, and each concentration was the mean values ± SD (n > 6).

### Table 3.4: Concentrations of test samples used to determine cytotoxicity

<table>
<thead>
<tr>
<th>Test Samples</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0.5%, 0.8%, 1%</td>
</tr>
<tr>
<td>Verapamil</td>
<td>5 µM, 10 µM, 20 µM, 30 µM</td>
</tr>
<tr>
<td>Metformin</td>
<td>5 µM, 10 µM, 20 µM, 30 µM</td>
</tr>
<tr>
<td>St. John's wort® (St. John's wort)</td>
<td>50 µg/ml, 100 µg/ml, 200 µg/ml</td>
</tr>
<tr>
<td>Aloe Ferox® (A. ferox)</td>
<td>50 µg/ml, 100 µg/ml, 200 µg/ml</td>
</tr>
<tr>
<td>Prosit® (A. ferox – major ingredients)</td>
<td>50 µg/ml, 100 µg/ml, 200 µg/ml</td>
</tr>
<tr>
<td>Aloes Powder® (Aloe species)</td>
<td>150 µg/ml, 200 µg/ml</td>
</tr>
<tr>
<td>Promune® (S. frutescens)</td>
<td>50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml</td>
</tr>
<tr>
<td>Probetix® (S. frutescens)</td>
<td>50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml</td>
</tr>
<tr>
<td>S. frutescens leaves – shade dry</td>
<td>50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml</td>
</tr>
<tr>
<td>P. africana bark – freeze dried powder</td>
<td>50 µg/ml, 100 µg/ml, 200 µg/ml</td>
</tr>
</tbody>
</table>

**Note:**
Species name of commercial herbal products was listed in brackets.
Same Concentrations for each sample were assessed in Caco-2 or Caco-2VBS10 cell lines.

### 3.2.3.4: Evaluation P-glycoprotein assay using vinblastine sensitivity

In the present study, effects of selected medicinal plants on P-gp activity were evaluated by assessing their impact on vinblastine anti-proliferation potency in Caco-2VBS10 cell system using MTT assay. To achieve this study aim, it was important to establish that the anti-proliferation potency of vinblastine directly correlates to P-gp activity in this Caco-2 cell based system. Indeed, preliminary experiments verified this correlation by demonstrating that the increased anti-proliferation potency of vinblastine was stimulated by decreased P-gp mediated efflux activity. Before discussing these experimental verifications, the basic assay principle is illustrated in Figure 3.3.
The principle of this assay is based on the reversal of drug resistance in modified Caco-2 cells specifically altered to express high efflux protein activity, and hence these cells display a multidrug resistance phenotype. In the absence of P-gp inhibitors, VBS is rapidly pumped out by P-gp. In contrast to Caco-2WT, Caco-2VBS10 cells exhibit higher P-gp expression with higher efflux activity and as a result these cells accumulate less VBS. Thus, Caco-2VBS cells can survive at higher concentrations of VBS. The addition of a plant extract containing a P-gp inhibitor or substrate will inhibit or compete with VBS and consequently reverse the drug resistance leading to a decrease in the concentration of the drug required to inhibit proliferation. The assay relies on the principle that a direct correlation between P-gp activity and the anti-proliferatory effect of VBS.

To evaluate the effects of medicinal plants on the anti-proliferation effect of vinblastine on Caco-2VBS10 cells, it was necessary to determine anti-proliferation effect of vinblastine in absence of the plant extracts first. For this purpose and to ensure that changes in anti-proliferation effect of vinblastine were due to variation in P-gp activity, IC_{50} values of vinblastine were determined in both Caco-2WT and Caco-2VBS10 cells. The dose response curves for the anti-proliferation effect of vinblastine are presented in Figure 3.4. As expected, the IC_{50} value obtained for the Caco-2VBS10 cells (87 nM) was significantly
higher than in Caco-2WT cells (4.9 nM), representing an 18 fold increase. This increased resistance to vinca alkaloids in P-gp over-expressing carcinoma cells is consistent with observations in earlier studies (Rassouli et al., 2009).

![Graph showing dose response curves of VBS in Caco-2WT and Caco-2VBS10 cells](image)

**Figure 3.4: Dose response curves of VBS in Caco-2WT and Caco-2VBS10 cells**

Each dose response curve represents the mean ± SD of three to five independent experiments. Percentage cells survival was determined based on the MTT assay. *: p<0.001, significant difference of VBS cytotoxicity between Caco-2WT and Caco-2VBS10 using Student’s T-test. VBS = Vinblastine sulphate; R = Correlation coefficient.

As mentioned previously, P-gp expression levels may vary depending on the passage number in Caco-2WT cells; but does not change significantly over a broad range of passage numbers in Caco-2VBS10 cells (Siissalo et al., 2007). The IC$_{50}$ values for vinblastine in Caco-2WT and Caco-2VBS10 cells at different passage numbers are presented in Figure 3.5. The data shows that the anti-proliferation effect of vinblastine in Caco-2WT cells is dependent on the time in culture (Figure 3.5: A); in contrast, anti-proliferation potency of vinblastine in Caco-2VBS10 remained relatively constant across numerous passages (Figure 3.5: B). This observation suggests that the anti-proliferatory potency of vinblastine is directly associated with an increased P-gp expression in Caco-2VBS10 cell model as demonstrated by Shirasaka et al., (2006).
The IC\textsubscript{50} values of VBS in Caco-2WT cells decreases as passage number increases (the range was from 11 to 30). (B) The anti-proliferation potency of VBS in Caco-2VBS10 cells remained fairly constant from passage number 30 to 70. Each bar presents the IC\textsubscript{50} values of VBS at a specified passage number and represents a single experiment. It should be noted that the passage number of Caco-2VBS10 cells is higher than Caco-2WT because a number of transfers were required before the growth rate became constant again. Therefore the passage number of Caco-2VBS10 cell line does not indicate the absolute number of transfers in presence of VBS (Passage 11-30 represents the development of resistance). VBS = Vinblastine sulphate

