INHIBITORY EFFECT OF SELECTED HERBAL SUPPLEMENTS ON CYP450 - MEDIATED METABOLISM - AN IN VITRO APPROACH.

by

CHARLIZE WHITE

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Master of Science (Pharmacology)

Division of Clinical Pharmacology

Department of Medicine and Health Sciences

University of Stellenbosch

Supervisor: Bernd Rosenkranz

Professor and Head, Division of Pharmacology, Faculty of Medicine and Health Sciences

Co-supervisor: Patrick Bouic

Professor, Division of Medical Microbiology, Faculty of Medicine and Health Sciences

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DECLARATION

By submitting this thesis, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

ABSTRACT

INTRODUCTION

Herbal products are popularly used as complementary and alternative medicines to treat a variety of conditions. Often patients use them in conjunction with conventional medicines. Herbal products contain many pharmacologically active phytochemicals that may interfere with the absorption, distribution, metabolism, and elimination of medicines. This interaction can lead to an increase of the plasma concentrations of other medicines to toxic levels, or to their decrease below therapeutic levels, resulting in lack of efficacy. The liver cytochrome P450 (CYP) enzymes are responsible for the metabolism of a large majority of medicines. In order to provide more information on the potential interaction between African herbal medicines and conventional medicines, the present study has investigated the inhibition of selected CYP enzymes by three popular South African medicinal plants, Buchu, African ginger, and *Warburgia salutaris*.

METHODS

Buchu capsules, African ginger, and *Warburgia salutaris* tablets were obtained in a local pharmacy. 60% methanol/water extracts were prepared and analysed by GC-MS to reveal the composition of the volatile components of each product. Fluorogenic inhibition assays were conducted using Vivid® recombinant CYP screening kits according to the manufacturer's protocol. This protocol included the pre-incubation of herbal extracts, recombinant CYP isoform and cofactor solution. The metabolic reaction was initiated by the addition of CYP-specific substrate and $NADP^+$; the solution was incubated for 30 minutes at 37°C, after which fluorescence was measured using a microplate reader. The percentage remaining activity was calculated and used to determine the IC_{50} values of each herbal product. Time - dependent inhibition (TDI) was evaluated using the normalized ratio, $NADP^+$-, concentration -, and time - dependent approaches.

RESULTS

The GC-MS analysis revealed monoterpenes, sesquiterpenes, and alkane hydrocarbons in the volatile component. *Warburgia salutaris,* African ginger, and Buchu inhibited CYP2C19 with IC_{50} values of 5.88 µg/ml, 32.38 µg/ml, and 53.52 µg/ml, respectively. Likewise, the IC_{50} values of 5.64 µg/ml, 1.09 µg/ml, and > 100 µg/ml were obtained for inhibition of CYP3A4 by *Warburgia salutaris,* African ginger, and Buchu, respectively. Using the normalized ratio, *Warburgia salutaris* and African ginger showed time- and concentration - dependent inhibition of CYP1A2, and Buchu showed intermediate TDI effects that were not concentration dependent. All three extracts showed TDI of CYP3A4; the inhibition displayed by Buchu and *Warburgia salutaris* was $NADP^+$ dependent. African ginger was the only extract to show $NADP^+$ dependent inhibition of CYP1A2. A kinetic TDI assay

showed that the IC_{50} value of African ginger decreased over time, indicating TDI. *Warburgia salutaris* was not a time-dependent inhibitor of CYP3A4, and Buchu may have a limited time-dependent inhibitory effect.

CONCLUSION

Warburgia salutaris, African ginger, and Buchu have the potential to cause clinically relevant herb-drug interaction, if sufficient concentrations are achieved *in vivo.* Further studies are needed to confirm this finding.

ABSTRAKT

INLEIDING

Kruie produkte word gebruik as komplementêre en alternatiewe medisynes om 'n verskeidenheid van gesondheidstoestande te behandel. Pasiënte gebruik dit dikwels tesame met konvensionele medisynes. Kruie produkte bevat verskeie farmakologiese aktiewe plant chemikalieë wat met die absorpsie, distribusie, metabolisme en eliminasie van medisyne inmeng. Hierdie interaksie kan aanleiding gee tot 'n toename in plasma konsentrasies van die ander medisynes tot toksiese vlakke of tot hul afname na onder terapeutiese vlakke wat dan aanleiding gee tot 'n gebrek aan doeltreffendheid. Die lewer sitochroom P450 (CYP) ensiemes is verantwoordelik vir die metabolisme van die meeste medisynes. Die huidige studie is onderneem in 'n poging om meer inligting aangaande die potensiële interaksies tussen Afrika kruie medisynes en konvensionele medisynes te bepaal. Die inhibisie van geselekteerde CYP ensiemes deur drie gewilde Suid Afrikaanse medisinale plante, Buchu, Afrika gemmer en *Warburgia salutaris* is ondersoek.

METODES

Buchu kapsules, Afrika gemmer, en *Warburgia salutaris* tablette is by 'n plaaslike apteek bekom. 60% methanol/water ekstrakte is voorberei en die samestelling van die vlugtige komponete van elke produk is deur die analises met GC-MS bepaal. Fluoroserende inhibisie bepalings is uitgevoer deur gebruik te maak van Vivid® rekombinante CPY siftings toetse. Hierdie protokol sluit in die pre-inkubasie van die kruie ekstrakte, rekombinate CYP isoform en ko-faktor oplossing. Die metaboliese reaksie word geaktiveer deur die byvoeging van CYP-spesifieke substrate en $NADP^+$; die oplossing is vir 30 minute by 37°C geinkubeer, waarna fluorosensie deur middel van 'n mikroplaatleser gemeet is. Die persentasie oorblywende aktiwiteit is bereken en daarna gebruik om die IC_{50} waardes van elke kruie produk te bepaal. Die tydafhanklike inhiberende uitwerking (TAI) is bereken deur gebruik te maak van die genormaliseerde verhouding, $NADP^+$-, konsentrasie-, en tyd afhanklike benaderings.

RESULTATE

Die GC-MS analises het monoterpiene, sekwiterpiene, en alkaan koolwaterstowwe aangetoon. *Warbugia salutaris*, Afrika gemmer, en Buchu het CYP2C19 geinhibeer met IC_{50} waardes van 5.88 ug/ml, 32.38 ug/ml, en 53.52 ug/ml onderskeidelik. Eweneens is IC_{50} waardes van 5.64 ug/ml, 1.09 ug/ml en > 100 ug/ml onderskeidelik verkry met inhibisie van CYP3A4 deur *Warburgia salutaris*, Afrika gemmer en Buchu. Deur gebruik te maak van die genormaliseerde verhouding wys *Warburgia salutaris* en Afrika gemmer tyd en konsentrasie-afhanklike inhibisie van CYP1A2. Buchu wys intermediêre TDI effekte wat nie konsentrasie afhanklik is nie. Al drie ekstrakte het 'n TDI van

CYP3A4 aangedui; die inhibisie aangetoon deur Buchu en *Warburgia salutaris* was $NADP^+$ afhanklik. Afrika gemmer was die enigste ekstrak wat $NADP^+$ afhanklike inhibisie van CYP1A2 aangetoon het. ‘n Kinetiese TDI toets het gewys dat die IC_{50} waarde van Afrika gemmer oor tyd afneem wat TDI aandui. *Warburgia salutaris* is nie ‘n tyd-afhanklike inhibitor van CYP3A4 nie en Buchu kan dalk ‘n beperkte tyd-afhanklike inhibitoriese effek hê.

GEVOLGTREKKING

Warburgia salutaris, Afrika gemmer en Buchu het die potensiaal om klinies relevante kruie-medisyne interaksies te veroorsaak indien genoegsame konsentrasies *in vivo* bereik word. Verdere studies is nodig om hierdie bevindinge te bevestig.

DEDICATION

To my parents, Erick and Suzaan,

who still continue to invest into life, my dreams, and my aspirations.

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LIST OF ABBREVIATIONS

Abbreviation	Description
[I]	Inhibitor concentration
[S]	Substrate concentration
5-HT3	5-hydroxytryptamine
AIDS	Acquired immunodeficiency syndrome
ARV	Antiretroviral drugs
AUC	Area under the curve
BOMCC	7-benzyloxymethyloxy -3- cyanocoumarin
CAM	Complementary and Alternative Medicines
Cmax	Maximum serum concentration
CYP	Cytochrome P450
CYP450	Cytochrome P450
DDI	Drug- drug interactions
DMSO	Dimethylsulfoxide
EMC	Erythromycin
EOMCC	7-ethyloxymethyloxy -3- cyanocoumarin
FFL	Furafylline
g	Gram
GC	Gas chromatography
GC-MS	Gas chromatography
GIT	Gastrointestinal tract
GST	Glutathione *S*-transferase
HDI	Herb-drug interaction
HIV	Human immunodeficiency virus
HLM	Human liver microsomes
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography tandem mass spectrometry
IC_{50}	Concentration of inhibitor that results in 50% inhibition of enzyme activity
KI	Inhibition constant (time-dependent inhibition)
K_i	Inhibition constant (reversible inhibition)

k_{inact}	Maximal rate of enzyme inactivation (time-dependent inhibition)
K_m	Intrinsic transporter affinity or Michaelis-Menten constant
KTZ	Ketoconazole
LC-MS	Liquid chromatography - mass spectrometry
m/z	Mass-to-charge ratio
MBI	Mechanism-based inhibition
MCC	Medicines Control Council
MCZ	Miconazole
MDR1	Multidrug-resistant protein 1/ P-glycoprotein
mg	Milligram
mg/ml	Milligram per millilitre
MIC	Metabolic intermediate complex
min	Minute
ml	Milliliter
ml/min	Millilitre per minute
mm	Millimeter
mM	Millimolar
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSD	Mass selective detector
n	Number of replicates
$NADP^+$	β Nicotinamide adenine dinucleotide phosphate
NADPH	β Nicotinamide adenine dinucleotide phosphate, reduced form
NAT	*N*-acetyl transferase
Nm	Nanometer
nM	Nanomolar
NSAID	Non-steroidal anti-inflammatory drug
P-gp	P-glycoprotein
PXR	Pregnane X receptor
rCYP	Recombinant cytochrome P450
rpm	Revolutions per minute
SD	Standard deviation
SEM	Standard error of the mean