To confirm that previous observations were not due to cellular changes other than functionality of P-gp in Caco-2 cell model system, it was decided to determine whether the anti-proliferation potency of vinblastine could be enhanced in presence of 20 µM verapamil (a known P-gp inhibitor). The dose response curve is shown in Figure 3.6. It was not surprising that the anti-proliferation potency of vinblastine was enhanced significantly by 544 fold in the presence of 20 µM verapamil. It is important to point out that 20 µM verapamil alone did not show any toxic effects on Caco-2VBS10 cells (Figure A.1: D in Appendix A). To further ensure that this enhanced anti-proliferation potency of vinblastine in presence of verapamil is P-gp specific, the study assessed the anti-proliferation potency of vinblastine in presence of 20 µM metformin, a non P-gp inducer and non P-gp substrate (Song et al., 2006). As expected, it had no effect on MTT dye accumulation (Figure 3.7) and metformin at 20 µM is non-toxic to Caco-2VBS10 cells (Figure A.1: F in Appendix A).
Each dose response curve represented the mean ± SD of three independent experiments. Percentage cells survival was calculated using the absorbance of treated group over the absorbance of untreated control group. In comparison to the IC$_{50}$ values of the control (x) (87 nM), the IC$_{50}$ values of VBS in the presence of 20 µM verapamil is 0.16 nM. VBS = Vinblastine Sulphate; R = correlation coefficient; * p<0.001, significant difference between tested sample in the presence of verapamil (20 µM) and control groups using Student’s T-test.

**Figure 3.6: Dose response curve of VBS in presence of 20 µM verapamil in Caco-2VBS10 cells**

Each bar presented the mean of IC$_{50}$ values ± SD (n=3, three independent experiments). In comparison to control group, there is no significant difference between the tested groups containing 20 µM metformin. p<0.05, significant difference calculated using Student’s T-test. VBS = Vinblastine sulphate.

**Figure 3.7: IC$_{50}$ values of VBS in presence of 20 µM metformin in Caco-2VBS10 cells**

Taken together, the higher IC$_{50}$ values in Caco-2VBS10 relative Caco-2WT, culture time dependent changes in the IC$_{50}$ values for vinblastine in Caco-2WT but not in Caco-2VBS10.
cells and the enhanced vinblastine anti-proliferation effect in presence of a P-gp inhibitor, confirm the correlation between the anti-proliferation potency of vinblastine and P-gp activity. In addition, these results demonstrate that the Caco-2VBS10 system used in the present study is stable and suitable for assessing the functionality of P-gp.

One major limitation of this assay is the compounding effect of any intrinsic cytotoxicity of test samples on cells, which may interplay with growth inhibitory action of vinblastine and lead to false results. To ensure that any measured anti-proliferation effect of vinblastine is caused by vinblastine rather than study samples, it is important to determine the sub-lethal concentrations of the study samples on cells first. Since, all stock solutions were prepared using 100% DMSO; cytotoxicity of DMSO at various concentrations was also assessed. The DMSO concentration used in the study never exceeded 1 %, which wasn’t toxic to both Caco-2WT and Caco-2VBS10 cell lines (Figure A.1: A-B in Appendix A). The data shown in Figure A.1 (G-N in Appendix A) indicated that St. John’s wort®, (positive control), Aloe Ferox®, Prosit® and Aloes powder® extracts at concentration 200 µg/ml were non-toxic to both cell lines. However, due to the toxic effect of DMSO (1.4%) on cells (Figure A.1: A-B in Appendix A), higher concentrations of study samples could not be tested. Further, the non-toxic concentration used in the study was 100 µg/ml for all three S. frutescens preparations and 50 µg/ml for P. africana (Figure A.1: O-V in Appendix A). The inhibitory effect on P-gp activity by medicinal plants with these determined sub-lethal concentrations is discussed in the chapter 4.
CHAPTER 4: RESULTS & DISCUSSION

This chapter discusses the in vitro interaction between test medicinal plants preparations (Aloe ferox, Sutherlandia frutescens and Prunus africana) and therapeutic drugs in terms of CYPs inhibition and P-gp activity. The results are discussed with focus on predicting the clinical importance of the observed activity.

4.1: In vitro inhibition of CYP2C9 and CYP3A4 metabolism by African medicinal plants

The objective of this experiment was to evaluate the in vitro inhibitory potency (IC_{50} values) of three African medicinal plants: A. ferox, S. frutescens and P. africana extracts on recombinant CYP2C9 and CYP3A4 enzyme activities. The aim of the assay was to predict the potential for likelihood of clinically relevant drug-herb interactions of these African medicinal plants with therapeutic drugs used in type II diabetic management through CYP2C9 and CYP3A4 metabolism. In general, the recombinant CYP in vitro assay is useful to distinguish compounds which exhibit no inhibition or potent inhibitory effects (Zou et al., 2002). For samples which show some, but not a strong inhibitory effect on the enzyme, predicting systemic effects in vivo is unreliable due to unknown bioavailability of the compound. However the possibility for pre-systemic drug-herb interactions through CYP2C9 and CYP3A4 in small intestine should not be ruled out as gut concentrations are likely to be several folds higher. As mentioned previously, CYP2C9 and CYP3A4, two most abundant iso-enzymes in the gut, play a significant role in the metabolism of xenobiotics in the intestine (section: 2.1.2 in chapter 2).

The inhibitory potency of a number of commercially available herbal products and in house prepared plant extracts are summarized in Table 4.1. In the Vivid\textsuperscript{®} CYP3A4 inhibitory assay, ketoconazole and St. John’s wort were used as positive controls. The IC_{50} value obtained for ketoconazole was 54.03 µg/ml which is consistent with previous in vitro studies.
Similarly, a potent inhibitory effect on CYP3A4 activity was observed for St. John’s wort extract with an IC50 value of 27.15 µg/ml. This finding is in good agreement with earlier in vitro investigations from Hellum & Nibsen, (2008). Their study observed that St. John’s wort is a potent CYP3A4 inhibitor with IC50 values of 15.4 µg/ml using human CYP3A4 supersomes (Hellum & Nibsen, 2008). It is important to mention that the CYP3A4 substrates used in the current study differs from that used by Hellum & Nibsen (2008). This is interesting because (especially for CYP3A4) it has been demonstrated that a medicinal herb may have different inhibitory potencies on CYP3A4 activity when evaluated using different substrates (Yale & Glurich, 2005). Observations of potent inhibitory effect by St John’s wort in both the current study and that of Hellum & Nibsen (2008) despite the use of different substrates may indicate that the potent inhibitory effect of St John’s wort towards CYP3A4 is substrate independent. Such substrate independent inhibition has also been shown for other CYP3A4 inhibitors such as quinidine (Crespi & Stresser, 2002).

CYP2C9 inhibition by a commercial G. biloba extract (positive control) showed a strong inhibitory effect with an IC50 value of 31.49 µg/ml. In an earlier study, Yale & Glurich, (2005) reported that G. biloba moderately inhibited CYP2C9 activity, using recombinant CYP2C9 in vitro assay. The variation between the IC50 values obtained in the present study compared to that observed by Yale & Glurich (2005), may be due to different extraction solvents and extraction methods, different sources of enzymes, different enzyme substrates and different analysis methods.
<table>
<thead>
<tr>
<th>Species Name</th>
<th>Trade Name</th>
<th>Preparation Type</th>
<th>CYP2C9</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. ferox</td>
<td>Aloe Ferox®</td>
<td>Undisclosed</td>
<td>54.31 ± 1.043</td>
<td>55.36 ± 1.046</td>
</tr>
<tr>
<td>A. ferox</td>
<td>Prosit®</td>
<td>Undisclosed</td>
<td>113.0 ± 1.031</td>
<td>151.4 ± 1.037</td>
</tr>
<tr>
<td>Aloe species</td>
<td>Aloes Powder®</td>
<td>Undisclosed</td>
<td>87.57 ± 1.070</td>
<td>163.2 ± 1.046</td>
</tr>
<tr>
<td>S. frutescens</td>
<td>Promune®</td>
<td>Undisclosed</td>
<td>189.9 ± 1.036</td>
<td>132.1 ± 1.064</td>
</tr>
<tr>
<td>S. frutescens</td>
<td>Probetix®</td>
<td>Undisclosed</td>
<td>182.2 ± 1.055</td>
<td>126.6 ± 1.046</td>
</tr>
<tr>
<td>S. frutescens</td>
<td>Fresh leaves (shade dry)</td>
<td></td>
<td>58.48 ± 1.019</td>
<td>81.71 ± 1.048</td>
</tr>
<tr>
<td>P. africana</td>
<td>-</td>
<td>Bark extracts (hot water)</td>
<td>20.21 ± 1.011</td>
<td>16.27 ± 1.013</td>
</tr>
<tr>
<td>St. John’s wort a</td>
<td>St. John’s wort®</td>
<td>Undisclosed</td>
<td>ND</td>
<td>27.15 ± 1.062</td>
</tr>
<tr>
<td>G. biloba a</td>
<td>Ginkgo Biloba®</td>
<td>Undisclosed</td>
<td>31.49 ± 1.063</td>
<td>ND</td>
</tr>
<tr>
<td>Ketoconazole a</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>54.03 ± 1.106</td>
</tr>
</tbody>
</table>

Note:
- a: Positives controls; ND = Not determined; The IC₅₀ values represent mean ± SD (n=3)

No previous data has been reported in the literature regarding the interaction between *A. ferox* and the activity of CYP2C9 and CYP3A4. Of all the *A. ferox* preparations, Aloe Ferox® was the most potent CYP2C9 inhibitor, with an IC₅₀ value of 54.31 µg/ml, followed by Aloes Powder®, which showed strong to moderate inhibitory effect on CYP2C9 activity (IC₅₀ ≈ 87.6 µg/ml). Prosit® was the weakest inhibitor of CYP2C9 activity with an IC₅₀ ≈ 113 µg/ml (Table 4.1). In the Vivid® CYP3A4 inhibitory assay, Aloe Ferox® extract showed potent inhibitory effect with the IC₅₀ value of 55.36 µg/ml. Prosit® and Aloes Powder showed moderate inhibitory effect, with IC₅₀ values of 151.4 µg/ml and 163.2 µg/ml respectively (Table 4.1).

Among the *S. frutescens* preparations, the leaf extract was the most potent inhibitor of CYP2C9 activity with an IC₅₀ value of 58.6µg/ml. Both commercial products Promune® and Probetix® showed much weaker inhibitory effects on CYP2C9 activity with IC₅₀ values of 189.9 µg/ml and 182.2 µg/ml respectively. A possible reason is that both commercial
products contain additional excipients, with the actual *S. frutescens* concentration being much lower than in the raw plant extract. This is in accord with the lower inhibitory effect on both CYP2C9 and CYP3A4 activity from both the commercial products relative to the raw plant extract (Table 4.1).

Mills *et al.* (2005) have reported that *S. frutescens* leaf extracts (both methanol and water) at a concentration of 100 mg/ml produced potent inhibitory effects on CYP3A4 (96%) in human CYP supersome *in vitro* assay and consequently these authors suggested that *S. frutescens* has the potential to interact with antiretroviral (ARV) medications. However the high concentration tested in that study (100 mg/ml) could be questioned with regards to the physiological relevance of these observations. The results presented in Table 4.1 provide a more realistic representation of the inhibitory potential of *S. frutescens* and further supports the significance of this inhibition to physiologically relevant concentrations.

In addition to the inhibitory effects of *S. frutescens* on the activity of CYP3A4, it has recently been reported that a *S. frutescens* extract at 300 µg/ml for 96 hours, elevated CYP3A4 expression in colon derived LS-180 cells (Mitra *et al.*, 2010). This *in vitro* finding was further confirmed by *in vivo* studies in which rats pre-treated with *S. frutescens* extract for 4 days and then dosed with nevirapine (a CYP3A4 substrate), had significantly diminished plasma concentration of nevirapine. In addition, they noted increased hepatic and intestinal CYP3A4 mRNA levels by 3-fold and 2-fold respectively (Mitra *et al.*, 2010). Taken together the *in vitro* data from present study and the findings reported by Mitra *et al.* (2010) suggest that *S. frutescens* extracts exert a dual effect on CYP3A4 activity. In other words, *S. frutescens* extract is capable of inhibiting CYP3A4 activity as noted in the CYP3A4 *in vitro* assays, and with chronic exposure it could enhance CYP3A4 expression levels in cell based assays and *in vivo*. A similar effect has been observed with St. John’s wort (CYP3A4), *G. biloba* (CYP2C9) and Garlic (CYP3A4) (Foster *et al.*, 2001; Foster *et al.*, 2002; Delgoda & Westlake, 2004; Sugiyama *et al.*, 2004; Hellum *et al.*, 2007).
In comparison to the inhibitory effect of St. John’s wort (IC$_{50}$ ≈ 27.15 µg/ml) on CYP3A4 enzyme activity, *P. africana* showed a significantly stronger inhibitory effect, with an IC$_{50}$ value of 16.3 µg/ml (p<0.001). In Vivid® CYP2C9 assay, *P. africana* was also the most potent CYP2C9 inhibitor amongst tested medicinal herbs with an IC$_{50}$ value of 20.21 µg/ml, which was also significantly stronger than the *G. biloba* extracts (positive control) (p<0.001) (Table 4.1).