SPME	Solid phase microextraction
SSRI	Selective serotonin reuptake inhibitor
ST	Sulfotransferase
TDI	Time - dependent inhibition
T_r	Retention time
TRIS	Tris(hydroxymethy)aminomethane
U/ml	Units per millilitre
UGT	Uridine diphosphate glucuronosyltransferase
$^{v}/_{v}$	Volume per volume
V_{max}	Maximal transporter activity or velocity
WHO	World Health Organization
$^{w}/_{w}$	Weight per weight
αNP	α-naphthoflavone
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microliter
µm	Micrometre
µM	Micromolar

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CHAPTER ONE

INTRODUCTION TO STUDY

1.1 Traditional medicine

Medicinal plants have long been used for the maintenance of health and the treatment of diseases. South Africa's rich biodiversity includes many indigenous and endemic plant species, some of which may possess various health benefits. The knowledge of these health benefits forms an integral part of traditional African medicine, a holistic practice developed over generations before the advent of modern medicine.

Traditional African medicine combines herbalism and spirituality, and its health practitioners are represented by three types of healers, the herbalist (*inyanga*), the diviner (*isangoma),* and the faith healer (*umthandazi)* (Kale, 1995). Even in the era of modern medicine, traditional medicine has remained a viable alternative of health care treatment as it is easily accessible (especially in rural areas), relatively affordable, and is culturally accepted (Babb *et al.,* 2007).

With the HIV/AIDS and tuberculosis pandemic facing the country, the accessibility of traditional medicine in relation to modern health care has resulted in a dual health care system. Indeed, the South African government has promoted the integration of traditional healers into its legislative framework (Gqaleni *et al.,* 2007). Various medicinal plants, such as *Lesseria frutescens,* have been claimed to possess therapeutic and even anti-HIV effects (Harnett *et al.*, 2005) and most medicinal plants are used as immune boosters or in the management of symptoms. Nonetheless, the increased interest in the potential of African herbal medicine has led to the commercialization of many indigenous plant species.

In some parts of the world, these commercialized herbal products are sold as "phytomedicines" and are subject to the same regulatory standard as regular medicine (Zhang, 1998). In South Africa, these products are sold as complementary medicines and like other medicines, are regulated by the Medicines Control Council (MCC) of South Africa. Under new legislation, certain products that contained banned or scheduled substances were withdrawn from the market. Therefore, products such as milk thistle, which contains silymarin, require a prescription from a general practitioner and products such as *Piper methysticum,* which is reported to cause liver damage (Ernst, 2007), is no longer available in South Africa. In addition, complementary medicines have to be labelled correctly and all products are to be reviewed by the MCC over the course of a few years (PSSA, 2014). Despite regulation, these complementary medicines are still not subject to the same safety and efficacy trials

required of prescription medications. Therefore the product information contains little information on safety issues, including warnings, contra-indications, or considerations regarding the concomitant usage of these complementary medicines with conventional medicines.

Complementary and alternative medicines (CAM) are defined as practices or medical products that are chosen as adjuncts or alternatives to Western medical approaches (Debas *et al.,* 2006). "Complementary" medicines refer to practices and products together with conventional medicines, while "alternative" medicines refer to approaches in place of conventional medicines. CAMs include natural products such as herbs, vitamins, minerals, and probiotics. These are usually regulated and sold as dietary supplements. Other CAMs include acupuncture, traditional medicine, naturopathy, massage therapy, meditation, and hypnotherapy.

1.2 Herb - drug interactions

The increase in the popularity of herbal products has prompted the investigation into the potential interaction between herbs and prescription medication (Fugh-Berman & Ernst, 2001). Most herbal products, as well as orthodox drugs, are metabolized by phase I metabolic enzymes, the liver cytochrome P450 enzymes (CYP450), and phase II metabolic enzymes, uridine diphosphoglucuronosyl transferase (UGT), N-acetyl transferase (NAT), glutathione S-transferase (GST) and sulfotransferase (ST) (Zhou *et al.,* 2003). As these enzymes possess a great deal of variation (Denisov *et al.,* 2005) and are able to metabolize a large variety of biomolecules, the overlapping substrate specificity also allows modulation of enzyme activity. Consequently, the binding of one substrate to the enzyme active site can influence the metabolism of another substrate.

This is also true for drug transporters such as P- glycoprotein (P-gp) that play a significant role in the absorption, distribution, and elimination of drugs. Interaction by herbal products at any stage of metabolism can therefore alter the pharmacokinetic profile of drug action.

Drug interactions have various outcomes. The interaction may result in enzyme induction, where the metabolism of one compound (drug, herbs, pollutants) may result in an increased biotransformation of another drug. As a result, the drug may exhibit decreased efficacy as it is metabolized too rapidly to produce the desired effect. Alternatively, induction may result in toxicity if the drug action requires active metabolites, since an increased metabolism of a drug would increase the amount of drug metabolite (Ogu & Maxa, 2000). Drug interactions may also result in inhibition, where metabolism of a drug is decreased. This may lead to elevated levels of the active drug – a potential

for drug toxicity – or it may lead to decreased efficacy if the drug requires biotransformation (Ogu & Maxa, 2000).

Unlike conventional medicines, herbal products contain many phytochemicals. As many of these phytochemicals are pharmacologically active compounds such as alkaloids, steroids, terpenoids, flavonoids and coumarins, ingestion of these products with conventional medicines increases the likelihood of herb-drug interactions (HDI) (Izzo, 2005). These phytochemicals have been shown to have biological significance and possess the potential to affect pathological states (Han *et al.,* 2007). In addition, most individuals assume the safety of natural products, which may be the reasoning behind an individual's polypharmacy. Therefore, there is a high risk of interactions between herbal products and prescription medicines. Adequate scientific knowledge of potential HDI is needed to aid in advising health care professionals in co-administration of herbal products to decrease the risk of toxicity or inefficacy of drug treatments. This is especially relevant for complex treatment regimens, such as highly active anti-retroviral treatment, anti-tubercular treatment, and chemotherapy.

1.2.1 Herb - drug interactions of African herbal medicine

In Africa, usage of herbal medicine in combination with Westernized medicine is a common, yet unadvised, practice that may have serious implications. Unlike the more popular herbal medicines used in developed countries, very little data on the potential of African herbal medicines to cause HDI exist. Therefore, there is a great need for further research in this specific field of HDI for these products. The current information on the HDI of African herbal medicines from both *in vitro* and *in vivo* studies is summarized below.

1.2.1.1 *Lessertia frutescens*

Lessertia frutescens, commonly known as Cancer bush, is traditionally used in the treatment of fever, diabetes, colds, influenza, cough, chest complaints, asthma, stress and anxiety, rheumatism, muscle-wasting, open wounds, gastric and intestinal complaints, dysentery, liver and kidney conditions, urinary tract infections, and cancer (Watt & Breyer-Brandwijk 1962). In addition, it is also used as an immune booster by HIV/AIDS patients (Prevoo *et al.,* 2004), and the South African Ministry of Health has even recommended its usage as a herbal treatment of HIV/AIDS (SADC, 2002).

However, *in vitro* studies have shown the potential of *L. frutescens* to cause HDI. *L. frutescens* demonstrates near complete inhibition (96%) of CYP3A4 and dose-dependent activation of the pregnane X receptor (PXR) (Mills *et al.,* 2005). A study conducted by Fasinu (2013) using human liver microsomes showed that not only does *L. frutescens* inhibit CYP3A4, but also that the inhibition increases with increased consumption of the herb. In addition, a study by Minocha *et al.* (2011) to determine the effects of a single dose and long-term administration of *L. frutescens* on nevirapine bioavailability in rats showed that although there was no significant difference in the oral bioavailability of nevirapine before and after the single dose administration, there was a 50% decrease in bioavailability parameters (AUC, C_{max}) after five days of treatment. In the same study, a two - threefold increase in CYP3A4 mRNA expression was observed in the small intestine and liver tissue, indicating that the decreased bioavailability of nevirapine may be due to the increased metabolism by CYP3A4. If this HDI were to occur in humans, sub-therapeutic levels of nevirapine may be achieved, which could result in treatment failure and drug resistance.

1.2.1.2 *Hypoxis hemerocallidea*

H. hemerocallidea, also known as the African potato, is used in the treatment of muscle-wasting diseases, cancer, tuberculosis, benign prostatic hypertrophy, urinary tract infections, cardiac diseases, impotency, intestinal parasites, cancer, headaches, burns, ulcers, colds, influenza, hypertension, diabetes, psoriasis, inflammation, and diabetes (Watt & Breyer-Brandwijk, 1962; Mills *et al.,* 2005). Like *L. frutescens, H. hemerocallidea* is also used as an immune booster in the management of HIV/AIDS and recommended by the South African Ministry of Health (SADC, 2002).

The main phytochemical constituent in African potato is hypoxoside, which is converted in the gastrointestinal tract to rooperol through bacterial β -glucosidase activity (Nair *et al.,* 2007). Assays employing the pure compounds of hypoxoside and rooperol showed that hypoxoside did not show any *in vitro* CYP inhibition but rooperol displayed potent inhibition (Laporta *et al.,* 2007; Nair *et al.,* 2007). In addition, hypoxoside was a potent inducer of P-glycoprotein, while rooperol did not have a significant effect (Nair *et al.,* 2007).