Unfortunately, it is difficult to estimate the true clinical significance of this inhibition on both CYP2C9 and CYP3A4 activity since recombinant CYP assays do not address bioavailability issues such as conjugating enzyme metabolism, drug efflux, protein binding and enzyme induction (Zou et al., 2002). Since gut concentrations are likely to be much higher than in the liver, CYP2C9 and CYP3A4 inhibitors may have significant impact on the absorption of drugs metabolized by these enzymes. Therefore, the study evaluated percentage inhibitory effect on CYP2C9 and CYP3A4 of tested samples at an estimated gut concentration (Table 4.2). Based on these findings, the likelihood of clinical interactions occurring in the gut can be assessed (Foti et al., 2007).

At estimated gut concentrations, all three aloe samples exerted potent inhibitory effect on CYP2C9 activity, most potent being Prosit® (96 %) followed by Aloe Ferox® (89 %) and Aloes powder® (71 %). Strong inhibitory effect on CYP3A4 activity was observed for both Prosit® (86 %) and Aloes Powder® (84 %). *S. frutescens* extracts exerted 79 % inhibitory effect toward CYP2C9 activity, followed by Probetix® (74 %) and Promune® (66 %). The study observed that Promune® showed 66% inhibitory effect on CYP3A4 activity and Probetix® caused 75 % inhibition on CYP3A4 activity (Table 4.2). Overall, all the investigated medicinal plants at their respective estimated gut concentration are likely to elicit drug-herb interaction with orally administered therapeutic drugs. It should be noted, the small difference in inhibitory effect on both CYP2C9 and CYP3A4 at estimated gut concentration from all plan preparations is related to the recommended dose and yield for each herbal extract, therefore these values do not necessarily reflect in the IC$_{50}$ values.
### Table 4.2: % Inhibition of test samples on CYP2C9 and 3A4 activity at estimated gut concentrations

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Trade Name</th>
<th>Daily Recommended dose&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Estimated Gut Concentration (µg/ml)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% inhibition CYP2C9</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. ferox</em></td>
<td>Aloe Ferox®</td>
<td>2 tab/day (1.269 g)</td>
<td>2538</td>
<td>89</td>
<td>88</td>
</tr>
<tr>
<td><em>A. ferox</em></td>
<td>Prosit®</td>
<td>2 cap/day (587.3 mg)</td>
<td>1175</td>
<td>96</td>
<td>86</td>
</tr>
<tr>
<td>Aloe species</td>
<td>Aloes Powder®</td>
<td>1000 mg/day&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2000</td>
<td>71</td>
<td>84</td>
</tr>
<tr>
<td><em>S. frutescens</em></td>
<td>Promune®</td>
<td>2 cap/day (458.9 mg)</td>
<td>918</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td><em>S. frutescens</em></td>
<td>Probetix®</td>
<td>2 cap/day (478.9 mg)</td>
<td>958</td>
<td>74</td>
<td>75</td>
</tr>
<tr>
<td><em>S. frutescens</em></td>
<td>-</td>
<td>800 mg/daily&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1600</td>
<td>69</td>
<td>77</td>
</tr>
<tr>
<td><em>P. africana</em></td>
<td>-</td>
<td>100 mg/d&lt;sup&gt;b&lt;/sup&gt;</td>
<td>200</td>
<td>≈100</td>
<td>≈100</td>
</tr>
<tr>
<td><em>G. biloba</em></td>
<td>Ginkgo Biloba®</td>
<td>1 cap/day (417 mg)</td>
<td>834</td>
<td>90</td>
<td>ND</td>
</tr>
<tr>
<td><em>St. John’s wort</em></td>
<td>St. John’s wort®</td>
<td>1 cap/dose (382.8 mg)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>765</td>
<td>ND</td>
<td>95</td>
</tr>
</tbody>
</table>

**Note:**

- **a:** Estimated gut concentration ranges were calculated using daily recommended dose to divide by 500 ml. The estimation was roughly present the concentration that occur in the gut (Foti et al., 2007)
- **b:** Daily recommended does for fresh *S. frutescens*, *P. africana* and Aloes powder were recommended by previous literatures (Johnson et al., 2007; Crinnion, 2002; Diamond, 2008)
- **c:** St. John’s wort is recommended to be taken in multiple daily doses. This has been taken into consideration when calculating estimated gut concentrations. Values for the remaining samples were calculated assuming a single daily dose. **ND** = Not Determined

### 4.2: In vitro modulation of P-gp activity by African medicinal plants

To date, no previous studies have been reported on the potential interaction between *A. ferox* and P-gp substrates/inhibitors. In the present study, three investigated Aloe samples significantly enhanced the anti-proliferation potency of vinblastine (p<0.05) in Caco-2VBS10 cell line (Figure 4.1). Of these, Prosit® was the most potent P-gp inhibitor with a mean IC<sub>50</sub> value for vinblastine being 39.9 µg/ml which represents a 2.3 fold difference compared to the control. It should be noted, beside the major ingredients *A. ferox*, Prosite® extract also contains other herbs although to a much lesser extent including *Zingiber officinale, Capsicum frutescens, Glycyrrhiza glabra* and *Allium sativum* (Table 3.1). Foster
et al. (2001) reported that constituents in *A. sativum* (garlic) may be a P-gp substrate and thus a potential inhibitor in vitro. This is a possible reason for the more potent anti-proliferation effect of vinblastine that Prosit® extracts triggered (Figure 4.1).