Aqueous and ethanolic extracts of *H. hemerocallidea* have shown to have an inhibitory effect of CYP3A4 activity and to activate PXR in a dose-dependent manner (Mills *et al.,* 2005). Studies done with Caco-2 intestinal cells showed that extracts prepared according to ethnomedicinal guidelines decrease efflux of nevirapine, indicating a possible increased bioavailability (Brown *et al.,* 2008).

1.2.1.3 Other medicinal herbs

Harpagophytum procumbens, also known as devil's claw, is used to treat arthritis, rheumatism, sore joints, diabetes, gastrointestinal problems, neuralgia, headache, gout, and loss of appetite (van Wyk, 1997). *In vitro* studies have shown that *H. procumbens* increases the expression of P-gp transporters and inhibits P-gp efflux of calcein AM from human kidney-2 (HK-2) proximal tubule cells (Nair *et al.,* 2007). *H. procumbens* also shows weak inhibitory action on CYP1A2 and CYP2D6, and moderate inhibitory activity on CYP2C8, CYP2C9, CYP2C19, and CYP3A4 (Ungar *et al.,* 2004).

Echinacea purpurea, commonly known as Echinacea, is used in the treatment of respiratory infections, colds, and influenza. Although not indigenous to sub-Saharan Africa, the popularity of Echinacea is increasing due to its immunomodulatory and antiviral properties. *In vitro* data suggests that Echinacea is a reversible inhibitor of CYP1A2, CYP2C19, and CYP3A4, and upregulates the expression of CYP1A2, CYP3A4, and multidrug-resistant protein 1/P-glycoprotein (MDR1) genes (Awortwe, 2015). *In vivo* studies indicate that Echinacea reduces the oral clearance of CYP1A2 substrates and selectively modulates both hepatic and intestinal CYP3A activity (Gorski *et al.,* 2004).

An *in vitro* study using human liver microsomes (Fasinu, 2013) screened 15 South African medicinal plants for CYP450 inhibitory activity. Of the 15 plants that were screened, 12 showed varying degrees of CYP1A2 inhibition with *Spirostachys africana* exhibiting the most potent inhibition. Eight of the 15 plants showed potent inhibition of CYP2C9, and seven plants, *Acacia karroo, Capparis sepiaria, Chenopodium album, Pachycarpus concolor, Ranunculus multifidus, Lessertia frutescens, and Zantedeschia aethiopica*, inhibited CYP2C19 to varying degrees. Of the 15 plants, only two herbs, *Alepidea amatymbica and Tulbaghia violacea,* did not possess CYP3A4 inhibitory activity. As these plants are commonly employed in the treatment of conditions such as respiratory problems, gastrointestinal problems, skin problems, bacterial and viral infections, urinary tract infection, diabetes, cancer, and venereal diseases, they often are used concomitantly with conventional medicines (Fasinu, 2013).

1.3 Inhibition of CYP enzymes

HDI and drug-drug interactions (DDI) are often the result of inhibition, activation, or induction of the phase I and II metabolic enzymes and the various drug transporters. This study focuses on herb-drug interactions as a result of CYP450 inhibition, and the following review focuses on the various mechanisms of inhibition.

The mechanisms of CYP inhibition can be broadly classified into reversible and irreversible inhibition (time - dependent inhibition). Reversible inhibition can be further classified into competitive, non-competitive, uncompetitive, and mixed inhibition (Berg, 2002). Irreversible (time - dependent) inhibition includes the subset of mechanism - based inhibition, which can be further classified into quasi - reversible inhibition and true irreversible inhibition.

1.3.1 Competitive inhibition

In competitive inhibition, a molecule (inhibitor) similar to the substrate competes with a substrate for the active site of the enzyme. The molecule binds to the enzyme in a reversible manner and inhibits the binding of the substrate. As the overall structure of the enzyme is not affected by the inhibitor, the enzyme is still able to catalyse any substrate that does bind to the active site. In addition, competitive inhibition can be overcome by raising the concentration of the available substrate. The metabolic rate of the reaction (v) can be expressed as:

$$v = V_{max}\,[S]/\,K_m(1+[I]/K_i) + [S] \qquad \text{Equation 1.1}$$

Where V_{max} is the maximum velocity of the reaction, [S] is the substrate concentration, K_m is the Michaelis-Menten constant-the substrate concentration at which the reaction rate is half of V_{max}, [I] is the inhibitor concentration, and K_i is the inhibition constant, which is the dissociation constant (Nelson & Cox, 2008).

1.3.2 Non-competitive inhibition

During non-competitive inhibition, the inhibitor does not bind to the active site, but rather binds to another site elsewhere on the enzyme. This binding affects the structure of the enzyme and therefore affects the ability of the substrate to bind to the active site. As a result, increasing the substrate concentration will not affect the degree of inhibition. The metabolic rate of the reaction can be expressed as:

$$v = \{V_{max}/\,(1+[I]/K_i)\}\,[S]/K_m + [S] \qquad \text{Equation 1.2}$$

1.3.3 Uncompetitive inhibition

In uncompetitive inhibition, the inhibitor only binds to the enzyme once the substrate has bound to the enzyme and cannot bind to the enzyme alone. Consequently, the likelihood of uncompetitive inhibition occurs at high concentrations of both substrate and enzyme. The metabolic rate of the reaction can be expressed as:

$$v = \{V_{max}/ (1+[I]/K_i)\} [S]/K_m/(1+[I]/K_i) + [S] \quad \text{Equation 1.3}$$

1.3.4 Mixed inhibition

Mixed inhibition can be thought of as a "mixture" of competitive inhibition and uncompetitive inhibition. The inhibitor can bind to the enzyme before the substrate binds (such as in competitive inhibition), or can bind to the enzyme substrate complex, after the enzyme has bound (such as in uncompetitive inhibition). However, the inhibitor has a greater affinity for one of the two states. The metabolic rate of the reaction can be expressed as:

$$v = V_{max}/ (1+[I]/K_i) + (1 + K_s/[S]) \times (1 + [I]/Ki) \quad \text{Equation 1.4}$$

1.3.5 Time - dependent inhibition

Time - dependent inhibition is a process whereby the inhibitor increases in potency upon prolonged exposure to the enzyme during the pre-incubation period. Potential mechanisms of this process include the formation of metabolites that possess stronger inhibitory activity than the parent molecule and mechanism-based inhibition (MBI) - the inactivation of an enzyme through the formation of metabolites that bind irreversibly to the enzyme. In this case, *de novo* synthesis of the enzyme is required to maintain the original level of enzyme activity.

MBI can further be classified into quasi-irreversible and true irreversible inhibition. In quasi-irreversible inhibition, the metabolite formed coordinates with the ferrous form of the CYP heme, forming a metabolic intermediate complex (MIC) that renders the enzyme catalytically inactive. In true irreversible inhibition, the substrate covalently binds to the enzyme.

1.4 Cytochrome P450 enzymes

Cytochrome P450s (CYP450) are a superfamily of monooxygenases responsible for the phase I oxidation of more than 95% of all drugs available on the market (Alden *et al.,* 2010). They are also

responsible for the biosynthesis of steroid hormones, cholesterol, prostaglandins, bile acids, and thromboxane A2 (Nebert & Russell, 2002). These enzymes are located in the endoplasmic reticulum of organs such as the liver, kidneys, skin, intestines, placenta, brain and lungs (Flockhart, 2007), but the majority of CYP450 - mediated metabolism occurs in the liver, the primary site for xenobiotic biotransformation (Ogu & Maxa, 2000).

The majority of drugs are metabolized at clinically relevant concentrations by one or a few isoenzymes (Zanger & Schwab, 2013). A single isoenzyme is therefore responsible for the oxidation of many different drugs, as CYP450s possess enough structural diversity to allow the binding of various substrates (Denisov *et al.,* 2005). As a result, 90% of all xenobiotics are metabolized by six isoenzymes, namely, CYP1A2, CYP2C9, 2C19, 2D6, and 3A4/5 (Wilkinson, 2005).

1.4.1 Cytochrome P450 1A2

Cytochrome P450 1A2 (CYP1A2) belongs to the CYP1 family of heme proteins, also consisting of CYP1A1 and CYP1B1. Expression of both CYPs 1A1 and 1B1 in humans is low and occurs mainly in extra-hepatic tissue. In addition, both enzymes contribute little to drug metabolism and clearance of xenobiotics, however, CYP1A1 does have a significant role in the activation of procarcinogens (Shimada *et al.,* 1992). CYP1A2 is the primary CYP1 enzyme and accounts for 13-15% of total CYP450 content (Sridhar *et al.,* 2012). It is predominantly expressed in the liver and is responsible for the phase I metabolism of drugs such as propranolol, tacrine, theophylline, clozapine, and verapamil (Flockhart, 2007).

CYP1A2 plays a significant role in the metabolism of endogenous substances (Guengerich, 1993) and environmental toxins. It is also responsible for the activation of many environmental carcinogens, including certain mycotoxins, dietary heterocyclic amines, and the nitrosamines found in cigarette smoke (Faber *et al*., 2005). Consequently, its activity may affect an individual's susceptibility to cancer (Sridhar et al, 2012; Faber et al., 2005) and higher enzyme activity, in some studies, has been associated with a higher prevalence of certain cancers (Lang *et al.,* 1994; Horn *et al.,* 1995).