![Graph](image_url)

**Figure 4.1: IC₅₀ values of VBS in the presence of Aloe Ferox®, Prosit® and Aloes powder® in Caco-2VBS10 cells**

Each bar presented the mean of IC₅₀ values ± SD(n=3, three independent experiments). IC₅₀ values of VBS were determined in the absence or presence of control or test samples using the MTT assay. St. John’s wort is the positive control at concentration 200 µg/ml; the concentration of Aloe Ferox®, Prosit® and Aloes Powder® used in the study is 200 µg/ml. * denote that the significant difference between test samples between the control was calculated using Student’s T-test (p<0.05). **VBS** = Vinblastine sulphate

The IC₅₀ values for vinblastine in the presence of Promune®, Probetix® and *S. frutescens* leaf extract are presented in Figure 4.2. The results shown that the IC₅₀ values were significantly lower in presence of *S. frutescens* samples compared to control group (p<0.05). The investigated concentration for Promune®, Probetix® and *S. frutescens* leaf extract was 100 µg/ml which represents an easily achieved concentration in the small intestine after oral intake. Thus, the potential for a significant in vivo interaction between *S. frutescens* and therapeutic agents (P-gp substrate/inhibitor) should be considered.
Each bar presented the mean of the IC\textsubscript{50} values ± SD (n=3, three independent experiments. IC\textsubscript{50} values of VBS were determined in the absence or presence of control or test samples using the MTT assay. St. John’s wort is the positive control at concentration 200 µg/ml; the concentrations of Promune\textsuperscript{®}, Probetix\textsuperscript{®} and fresh \textit{S. frutescens} leaf extracts used in the study were 100 µg/ml. * denotes a significant difference relative to the control (p<0.05), calculated using Student’s T-test. VBS = Vinblastine sulphate

Studies on modulation of P-gp activity by \textit{S. frutescens} have been reported, but results are contradictory. Brown \textit{et al.} (2008) reported that \textit{S. frutescens} water extract did not significantly affect the uptake of nevirapine in normal Caco-2 cells. However, this observation does not rule out the potential interaction between \textit{S. frutescens} and drugs effluxed by P-gp. Even though their study observed that verapamil decreased nevirapine transport significantly, thereby suggesting that nevirapine was actively transported by P-gp (Brown \textit{et al.}, 2008); other transporters may also have been involved. The affinity of nevirapine for P-gp is presumably quite low as evidenced by the fact that nevirapine failed to inhibit P-gp activity in a Caco-2 transport study (Stormer \textit{et al.}, 2002). Furthermore, Janneh \textit{et al.} (2009) reported that the transport of nevirapine is independent of P-gp and may be influenced by other transporters. Moreover, although verapamil is a known P-gp inhibitor, it has also been shown to inhibit other drug transporters (Siissalo \textit{et al.}, 2009). Therefore one can’t rule out the possibility that verapamil might, to some degree, inhibit
other transporters in the Caco-2 cell system as well and that the study by Brown et al. (2008) does not provide exclusive proof that \textit{S. frutescens} does not affect P-gp activity.

In 2010, Mitra \textit{et al.} reported that \textit{S. frutescens} (300 µg/ml) elevated P-gp expression level in LS-180 cell line, and these changes were confirmed by an increased P-gp efflux activity measured from decreased cellular digoxin (a P-gp substrate) level. One explanation for the difference in the observed effects of \textit{S. frutescens} on P-gp expression in the two cell lines (LS-180 and Caco-2) could be that \textit{S. frutescens} elevates P-gp expression level in the LS-180 cell line (human colonic cell) via activation of PXR receptors since LS-180 cells express PXR, while Caco-2 cells have been reported to express little or no PXR (Mitin \textit{et al.}, 2004). In addition, since, PXR controls both P-gp and CYPs gene expression, this explanation is consistent with the increase in CYP3A4 expression in LS-180 cells after exposure to \textit{S. frutescens} extract reported by Mitra \textit{et al.} (2010) in the same study. A similar difference in the effect of a P-gp substrate on P-gp expression in these two cell lines is exhibited by verapamil, a known P-gp inhibitor (Collett \textit{et al.}, 2004). It has been reported that verapamil at concentration of 50 - 300 µM stimulated a four to six fold increase in P-gp mRNA level in LS-180 cell line within three hours with maximal stimulation. As an explanation, the study proposed that high-permeability P-gp substrates may have the capacity to rapidly up-regulate P-gp in intestinal cells \textit{in vitro} (Collett \textit{et al.}, 2004). Further, these highly permeable compounds can easily achieve high hepatic concentrations, and these are often sufficient to elevate P-gp level in the liver as well (Collett \textit{et al.}, 2004; Hitzl \textit{et al.}, 2003). This could explain the observation of Mitra \textit{et al.}, (2010) in a rat model, after treatment with \textit{S. frutescens} extracts for 4 days, that hepatic P-gp mRNA level increased by two-fold and intestinal P-gp mRNA by three-fold (Mitra \textit{et al.}, 2010). Therefore, it is suspected that at least one compound in \textit{S. frutescens} extract may have a similar characteristic as verapamil, with the potential to induce P-gp expression level \textit{in vitro} and \textit{in vivo} by a mechanism that possibly involved with PXR (Collett \textit{et al.}, 2004). However, it was not a goal to of this work to investigate the molecular mechanism of P-gp alteration by \textit{S. frutescens}, further refined \textit{in vitro} experiments and clinical trials are essential for clarifying this important aspect.
To date there are no reported literatures regarding interaction between *P. africana* and clinical medicines. *P. africana* at a concentration of 50 µg/ml enhanced the anti-proliferation effect of vinblastine significantly as evidenced by the decrease in IC$_{50}$ value to 50.9 µg/m, representing a 1.8 fold difference compared to the control (Figure 4.3). Although this is higher than the IC$_{50}$ values of St. John’s wort, it should be noted that the weaker *P. africana* inhibitory effect on P-gp probably reflects the difference in concentration between these two samples. Due to inherent toxicity of *P. africana* extract, this sample could not be tested at the desired concentration in the Caco-2 cell model, and hence different concentrations of test sample were used. Nonetheless, this concentration of *P. africana* is likely to occur in the small intestine, suggesting, that there is a high potential for interaction between *P. africana* and P-gp substrates.