The most common probes for evaluation of CYP1A2 metabolism are phenacetin, caffeine, and melatonin. Caffeine is not often used as an *in vitro* probe, as its metabolism is extremely complex and difficult to monitor (Spaggiari *et al.,* 2014). It is however, useful in *in vivo* phenotyping, as it is relatively safe and can be detected in non-invasive manner. It is also the probe recommended by regulatory authorities. Phenacetin O-deethylation is the most frequently employed marker reaction for *in vitro* metabolism, as it is highly specific at low substrate concentrations (Spaggiari *et al.,* 2014).

Expression of CYP1A2 in the human liver is highly variable among individuals (Shweikl *et al.,* 1993) and has shown to have a corresponding variability in enzyme activity and drug metabolism (Potkin *et al.,* 1997). However, induction and inhibition of CYP1A2 has shown to be the most prominent factors influencing enzyme activity. CYP1A2 may be induced by cruciferous vegetables, chargrilled meat, polycyclic aromatic hydrocarbons, heavy exercise, smoking, caffeine intake (Faber *et al.*, 2005), and by certain drugs such as carbamazepine, insulin, omeprazole, and rifampin (Flockhart, 2007). In addition, certain factors have shown to have an inhibitory effect of CYP1A2. These include drugs such as ciprofloxacin, fluvoxamine, furafylline, quinolone antibiotics, methoxsalen, and oral contraceptives (Flockhart, 2007; Gardner *et al.,* 1983; Abernethy & Todd, 1985).

1.4.2 Cytochrome P450 2D6

Despite its relatively low abundance in the liver, cytochrome P450 2D6 (CYP2D6) is responsible for 25% of all drug metabolism, and it metabolizes drugs from many different therapeutic classes. Substrates of CYP2D6 include opioid analgesics like codeine and tramadol, β-blockers like bufuralol and metoprolol, antidepressants like amitriptyline, paroxetine, and venlafaxine, anticancer drugs like tamoxifen, antipsychotics like aripiprazole and risperidone, and antiarrhythmics like propafenone, mexiletine, and flecainide (Ionnides, 2008; Stingl *et al.,* 2012).

Of all the CYP450 isoforms, CYP2D6 shows the most variation in the efficiency and abundance of enzyme expressed between individuals (Wang *et al.,* 2009). This variation is largely due to genetic factors, but may also be a result of the broad substrate specificity of the enzyme. Genetic variation may result in increased or decreased protein expression, or even no protein or non-functional protein expression. Individuals may possess non-functional or partially defective alleles that result in a phenotype known as “poor metabolizer,” which is present in about 7% of Caucasians, 2-7% of Africans, and less than 1% of Asians (Abraham & Adithan, 2001).

The majority of the population exhibit the “extensive metabolizer” phenotype, while a small percentage of the population possesses various copies of the gene. These are known as “ultra-rapid metabolizers,” as they possess strong enzyme activity. Consequently, CYP2D6 activity ranges from complete deficiency to excessive metabolism (Wang *et al.,* 2009; Bradford, 2002).

The large variation in CYP2D6 substrates and inhibitors may also contribute to its variation in enzyme activity. The enzyme may be inhibited by drugs such as celecoxib, cocaine, ritonavir, ticlopidine, bupropion, paroxetine, and quinidine, whereas dexamethasone and rifampin are known

inducers of CYP2D6 (Flockhart, 2007). Bufuralol 1' - hydroxylation and dextromethorphan O-demethylation are two commonly used marker reactions for enzyme activity.

1.4.3 Cytochrome P450 2C9

The cytochrome P450 2C subfamily is the second largest P450 subfamily after CYP3A4 and contains three active members, 2C8, 2C9, and 2C19. It accounts for approximately 18% of all hepatic CYP450s (Lee *et al.,* 2002). Cytochrome P450 2C9 (CYP2C9) is the most prolifically expressed isoform of the 2C subfamily, responsible for the clearance of 15 - 20% of all drugs undergoing phase I metabolism (Sridhar *et al.,* 2012; Lee *et al.,* 2002). Substrates of CYP2C9 include the nonsteroidal anti-inflammatory drugs celecoxib, diclofenac, ibuprofen, and naproxen, the alkylating anti-cancer prodrug cyclophosphamide, the sulfonylureas tolbutamide, glipizide, glimepiride, and the anticoagulant warfarin (Flockhart, 2007).

Like CYP2D6, the gene encoding CYP2C9 enzymes is polymorphic, resulting in great variation of CYP2C9 activity between individuals (Lee *et al.,* 2002). Poor metabolizers are therefore at greater risk of toxicity, especially as many CYP2C9 substrates have a narrow therapeutic index. CYP2C9 is inhibited by drugs such as fluconazole, amiodarone, fenofibrate, fluvastatin, fluvoxamine, isoniazid, lovastatin, sulfamethoxazole, and sulfaphenazole (Flockhart, 2007). Its enzyme activity may also be induced by rifampicin.

The biotransformation of diclofenac to 4-hydroxydiclofenac and tolbutamide to 4-hydroxytolbutamide are both suitable marker reactions for the evaluation of CYP2C9 activity (Spaggiari *et al.,* 2014).

1.4.4 Cytochrome P450 2C19

Cytochrome P450 2C19 (CYP2C19) is a polymorphic enzyme that accounts for less than 5% of expressed CYP450 content in the liver, and 2 - 3% expressed CYP450 content in the small intestine (Paine *et al.,* 2006). As with CYP2C9, it also belongs to the CYP2C family and is responsible for the primary metabolism of 10% of marketed drugs including, omeprazole, mephenytoin, and diazepam. Polymorphisms within the encoding genes of CYP2C19 result in variation of enzyme activity, leading to poor or enhanced metabolism of drugs.

Inducers of CYP2C19 include rifampicin, prednisone, and artemisinin, and inhibitors include fluvoxamine, chloramphenicol, and ticlopidine (Foti & Wahlstrom, 2008). CYP2C19 exclusively

metabolises (S) - mephenytoin to 4'-hydroxymephenytoin, which is therefore an ideal probe substrate for CYP2C19 enzyme activity (Spaggiari *et al.,* 2014).

1.4.5 Cytochrome P450 3A4

Cytochrome P450 3A4 (CYP3A4) belongs to the CYP3A family, which also includes CYP3A5 and CYP3A7 isoforms (Guengerich, 1999). CYP3A4 is primarily expressed in the liver and the small intestine, accounting for approximately 40% of total hepatic cytochrome content and 50% of all CYP-mediated drug metabolism (Guengerich, 1999). CYP3A4 expression is shown to be extremely variable within the population, displaying greater than 100 fold variation between individuals (Zanger & Schwab, 2013).

Cytochromes belonging to the CYP3A subfamily have large active sites that bind to multiple structurally different substrates including antiarrhythmics such as quinidine, immune modulators such as cyclosporine A and tacrolimus, macrolide antibiotics such as clarithromycin and erythromycin, and benzodiazepines such as alprazolam, midazolam, triazolam, and diazepam (Shimada & Guengerich, 1989; Flockhart, 2007). In addition, other substrates include various antihistamines, anaesthetics, antifungals, analgesics, and endogenous substances like testosterone, cortisol, progesterone, and estradiol (Shimada & Guengerich, 1989; Flockhart, 2007). The broad specificity of CYP3A4 therefore prompts the use of two substrates for evaluation of enzyme activity.

Midazolam, testosterone, felodipine, dextromethorphan, and nifedipine are commonly selected as marker substrates for CYP3A4 enzyme activity. Dextromethorphan, however, is also metabolised by CYP2D6, and as midazolam and testosterone are controlled substances, nifedipine is often selected as an alternative substrate (Spaggiari *et al.,* 2014). However, nifedipine is light sensitive in solution, which makes it difficult to handle.

CYP3A4 is induced by HIV antiretroviral drugs such as efavirenz and nevirapine, carbamazepine, glucocorticoids, phenobarbital, phenytoin, dexamethasone, rifampin, and St. John's wort (Flockhart, 2007). Inhibitors of CYP3A4 include ketoconazole, grapefruit juice, erythromycin, itraconazole, ritonavir, telithromycin, and verapamil (Flockhart, 2007).

1.5 *in vitro* liver models for drug metabolism studies

For studies in drug metabolism, an appropriate liver model is required, as the liver is the primary organ responsible for drug biotransformation. It contains many drug-metabolizing enzymes that

form part of phase I, phase II, and phase III biotransformation reactions. Phase I reactions consist of hydrolysis, oxidation, and reduction, and are mainly conducted by CYP450. Phase II reactions are mainly conjugation reactions executed by UGT, NAT, GST, and ST. Phase III reactions, which consist of efflux and excretion, are mediated by the drug transporters.

An appropriate *in vitro* model should resemble the conditions expected to be found in an *in vivo* model. In addition, the selection of a model is dependent primarily on the purpose of the intended study. Various factors that need to be considered in the selection process include, expense, ethical considerations, availability, and *in vivo* similarity (Brandon *et al.,* 2003). Each model has various uses, advantages, and disadvantages which are outlined below.

1.5.1 Perfused liver

The isolated perfused liver model gives the most accurate reflection of *in vivo* conditions as it maintains the cellular architecture of the liver and contains all cells types found in hepatic tissue. In addition, the presence of functional bile canaliculi allows for the collection and analysis of bile (Groneberg *et al.*, 2002). There are however, several disadvantages to this model. There are no human livers available for perfusion and as the method of perfusion is both labour intensive and delicate to perform, it is difficult to reproduce. In addition, the liver is only functionally viable for a couple of hours after isolation (Wu *et al.,* 1999).