![Figure 4.3: IC$_{50}$ values of VBS in the presence of *P. africana* in Caco-2VBS10 cells](image)

Each bar presented the mean of the IC$_{50}$ values ± SD (n=3, three independent experiments). IC$_{50}$ values of VBS were determined in the absence or presence of control or test sample using the MTT assay. St. John’s wort is the positive control at concentration 200 µg/ml; the concentrations of *P. africana* extract used in the study were 50 µg/ml. * denotes a significant difference relative to the control group (p<0.05), calculated using Student’s T-test. VBS = Vinblastine sulphate

### 4.2.1: Considerations for this P-gp assay

Vinblastine is a potent inhibitor of cell division and a known substrate for P-glycoprotein. Previous studies have demonstrated that vinblastine is a good candidate in P-gp mediated
transport studies, because its transport is predominantly, if not completely, P-gp mediated (Horio et al., 1989). Furthermore, Zhou-Pan et al., (1993) showed that vinblastine is mainly metabolized by CYP450 3A, and other CYPs do not seem to metabolize it. The expression of CYP3A4 in Caco-2 cell is insignificant (Siissalo et al., 2007), so it is unlikely that the results will be influenced by metabolism of vinblastine.

From a theoretical point of view co-treatment with any P-gp inhibitor should augment the anti-proliferation effect of vinblastine due to increased cellular concentration. With this in mind it was decided to assess whether a change in the anti-proliferation effect of vinblastine can be used as a measure for the presence of P-gp inhibitors in the selected herbal remedies.

For comparison, the study first examined the characteristics of untreated, with respect to vinblastine resistance, Caco-2 cells (Caco-2WT). The results are presented in Figure 4.4. It was shown that there is no significant difference between the IC$_{50}$ values of vinblastine in presence or absence of test samples. Although, there was a decrease in IC$_{50}$ values of vinblastine in presence of St. John’s wort (200 µg/ml), the difference failed to reach statistical significance. It is speculated that failure in detecting any considerable changes in IC$_{50}$ values could be a consequence of low P-gp levels in these cells, as discussed in Section: 3.2.3.4 in chapter 3. It has been reported that St. John’s Wort inhibits P-gp in vitro (Patel et al., 2004; Hellum & Nilsen, 2008), after short term exposure and therefore serves as a positive control. As shown in Figure 4.1, St John’s wort enhanced anti-proliferation effect of vinblastine significantly (p<0.001), with an IC$_{50}$ values of 31.9 µg/ml representing a 2.8 fold decreases compared to the control in Caco-2VBS10 cells thus demonstrating the suitability of the current approach to measure P-gp activity. Furthermore, unlike in the wild type cells, the IC$_{50}$ values of vinblastine in the presence of the test samples were decreased significantly in Caco-2VBS10 cell line, compared to the control (Figure 4.1, Figure 4.2 & Figure 4.3). It could be added that this enhanced anti-proliferation effect of vinblastine in Caco-2VBS10 cells but not in Caco-2WT cells (Figure 4.4) further indicates that such enhancement is P-gp specific.
Each bar presented the mean of IC$_{50}$ values ± SD (n=3, three independent experiments). In comparison to the control, there is no significant difference between the tested groups containing various tested samples; p<0.05 the significant difference was calculated using Student's T-test. St. John's wort (200 µg/ml) is the positive control. The concentrations for Aloe Ferox®, Prosit® and Aloes Powder® were 200 µg/ml; the concentrations for Promune®, Probetix® and S. frutescens leaf extract were 100 µg/ml; and the concentration for P. africana extract was 50 µg/ml. VBS = Vinblastine sulphate

4.2.2: The intrinsic cytotoxicity of medicinal herbs on cell lines

Before assessing the enhanced anti-proliferation effect of vinblastine in the presence of medicinal herbs, the intrinsic cytotoxicities of medicinal herbs on both Caco-2WT and Caco-2VBS10 cell line were evaluated using MTT assay. In the present study, Caco-2VBS10 cells were able to tolerate higher concentrations of some medicinal herbs, compared to Caco-2WT. For instance, the highest sub-lethal concentration for Promune® extracts was 100 µg/ml in Caco-2WT and 150 µg/ml in Caco-2VBS10 cells (Figure A.1: O-P in Appendix A). Similarly for P. africana, the highest sub-lethal concentration was 50 µg/ml in Caco-2WT and 100 µg/ml in Caco-2VBS10 cells (Figure A.1: U-V in Appendix A).

The MTT assay is widely used as a general criterion for investigating cell density and cytotoxicity by measuring mitochondrial activity of living cells (Mosmann, 1983; Vellonen et al., 2004; Kapitza et al., 2007). In 2004, Vellonen et al. (2004) suggested that MTT may be transported by P-gp and any inhibition of P-gp may increase MTT reduction, leading to an apparent increase in cell viability. In other words, if an investigated medicinal herb is a P-
gp substrate or inhibitor, its interference with MTT assay will mask to some degree its intrinsic cytotoxicity. Therefore, there is debate on the MTT assay’s accuracy in detecting the cytotoxicity of investigated herbs (especially P-gp substrates or inhibitors). In future, inaccurately lowered cytotoxicity measurements of tested samples due to such an interaction can be avoided by using more specific cytotoxicity methods such as Lactate dehydrogenase (LDH), which detects release of cytoplasmic LDH out of the membrane, indicating cell membrane dysfunction (Vellonen et al., 2004; Kapitza et al., 2007).

The present study determined anti-proliferation potency of vinblastine in the presence of investigated medicinal herbs at their highest sub-lethal concentrations. The effects of varying the concentrations of the medicinal herbs on the anti-proliferation potency of vinblastine have not been investigated. More studies are needed to shed light on this important area. In addition, it is important to test even more compounds that interact with P-gp to obtain a “P-gp interaction profile” of the assay with respect to vinblastine proliferation potency. Furthermore, the current study was limited to the anti-proliferation effect of vinblastine. Multidrug resistance is associated with cross-resistance to many other cytotoxic drugs including anthracyclines, other vinca alkaloids and colchicines (Hunter et al., 1993). Therefore, more structurally unrelated cytotoxic agents can be used to replace vinblastine. It was shown that P-gp contains multiple binding sites. As a result, a P-gp substrate/inhibitor may lack of competitive interactions with another specified P-gp substrate (Schwab et al., 2003). The significance of this must be investigated by determining the interactions between the test samples and different P-gp substrate.

4.3: Clinical relevance of in vitro models

In the early stage of new drug development, both the in vitro models and in vivo experiments in various animals are used to investigate the main pharmacokinetic, pharmacodynamic and toxicity profile of a new drug. The Food and Drug Administration’s (FDA) guideline recommends that when preclinical in vitro experiments rule out the importance of a metabolic pathway, in other words, lack of drug-drug interaction at
metabolic level, it is unnecessary to carry on with further clinical *in vivo* studies (Guidance for industry, 1997).

Ideally, the purpose of *in vitro* models for drug biotransformation is to closely simulate the true biotransformation *in vivo*. The general advantage of these *in vitro* models is that the complexity of the study system is reduced and they are relatively inexpensive and easy to carry out. Several hepatic *in vitro* models have been used extensively for drug biotransformation studies including supersomes, microsomes and cytosol, S9 fraction, cell lines, transgenic cell lines, primary hepatocytes, liver slice and perfused liver (Figure 4.5).

Each *in vitro* model has its advantages and disadvantages (Figure 4.5). Before choosing an optimal model in a defined situation, a number of factors have to be taken into account such as expense, the availability of the model, complexity of the experiment, *in vivo* resemblance and ethical considerations (Brandon et al., 2003). The following subsections will discuss the two *in vitro* models used in present study.

![Figure 4.5: Models have been used in the development of drug discovery](image)

*In vitro* and *in vivo* models are used in the development of new drugs, ranging from human model to isolated enzymes (supersomes) model, in order of *in vivo* resemblance. The figure was taken from Brandon et al., 2003.
4.3.1: Recombinant human hepatic supersome based CYPs assay model

The recombinant human hepatic microsome based CYPs in vitro assay is widely used to identify substances which are metabolized by CYP enzymes and to predict potential drug-herbal interaction. However, this assay model cannot adequately define the significance of a metabolic pathway as it does not address conjugating enzyme metabolism, switching of metabolic pathways or enzyme induction (Zou et al., 2002). In addition, this microsome based model is enriched with CYPs so the results obtained from these model assays have a much higher biotransformation rate when compared to the intact cell models and the human in vivo system (Donato et al., 2004). These imitating factors could explain that data obtained from the in vitro assays may be contradictory to in vivo data. In order to strengthen the findings of the current study, the inhibitory potency of two well studied medicinal herbs (G. biloba/CYP2C9 and St. John’s wort/CYP3A4) on CYP450s activities were included as a reference for herbal remedies with known clinical importance. The mechanism of drug-herb interactions of G. biloba (CYP2C9) and St. John’s wort (CYP3A4) have been well evaluated in vitro, in vivo, and in clinical setting (section: 2.3 in chapter 2). By comparing the in vitro CYPs inhibitory potency of tested African herbs to herbs known to produce clinically significant drug–herb interactions, the suitability and sensitivity of this assay is confirmed, however it is important to mention that it is incorrect to correlate study findings of different herbs directly, further refined in vitro and clinical studies are necessary.

4.3.2: Cell line models

As mentioned previously, Caco-2 cells represent a well characterised model for drug interaction studies. However, the application of Caco-2 cells requires careful characterization and the data needs to be interpreted with caution. Caco-2 cell line contains a few carrier-mediated mechanisms, which influence each other (Ungell, 2004). Thus, careless use of the data without knowledge of other mechanisms may mislead the information. In order to develop a reliable in vitro model to study the P-gp substrates, an extensive literature review has been carried out to understand the characteristics of Caco-
2WT and the advantages of selecting vinblastine for P-gp induction. Further, the assay principle was carefully validated by different experiments (Section: 3.2.3.4 in chapter 3) to ensure that the detected anti-proliferation potency of vinblastine is P-gp specific.

Like working with all other cell lines, however, correct aseptic technique is important as the cells are susceptible to contamination. This is especially true when Caco-2 is used for studying P-gp functionality, as the P-gp levels in wild type Caco-2 cells is highly variable. This variability is seen even for Caco-2 cells from the same cell origin within the same laboratory (Ungell, 2004; Siissalo *et al.*, 2007). Caco-2VBS10 cells, on the other hand, have fairly stable P-gp expression levels within individual batches. Though, Caco-2VBS10 cultures created from different Caco-2WT cell origins may have different P-gp levels. This is important to consider when interpreting data.
CHAPTER 5: CONCLUSION

African traditional healers are highly respected members of their communities and play a vital role in the healthcare of a large percentage of the South African population. Consequently traditional remedies have emerged as an integral part of health care in South Africa and many other African countries. The formalization of African traditional healing system has been recently legislated by the South African government (Traditional Health Practitioners Act (No 35 of 2004)). Unlike other countries such as China and India, information regarding the safety and efficacy of African traditional medicines is scarce. The present study was thus initiated to address the lack of information regarding the safety of African traditional medicines.

Unfortunately many people suffer from the misconception that herbal products are totally safe and without any side-effects because they are derived from natural sources and have been used for many years. Components of medicinal plants can alter the absorption and/or metabolism of conventional drugs leading to reduced efficacy or systemic drug toxicity (Delgoda & Westlake, 2004; Colalto, 2010). Furthermore, drug-herb interaction could lead to treatment failure and increased drug toxicity (Hu et al., 2005). Although there exists numerous examples of clinically significant effects of herbal remedies on prescription drugs, little is known on the risk of African traditional medicine, despite its wide spread use. Patients at greatest risk for interactions are those who suffer from chronic disease such as diabetes and who use multiple medications, particularly those drugs with a narrow therapeutic range (Chavez et al., 2006). The present study was thus initiated as a starting point to address the issue of possible interactions between African traditional medicines and current prescription drugs used in type II diabetic care. Highlighting the potential for drug-herb interaction would also create awareness among physicians and traditional healers and provide a platform to advise their patients on potential adverse effects of herbal products when used in combination with conventional drugs. While this study focused exclusively on diabetes, many of the findings are equally applicable to other diseases which share common enzymes or efflux proteins in their drug metabolism.
The high cost of clinical studies and the risk of toxic drug interactions make the use of initial *in vitro* assays imperative. Primary human hepatocytes are considered the gold standard, however these cells are problematic in that they do not divide significantly and thus require constant fresh isolation. Human liver suitable for the preparation of hepatocytes is extremely scarce and the metabolic capacity may vary from donor to donor (Tingle & Helsby, 2006). Though it is difficult to precisely predict the potential for medicinal plant products to interact with prescribed drugs, there exist numerous well characterized *in vitro* screening assays developed for the pharmaceutical industry to test drug-drug interactions of new drug candidates (Zhou *et al.*, 2007). As such, *in vitro* screening represents a well excepted strategy to investigate the preclinical safety and efficacy of new therapeutics. Due to species differences in drug metabolism, *in vivo* animal models are not suitable for predicting potential drug interactions (Tingle & Helsby, 2006).