1.5.2 Liver slices

The liver slice model for drug metabolism was originally introduced as a model to study organ function. This model offers maintains cellular architecture and the multicellular characteristics of the liver (Olinga & Schuppan, 2013). Liver slices contain functional drug metabolizing enzymes, however the expression of these enzymes are considerably lower when compared to other models such as human liver microsomes. Disadvantages of this model include the sourcing of liver samples, the lack of inter-individual variation, and maintenance of cell culture viability (Thohan and Rosen, 2002).

1.5.3 Primary hepatocytes

The primary hepatocyte model is the most similar to *in vivo* conditions as it reflects the diverse expression of drug metabolizing enzymes (Hewitt *et al.,* 2007). Culturing isolated hepatocytes in monolayer results in the de-differentiation of cells. The hepatocytes lose their morphology and

function after a few days in culture and consequently have a limited viability (Bi *et al.,* 2006). To overcome this loss of function, hepatocytes can be cultured between two layers of matrix (usually collagen or Matrigel) (Dunn *et al.,* 1989). Maintaining hepatocytes in this sandwich configuration restores the function and morphology of hepatocytes similar to that of *in vivo* hepatocytes.

1.5.4 Human liver S9 fractions

Human liver S9 fractions are subcellular fractions obtained by the differential centrifugation of liver homogenates. S9 fractions contain both phase I and II enzymes and therefore provide a better representation of *in vivo* drug metabolism. S9 fractions can be used to determine whether a drug undergoes oxidative metabolism as they provide a simple and convenient alternative to other complex liver models. However, the enzyme activity in S9 fractions is substantially lower when compared to human liver microsomes (Brandon *et al.,* 2003), which may result in undetected metabolite formation (Hakura *et al.,* 1999).

1.5.5 Human liver microsomes

Human liver microsomes (HLM) consist of vesicles from the endoplasmic reticulum of hepatocytes. They can be prepared by the homogenized and differential centrifugation of liver preparations or obtained commercially. HLM are the most popular model for *in vitro* studies as they contain a vast variety of phase I enzymes including the various isoforms of CYP450, flavin monoxygenases, UDP glucuronyl transferases (UGT) (Newton *et al.,* 2005) and are relatively affordable. HLM are employed in studies such as HDI and DDI, reaction phenotyping, metabolite identification, gender-based differences in drug metabolism, inter-individual variability in drug metabolism, and are used to predict *in vivo* clearance (Bourrie *et al.,* 1996; Li, 2001; Venkatakrishnan *et al.,* 2001).

1.5.6 Cell lines

Liver cell line are a less popular option for *in vitro* studies as the majority of available cell lines do not possess the phenotypic characteristics of liver tissue (Soldatow *et al.,* 2013) – the hepatocytes are de-differentiated and do not completely express all drug metabolizing enzymes. The most commonly used cell line for drug metabolism studies is Hep G2 (Brandon *et al.,* 2003). The more recently established hepatoma cell line, HepaRG, retains the phenotypic characteristics of liver tissue and has a stable expression of drug metabolizing enzymes (Aninat *et al.,* 2006). However, the expression of these enzymes are still lower than in any other model (Marion *et al.,* 2010) and require sensitive analytical methods to detect enzyme activity.

1.5.7 Recombinant cytochrome P450s

The recombinant cytochrome P450 (rCYP) model consists of microsomes prepared from insect or bacterial cells that express a single human CYP450 isoform and NADPH reductase (Brandon *et al.,* 2003). rCYPs are most commonly employed in fluorometric assays using fluorogenic probe substrates that yield highly fluorescent metabolites in CYP-specific reactions. The probe substrates are composed of a blocked dye that upon metabolism is released to become highly fluorescent in aqueous solution (see figure 1.1). As the fluorescent metabolites are excited in the visible light spectrum, there is little interference from UV-excitable compounds and NADPH (Invitrogen Corporation, 2005).

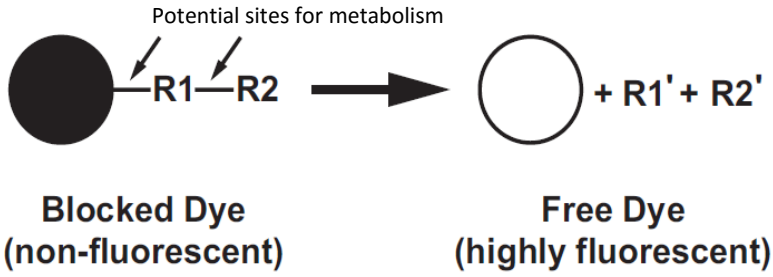


Figure 1.1: Schematic of the blocked non - fluorescent substrate metabolized to release a fluorescent metabolite (Source: Invitrogen Corporation, 2005)

Commercial fluorogenic assays follow a basic protocol that most often consists of the pre-warming of test compounds with the enzyme, addition of $NADP^+$ and the substrate that initiates the reaction, incubation, and finally the termination of the reaction. Fluorometric reading at this point is termed "endpoint mode," which measures the total fluorescence produced. However, fluorescent assays also allow for continuous reaction monitoring, also known as "kinetic mode," where fluorescence is measured at scheduled time intervals immediately after the reaction is initiated (see figure 1.2).

An advantage of this model is that the substrate is exclusively metabolized by a single isoform, unlike other models that express all phase I and II enzymes (Invitrogen Corporation, 2005). This model is therefore useful when studying the contribution of one isoform to the biotransformation pathway of a particular compound (Brandon *et al.,* 2003). As various genotypes are also available, the effects of polymorphisms on drug metabolism can also be studied. Recombinant enzymes are therefore used in the screening of new drug candidates to assess the contribution of each isoform to the compound's metabolism and can be used to study HDI and DDI. However, the expression of a single isoform has the disadvantage that it does not give an indication of the possible contribution of other metabolic enzymes.

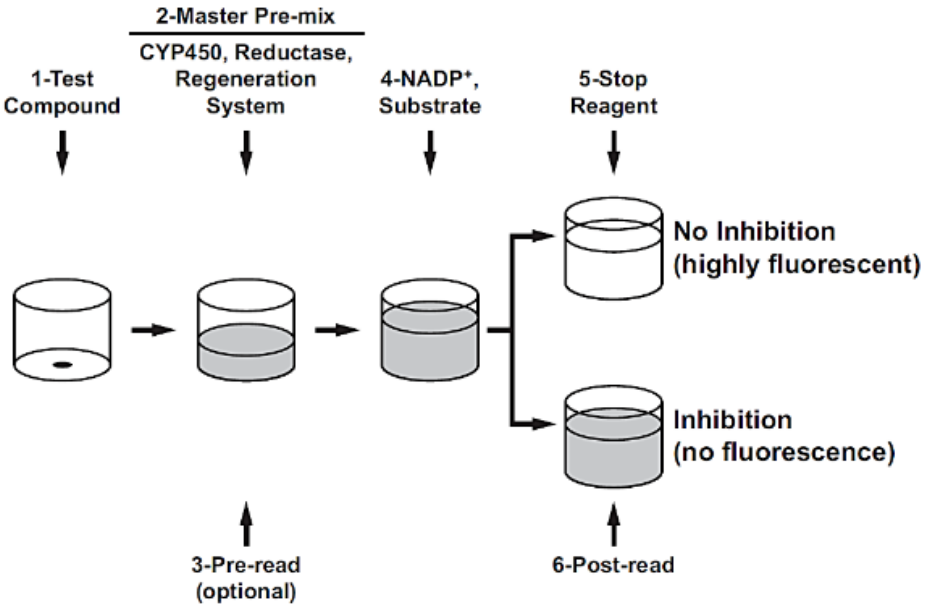


Figure 1.2: Schematic of the fluorometric assay protocol in endpoint and kinetic mode (Source: Invitrogen Corporation, 2008).

1.6 Selected herbal medicines

As this study aims to add to the current knowledge of traditional African medicine, it was necessary to include medicinal plants indigenous to South Africa. *Agathosma betulina, Siphonochilus aethiopicus,* and *Warburgia salutaris* were selected for this study based on their traditional medicinal uses and their popularity amongst consumers.

1.6.1 *Agathosma betulina*

Agathosma betulina, commonly known as Buchu, is an aromatic woody shrub endemic to the Cederberg region in the western Cape of South Africa (figure 1.3) (van Wyk *et al.,* 1997). It has a distinct blackcurrant fragrance and its leaf material is commonly used for medicinal purposes. Traditionally, Buchu leaves were chewed or taken orally as an infusion and used as a general health tonic, a diuretic, an antiseptic in urinary tract infections, an antispasmodic, an antipyretic, a cough remedy, and a treatment for colds and flu (Watt & Breyer-Brandwijk, 1962). It can also be mixed with fat and applied to the skin to treat bruises, act as an antibacterial and antifungal agent, and function as an insect repellent (Watt &

Figure 1.3: *Agathosma betulina* leaves (from van Wyk, 2008).

Breyer-Brandwijk, 1962). Today it is marketed primarily for the treatment of urinary tract infections, prostatitis, and as a diuretic (Flora Force, Cape Town, South Africa).

The two major volatile compounds in Buchu extract are menthone/isomenthone (31%) (British Herbal Medicine Association, 1996; British Herbal Codex, 1963) and diosphenol/Ψ-diosphenol (41%) (Fluck *et al.,* 1961). Other compounds include 11-17% limonene, 8% pulegone/isopulegone, and approximately 3% of both cis- and trans-8-mercapto-p-menthane-3-ones, which are believed to be responsible for the characteristic blackcurrant odour of Buchu (Kaiser *et al.*, 1975).

Buchu extract has possesses weak *in vitro* antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus,* and *Saccharomyces cerevisiae* (Lis-Balchin *et al.,* 2001), and anti-inflammatory and antispasmodic activity has been demonstrated *in vivo* (Steenkamp *et al.,* 2006; Lis-Balchin *et al.,* 2001).