Taken together, the findings from this study indeed suggest a concerning probability that many of the African traditional remedies will affect the metabolism of prescription drugs and may therefore have clinical significance. Furthermore, it is important to emphasize that the information presented here regarding the potential for drug interactions greatly under represents the total possible-domain of interactions, given the limited number of molecular targets assessed in this study. On the other hand, the lack of controlled clinical trials and the absence of any requirement on manufacturers of these supplements to evaluate the potential for interactions before marketing their products highlights the urgency to assess the biochemical impact of traditional African medicine on conventional drug therapy.

Both the Vivid® CYP3A4 and CYP2C9 enzymes were inhibited by all the plant preparations, but to different extents. *P. africana* extract was the most potent inhibitor of CYP2C9, with an inhibitory potency stronger than that of *G. biloba*, a well documented inhibitor of this enzyme in cDNA expressed recombinant enzyme assay (Delgoda & Westlake, 2004). Similarly *P. africana* extract was also the most potent inhibitor of CYP3A4. Its inhibitory potency was also stronger than St. John’s wort a medicinal herb known to influence the bioavailability of CYP3A4 metabolised drugs (Delgoda & Westlake, 2004; Zou *et al.*, 2002).
Furthermore at the estimated gut concentrations, all of the investigated herbal preparations have shown potent inhibitory effect toward both CYP2C9 and CYP3A4 activity suggesting that, these medicinal herbs may significantly impair the bioavailability of co-administered drugs that are metabolised by these enzymes, such as sulfonylureas, nateglinide, losartan and calcium channels blockers (Triplitt, 2006). Unfortunately it is difficult to estimate the true clinical significance of this inhibition since recombinant CYPs assays do not address bioavailability issues such as conjugating enzyme metabolism, drug efflux, protein binding and enzyme induction (Zou et al., 2002).

In the present study a new approach was taken to screen plant extracts for P-gp inhibition. The principle of this assay is based on the reversal of drug resistance in modified Caco-2 cells specifically altered to express high efflux protein activity, and hence these cells display a multidrug resistance phenotype. The addition of a plant extract containing a P-gp inhibitor or substrate will inhibit or compete with any cytotoxic drug and consequently reverse the drug resistance leading to a decrease in the concentration of the drug required to induce a set response (for example IC\textsubscript{50} value). A similar principle has previously been used to develop a cell-based high throughput assay to screen chemical libraries (Duan et al., 2009). In the present study however, drug resistance was induced using the anti-cancer drug (vinblastine). Previous studies have established that vinblastine induces drug resistance by specifically increasing the P-gp expression levels in Caco-2 cells (Shirasaka et al., 2006) While the results presented in this document support the utility of this assay to screen plant extracts for potential P-gp inhibitory activity, it is important to realise that the intrinsic toxicity and/or anti-proliferation activity of the test sample needs to be established before a definitive conclusion is reached. In order to validate this assay it is recommended that a larger number of known P-gp inhibitors are tested to confirm the accuracy of the model.

In the current study, it is difficult compare the inhibitory potency between investigated herbs, since the obtained highest lethal-concentration for each herbal preparation differs from each other. However, P. africana possessed similar potency relative to the other
products tested, but at a much lower concentration. Most importantly, it should be remembered, in order to determine whether medicinal herbs are transported by P-gp or has potential to inhibit or induce P-gp functionality, further refined *in vitro*, *in vivo* and clinical studies for individual herbs are fundamental to clarify this issue; it would be inaccurate to draw final conclusions relying on data from a single experiment.

It is difficult to predict clinical relevance using *in vitro* data alone. The clinical importance of drug-herb interactions is also influenced by patient-related factors such as age, gender and presence of other pathological conditions and genetic polymorphism (Zhou *et al*., 2007). The later is one of the most important features of CYPs (especially CYP2C9 and CYP3A4) and P-gp (Pal & Mitra, 2006). Due to various inter-individual and interethnic factors, several CYP450 iso-forms or P-gp are expressed in multiple forms, which causes variations in the enzyme/transporter activity. As a result of the genetic polymorphisms, capability of eliminating and/or responding to therapeutic drugs is largely variable among individuals (SØrensen, 2002; Correia, 2009). Due to such polymorphism, the potential for drug-herbal interaction varies among patients. In addition to patient related factors, drug related factors (such as dosing, dosage regimen and administering route) and medicinal herbal related factors (such as species, dose, and dosage regimen) play important roles in determining the clinical outcome of drug-herb interactions (Hu *et al*., 2005; Zhou *et al*., 2007)

It is important to note that the results in this study were obtained by testing crude extracts prepared for different commercial and medicinal plants and consequently the activity of traditionally prepared medicines may differ from the activity observed in these experiments due to differences in their phytochemical yield as a result of the different preparation methods (Foster *et al*., 2005). Additionally, healers may personalize their remedies or combine two or more plants which could potentiate the effects noted in this study. Manufactures of herbal remedies also have the tendency to combine different plant extract and to supplement their products with antioxidants, vitamins and preservatives, which may further complicate primary herb-drug interactions. Furthermore, *in vitro* findings are
substrate and condition specific and cannot necessarily be extrapolated to the clinical situation. Nevertheless, the results indicate that there is a potential risk of interactions if these traditional medicines are used with conventional therapeutic products and provide a starting point for more detailed studies with the ultimate goal of establishing clinical safety of these African medicinal products.
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APPENDIX: A
**Figure A.1: The Cytotoxicity of tested samples in Caco-2WT and Caco-2VBS cell lines**

Tested samples at various concentrations were plotted against its absorbance, at wave length 560 nm. Each bar represented as mean value ± SD (n>8). p<0.05, p<0.001: significant difference between test samples and control using Student’s T-test.