Buchu is sold commercially as supplements, teas, water infusions, extracts, and oils. Due to its flavour and fragrance, it is also used in the food industry as a flavouring agent and in the perfume and cosmetic industry (van Wyk *et al., 1997*).

1.6.2 *Siphonochilus aethiopicus*

Siphonochilus aethiopicus, also known as African ginger (figure 1.4), is one of the most popular of the traditional medicinal plants in South Africa. Consequently, it has been over-exploited to near extinction in the wild, found only in Mpumalanga and the Northern Province of South Africa. Currently it is preserved by cultivation. It is primarily indicated for the treatment of colds and flu, asthma, and sinusitis, sore throats, dysmenorrhea, candida, pain, hysteria and malaria (Watt & Breyer-Brandwijk, 1962; van Wyk *et al.,* 1997), where the roots and rhizomes can be chewed, prepared by infusions, or steamed and the vapours inhaled (van Wyk *et al.,* 1997).

Figure 1.4: *Siphonochilus aethiopicus* plant with flowers (from van Wyk, 2008).

African ginger has shown to have *in vitro* antifungal and antibacterial effects (Coopoosamy *et al.,* 2010), anti-inflammatory and immune-suppressing properties (Fouche *et al.,* 2013), and may be used as an anti-parasitic in the treatment of sleeping sickness (Igoli *et al.,* 2012). It has also shown to have a cytotoxic effect, but further investigation into its possible anti-cancer effects are required (Igoli *et al.,* 2011).

African ginger contains a high percentage of sesquiterpenes (Igoli & Obanu, 2011). One in particular, siphonochilone, is responsible for the plant's anti-inflammatory properties in the treatment of asthma (Gericke, 2011). Other major volatile compounds include diterpenes and furanoterpenes (Igoli & Obanu, 2011; Holzapfel *et al.,* 2002).

1.6.3 *Warburgia salutaris*

Warburgia salutaris is the fourth most popular medicinal plant sold in the South African traditional health sector (Mander, 1998). Its English name, the pepperbark tree, is derived from the peppery taste of its bark that is most commonly used in traditional practices (figure 1.5). *W. salutaris* is believed to be a remedy for all ailments and because of its popularity, is in danger of extinction in the wild. The distribution of *W. salutaris* is now restricted to the evergreen forests and wooded ravines on the eastern coast of South Africa.

Figure 1.5: *Warburgia salutaris* leaves (from van Wyk, 2008).

W. salutaris is believed to be a panacea of all health conditions and some traditional healers even use the bark in almost all their prescribed concoctions (Mukamuri and Kozanayi, 1999). It is used in the treatment of headaches, colds, coughs, throat infections, chest complaints, venereal diseases, abdominal pain, rheumatism, malaria, sores, stomach ulcers, backache, blood disorders, skin problems, and yeast, fungal, microbial, and protozoal infections (Gericke, 2001). The preparation and administration of the herbal medicine varies for each ailment. For burns, irritations, wounds and skin complaints, the powdered bark is mixed with animal fat and applied topically. For respiratory infections, the bark is sometimes smoked, or taken orally with water.

W. salutaris contains several drimane sesquiterpenes such as warburganal (Appleton *et al.,* 1992), polygodial (Mashimbye, 1993), salutarisolide (Frum *et al.,* 2005; Frum & Viljoen, 2006; Jaansen & de Groot, 1991; Kioy *et al.,* 1990)., muzigadial (Rabe and van Staden, 2000), ugandensidial, isopolygodial (Mashimbye *et al.,* 1999a), and mukaadial (Mashimbye *et al.,* 1999b), all of which are responsible for its antibacterial, antifungal, cytotoxic, and molluscicidal properties (Jansen, 1993). *W. salutaris* is also said to contain mannitol (Watt & Breyer-Brandwijk, 1962), which can be used as an artificial sweetener.

Traditionally, the bark is used for remedies. However, as this tree is threatened with extinction in the wild, more attention has recently been given to the leaf material, as it also contains approximately

the same amount of warburganal and polygodial as the bark (Drews *et al.,* 2001) and therefore is a more sustainable source of *W. salutaris*.

1.7 Justification of this study

Recently, there has been an increase in the interest in medicinal plants and their therapeutic effect, specifically as a natural alternative to orthodox medication. This was marked by the increase of the availability of herbal products sold commercially in health care stores in South Africa (Health Product Association, 2008). These products are easily accessible in the form of supplements, teas, extracts, and essential oils, and can be obtained from any pharmacy, health store, or local market.

Many of these natural products possess various therapeutic effects (Treurnicht, 1997; Rabie & van Stadan, 1997; Viljoen *et al.*, 2006). As the majority of supplements contain natural substances, individuals assume the safety of these products. As discussed above, this may result in the co-administration of supplements with prescription medication, especially in the management of side effects, as is often the case for chronic conditions such as HIV/AIDS and tuberculosis.

The pharmacokinetic profiles of drugs are taken into consideration for the determination of the appropriate dosage regimens. However, many of these drugs are prone to pharmacokinetic interactions, which may lead to reduced efficacy or toxicity and the need for a change of the recommended dose. Many African traditional medicines contain no information on the likely risk of drug interactions, and the safety of these products, especially when used concomitantly with conventional medications, has not been established. Therefore, this study aims to determine the likely risk of drug interactions of three popular South African medicinal plants using fluorescence - based assays and recombinant cytochromes.

1.8 Potential benefits of this study

As this research provides an indication of the *in vivo* inhibitory potential of certain medicinal plants, the knowledge gained from this study can be used to plan clinical pharmacokinetic studies and health care practitioners can be advised on the co-administration of conventional medication and herbal products. The results of this and subsequent studies can be used to formulate policy documents regarding the safety of herbal supplements. This study therefore provides important new knowledge of HDI in the field of African traditional medicines.

1.9 Hypothesis

The hypothesis to be tested in this study is that *Siphonochilus aethiopicus*, *Warburgia salutaris*, and *Agathosma betulina* taken as herbal supplements in addition to conventional medication, can interact with the metabolism of conventional drugs.

- Medicinal plants contain biologically active molecules that may interact with CYP450 - mediated metabolism.
- Common medicinal herbs have been shown to activate, induce, or inhibit CYP450 enzyme activity.

1.10 Aim of this study

The aim of this study is to investigate the potential inhibitory effects of herbal supplements on five major drug metabolizing CYP450 enzymes.

The specific aims are to:

- Perform *in vitro* inhibition studies with the three herbal supplements, namely *Siphonochilus aethiopicus* (African ginger), *Warburgia salutaris*, and *Agathosma betulina* (Buchu).
- Interpret the observed influence of the selected herbal supplements on the metabolic processes in terms of concentrations required to inhibit 50% enzyme activity (IC_{50}).
- Determine the phytochemical composition of the volatile component of each herbal product.

1.11 Ethical consideration

This study was approved by the University of Stellenbosch Health Research Ethics Committee (Reference number X14/07/014, Appendix 1).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

α-naphthoflavone, sulfaphenazole, ketoconazole, furafylline, erythromycin, quinidine, dimethyl sulfoxide (DMSO), and acetonitrile were obtained from Sigma Aldrich (St. Louis, USA). 96-well plates were purchased from Corning Costar Corp. (Corning, NY, USA). All reagents employed were of analytical grade.

2.1.2 Drug metabolizing enzymes

Vivid® CYP450 Screening kits for CYP1A2, CYP2D6, CYP2C9, CYP2C19, and CYP3A4 were purchased from Thermofisher Scientific (Thermofisher Scientific, MA, USA). Each kit contained P450 reaction buffer, P450 BACULOSOMES reagent, a fluorescent substrate, a fluorescent standard, the regeneration system (333 mM glucose-6-phosphate and 30 U/ml glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate, pH 8.0), and 10 mM $NADP^+$ in 100 mM potassium phosphate, pH 8.0.

2.1.3 Herbal supplements

The herbal medicines were purchased from a local pharmacy. The Buchu capsules were manufactured by Flora Force (Cape Town, South Africa) (batch no: FCC01390), and the Warburgia and African ginger tablets, sold under the names Bio-Warburgia and Bio-African ginger, respectively, were manufactured by Bioharmony (Durban, South Africa) (batch no: Bio-Warburgia - FT00582, Bio-African ginger - FT000349).

2.2 Methods

The initial work of this project included *in vitro* assays with human liver microsomes and the analysis of samples with high performance liquid chromatography (HPLC). The method development portion of the work proved to be challenging and we were unsuccessful in achieving adequate, consistent separation of metabolites in the samples. The method of analysis was therefore changed from HPLC to high performance liquid chromatography tandem mass spectrometry (HPLC-MS). After each

assay, samples were frozen at - 80°C until the time of analysis, which could not be done on site. The mass spectrometry capabilities of the HPLC-MS allowed the identification and quantification of analytes by mass. Despite this, there was much variation between samples and duplicate samples. Due to time constraints, determination of the cause of variation and correction thereof was not possible. Consequently, the study model was changed from human liver microsomes to recombinant cytochromes which led to robust results on the potential of herb-drug interactions for the three selected plants.

2.2.1 Extraction of herbal supplements for bioassays

2.2.1.1 Tablet extraction

10 Bio-Warburgia and Bio-African ginger tablets were powdered in a mortar and 1 g of each powder was dissolved in 5 ml of 60% methanol/distilled water and sonicated for three minutes. The solution was centrifuged at 4000 rpm (Allegra X-22, Beckman Coulter, USA) for five minutes and the supernatant transferred to a new tube. The pellet was again dissolved in 60% methanol/ water and the extraction process was repeated four times. The supernatants were all pooled in a single tube, filtered (0.22 µm; Whatman International LTD, Maidstone, England), and dried at 60 °C using a miVac concentrator (GeneVac Ltd, Suffolk, UK). The extract was reconstituted in 50% methanol/distilled water to a concentration of 12.5 mg/ml and stored at - 80 °C for further use.

2.2.1.2 Capsule extraction

The contents of one Buchu capsule (approximately 370 mg) was transferred to a centrifuge tube, weighed, and extracted with 5 ml of 60% methanol/ water, sonicated, centrifuged, and the supernatant transferred to a new tube according to the method described above. The extract was reconstituted in 50% methanol/distilled water to a concentration of 12.5 mg/ml and stored at -80 °C for further use.

2.2.2 Gas chromatography/mass spectrometry

Gas chromatography/mass spectrometry (GC-MS) analysis of herbal extracts was conducted to determine the presence of possible volatile compounds in each herbal supplement that are lost during an extraction process. The analysis was conducted together with Mr L Mokwena at the Central Analytical Facility GC-MS Unit.

Three tablets and the contents of one capsule were crushed and added to separate solid phase microextraction (SPME) headspace vials. The samples were dissolved in 5 ml 70% methanol/distilled water and a 20% salt solution was added to facilitate evaporation of volatile components into the headspace. The samples were vortexed for 30 seconds, sonicated for 30 minutes, and the headspace of the sample analysed following the method described below using a PDMS/DVB/Carboxen SPME fibre (grey).

GC-MS analysis was performed on an Agilent 6890 N gas chromatograph (Agilent, Palo Alto, CA) coupled to a Agilent 5975 MS mass spectrometer detector. Chromatographic separation was achieved using a Supelco 76023AST Astec Chiraldex B-PM (30 m, 0.25 mm ID, 0.12 µm film thickness) column.

The operating conditions were as follows: carrier gas, helium, with a flow rate of 1.2 ml/min; oven temperature program was maintained at 40 °C for 2 minutes, ramped at 5 °C/min to 80 °C, held for 2 minutes, and finally at 20 °C/min to 180 °C and held for 5 minutes (total run time = 22 min); injector temperature, 200 °C; ion source temperature, 240 °C; quadrupole temperature, 150 °C; transfer line temperature, 200 °C; solvent delay, 4 minutes. The mass spectral data was recorded on a mass selective detector (MSD) operated in full scan mode (35-600 m/z).

The relative retention index (RRI) was calculated for all compounds. Compound identification was performed by comparing the mass spectral data to the Wiley 275 MS library. Quantitative data (percentage area) was determined from the GC peak area.

2.2.3 Determination of P450 activity with Vivid® P450 assay kits

The inhibitory effects of three herbal supplements on recombinantly expressed human P450 enzymes were determined using Vivid® P450 screening kits (Vivid® EOMCC substrate CYP2C19 blue, Vivid® EOMCC substrate CYP1A2 blue, Vivid® EOMCC substrate CYP2D6 blue, Vivid® BOMCC substrate CYP3A4 blue, Vivid® BOMCC substrate CYP2C9 blue). The stability, robustness, reproducibility, and sensitivity of this model is described by Trubetskoy *et al.* (2005), Marks & Larson (2006), and Cohen *et al.* (2003). The assays were conducted using the manufacturers protocol (Invitrogen Corporation, 2005). The assays were performed in Costar opaque, black, 96-well plates (Corning Inc, Corning, NY, USA) in endpoint mode according to the experimental conditions listed in table 2.1. All assays, unless otherwise stated, were conducted in duplicate, and fluorescence was measured on a Cary Eclipse multi-plate reader.

Table 2.1: Concentration of the various CYPs in the incubate

	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Enzyme concentration (nM)	2	4	2	4	2
Fluorescent substrate	EOMCC	BOMCC	EOMCC	EOMCC	BOMCC
Substrate concentration (µM)	0.3	1.0	1.0	1.0	1.0
$NADP^+$ (µM)	3.0	10	10	10	10
Excitation/emission wavelength (nm)			406/460		

BOMCC = 7-benzyloxymethyloxy -3- cyanocoumarin
EOMCC = 7-ethyloxymethyloxy -3- cyanocoumarin

2.2.4 Inhibition assays

2.2.4.1 Two - point screening

The herbal products were initially screened at two concentrations. Each well contained 40 µl of the herbal product (10 µg/ml and 100 µg/ml), solvent, or positive control (1.11µM and 10 µM α-naphthoflavone for CYP1A2, 1.11µM and 10 µM sulfaphenazole for CYP2C9, 3.33 µM and 10 µM miconazole for CYP2C19, 1.11 µM and 10 µM quinidine for CYP2D6, and 0.05 µM and 0.5 µM ketoconazole for CYP3A4) diluted in reaction buffer, and 50 µl of a pre-mixture containing the recombinant CYP isoform and regenerating system diluted in reaction buffer. The plates were pre-warmed for 15 minutes at 37°C before a pre-reading to determine the potential background fluorescence of herbal extracts, as some phytochemicals have the tendency to behave as fluorogenic compounds.

Thereafter, the reaction was initiated by the addition of 10 µl of the appropriate Vivid® substrate and $NADP^+$ into each well and the plate were incubated for 30 minutes at 37°C. The reaction was terminated by the addition of 10 µl ice-cold 20% tris(hydroxymethy)aminomethane (TRIS) base in acetonitrile and the enzyme activity was monitored by measuring the formation of fluorescent metabolites at 406nm/460nm excitation/emission spectra. The final organic solvent concentration in the assay wells did not exceed 1%. The amount of metabolite formed relative to a control in the presence and absence of an inhibitor was expressed in percentage remaining activity and calculated as:

$$\%\ \text{residual activity} = \frac{\text{Test - test control}}{\text{Control - control blank}} \times 100\% \qquad \text{Equation 2.1}$$

Analysis of the two-point screening results indicated a low signal to noise ratio for certain CYP isoforms. In order to avoid analytical errors and misinterpretation of data, the IC_{50} values for CYP2C9, CYP2D6, and CYP1A2 were not determined. Further assays were conducted on CYP3A4, CYP2C19, and CYP1A2 (TDI).

2.2.4.2 IC_{50} determination

Herbal products with an initial concentration of 100 µg/ml were serially diluted in a 1:3 ratio. As in the two-point screening, each well contained 40 µl of the herbal product, solvent, or positive control, and 50 µl of a pre-mixture containing the recombinant CYP isoform and regenerating system diluted in reaction buffer. The plates were pre-warmed for 15 minutes at 37°C before a pre-reading to determine the potential background fluorescence of herbal extracts.

The reaction was initiated by the addition of 10 µl of the appropriate Vivid® substrate and $NADP^+$ into each well and the plates were incubated for 30 minutes at 37 °C. The reaction was terminated by the addition of 10 µl 20% TRIS base in acetonitrile and the enzyme activity was monitored by measuring the formation of fluorescent metabolites.

2.2.4.3 Time - dependent inhibition screening-normalized ratio

Time - dependent inhibition (TDI) screening of CYP3A4 and CYP1A2 was conducted using a protocol adapted from Yamamoto *et al.* (2002) and Thelingwani *et al.* (2009). Enzyme activity at two concentrations in either the presence or absence of $NADP^+$ was measured.

Each well contained 40 µl of a high or low concentration of herbal product (10 µg/ml, 100 µg/ml), positive control (2.5 µM, 25 µM erythromycin), or negative control (2.5 µM, 25 µM ketoconazole), and 50 µl of a pre-mixture that contained either the recombinant CYP isoform, regenerating system, reaction buffer including $NADP^+$, or a pre-mixture containing the same constituents without $NADP^+$. The plates were pre-warmed for 30 minutes at 37 °C and the reaction was initiated by the addition of 10 µl of the appropriate Vivid® substrate and $NADP^+$ thereafter. The plates were further incubated for 30 minutes at 37 °C. The reaction was terminated by the addition of 10 µl 20% TRIS base in acetonitrile and the enzyme activity was monitored by measuring the formation of fluorescent metabolites at 406nm/460nm excitation/emission spectra. The time-dependent inhibitory effect of each herbal product was expressed as the normalized ratio as shown in the equation below:

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

Where $R + 1^{NADP+}$ is rate of the reaction in the presence of an inhibitor (herbal product, positive, or negative control) and $NADP^+$, and $R - 1^{NADP+}$ is the rate of the reaction without an inhibitor in the presence of $NADP^+$; $R + 1^{-NADP+}$ is rate of the reaction in the presence of an inhibitor and absence of $NADP^+$, and $R - 1^{-NADP+}$ is the rate of the reaction in the in the absence of both inhibitor and $NADP^+$. As described by Atkinson *et al. (*2005), a normalized ratio value below 0.7 indicates clear TDI, a value between 0.7 and 0.9 indicates an intermediate TDI, and a value above 0.9 does not cause TDI.

2.2.4.4 Kinetic TDI of CYP3A4

A kinetic TDI assay was conducted on CYP3A4 to determine the change in IC_{50} values over time. The assay was conducted using a protocol adapted from Krippendorff *et al.* (2009). The herbal products, positive control, and negative control were pre-warmed at 37 °C with $NADP^+$ and regenerating system for 15 minutes. The reaction was initiated by adding a pre-warmed (37 °C) enzyme and substrate mixture. The microplate was placed in the plate reader and fluorescence was measure at 5-minute intervals for 30 minutes.

2.2.5 Determination of IC_{50}

Data was exported to an Excel spreadsheet (Microsoft, USA) where the signal-to-noise ratio and percentage residual activity were calculated. To determine the IC_{50} value – the concentration of inhibitor required to inhibit 50% of enzyme activity – the percentage residual activity was plotted against the log-transformed concentrations of the herbal product or the positive control. A sigmoidal curve was fitted using non-linear regression and the IC_{50} value was calculated using GraphPad® Prism (GraphPad Software Inc., San Diego, CA).

2.2.6 Statistical analysis

All data were expressed as mean ± SD. An unpaired t-test was used to determine the significance of enzyme activity in the presence and absence of $NADP^+$, where a p - value < 0.05 indicated statistical significance.

CHAPTER THREE

RESULTS

3.1 Extraction yield of herbal products

The calculated yields of the 60% methanolic extracts of the herbal products are shown in table 3.1. The table shows a high extraction yield for Buchu than the other products. This may be because the Buchu capsules contained approximately 370 mg of pure leaf material, while the other products contained 100 mg of leaf (*Warburgia salutaris*) or root and rhizome material (African ginger), along with excipients such as binders and fillers.

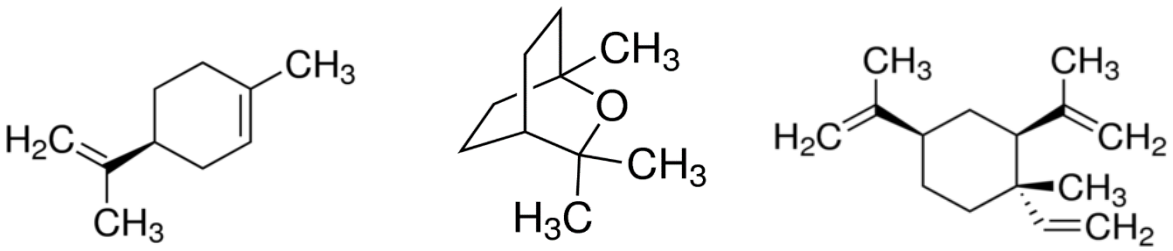
Table 3.1: Extraction yield of herbal products

Herbal products	**Yield (% $^{w}/_{w}$)**
Buchu	12.84%
African ginger	1.55%
Warburgia salutaris	4.47%

3.2 GCMS analysis of herbal products

3.2.1 Major components in African ginger

The gas chromatogram (GC) profile of the solvent extract of African ginger is shown in figure 3.2. 33 compounds were detected in the volatile component of African ginger and are tentatively identified in table 3.2. 19.47% of the compounds were monoterpene hydrocarbons and 8.28% of the compounds were sesquiterpenes hydrocarbons. The major compounds present in the volatile component of African ginger were limonene (3.28%), eucalyptol, (3.10%), and β-elemene (3.37%) (figure 3.1).



Limonene Eucalyptol β-elemene

Figure 3.1: The major compounds present in the volatile component of African ginger.

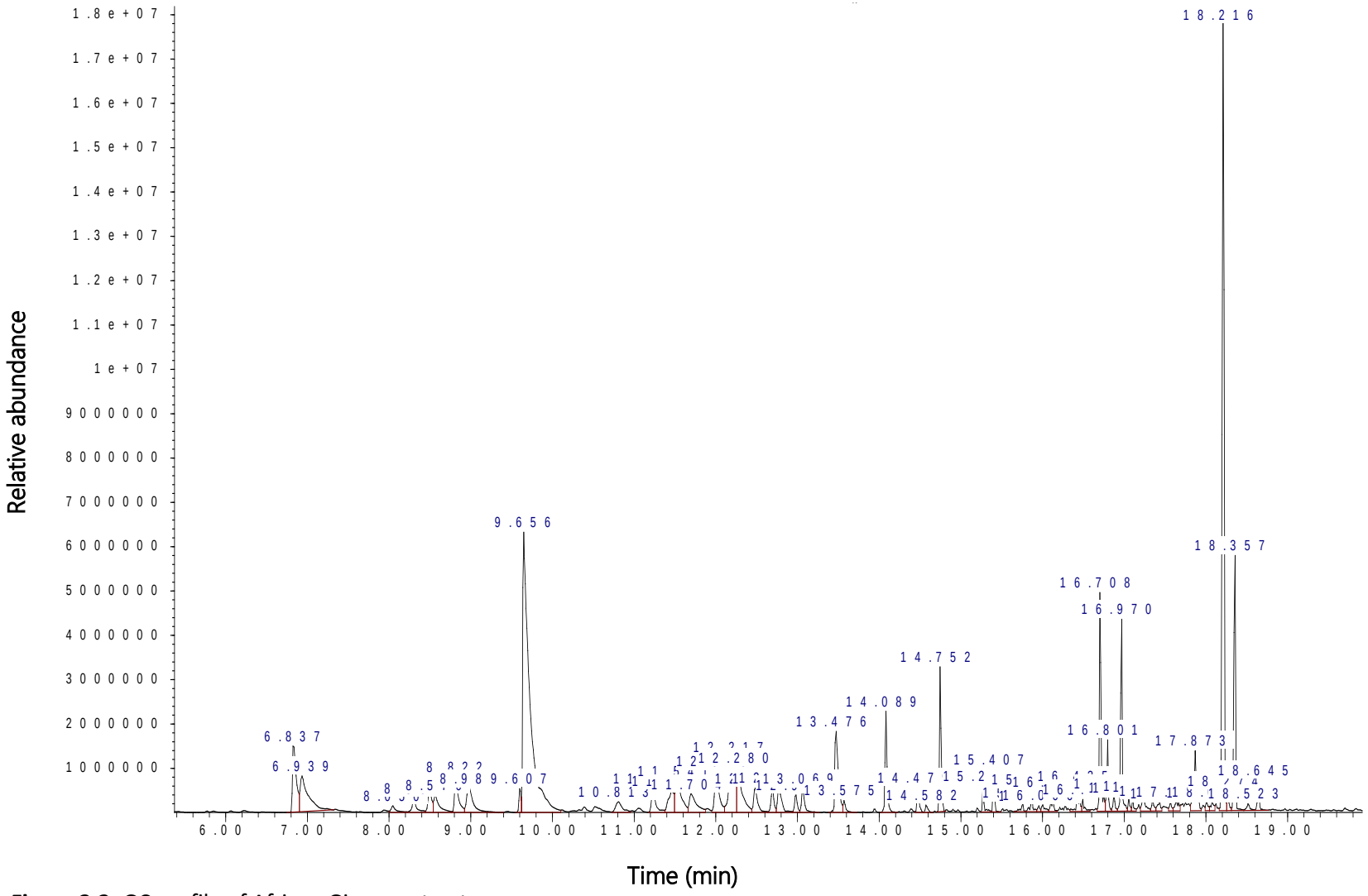


Figure 3.2: GC profile of African Ginger extract.

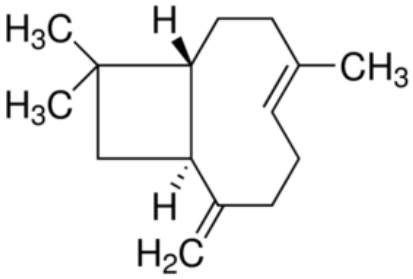
Table 3.2: Volatile components of African ginger extract

RRI	Compound	Quality %	Peak area %
903	Trimethylphosphine	33	3.22
962	α-Thujene	94	1.04
998	α-Pinene	97	0.45
1035	β-pinene	94	0.77
1054	β-pinene	97	1.21
1077	**L-limonene**	**93**	**3.28**
1079	p-cymene	95	2.65
1085	Trans-β-ocimene	95	1.07
1094	Sabinene	94	1.13
1105	γ-terpinene	95	0.82
1128	**Eucalyptol**	**99**	**3.10**
1133	α-terpinene	97	0.33
1162	Allo-ocimene	98	2.18
1184	Allo-ocimene	98	0.57
1290	1,3,5-p-menthatriene	95	0.19
1290	6-methylthio[1]benzothieno[2,3-c]quinoline	72	0.25
1310	9H-pyrrolo[3',4':3,4]pyrrolo[2,1-a]phthalazine-9,11(10H)-dione,10-ethyl-8-phenyl	72	0.13
1330	Terpinen-4-ol	96	0.45
1367	Pulegone	50	0.69
1412	(+) Cycloisosativene	92	1.20
1424	3-(3,4-dimethoxyphenyl)-6,7-dimethoxy-1-methyl-isochromene	72	0.37
1436	**β-elemene**	**95**	**3.37**
1473	Trans - α- bergamotene	90	0.58
1488	β-caryophyllene	99	0.30
-	2-hydroxymandelic acid	59	0.21
-	α- cadinene	98	0.39
-	β-selinene	99	1.82
-	γ- cadinene	83	0.23
-	β-sesquiphellandrene	45	0.29
-	Eudesma-3,7 (11) -dien	96	0.43
-	Pyrimidine, 2-(4-nitro-3-thienyl)	59	4.71
-	Germacrene B	96	0.24
-	Epi-ligulyl oxide	80	0.64

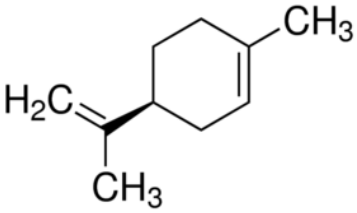
RRI = Relative Retention Index

3.2.2 Major components in *Warburgia salutaris*

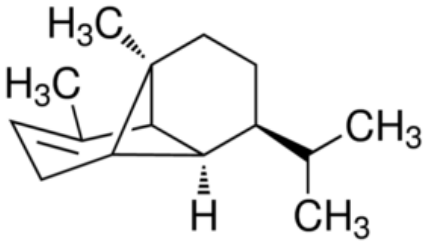
The GC profile of the solvent extract of *Warburgia salutaris* is shown in figure 3.4. 30 compounds were detected in the volatile component of *Warburgia salutaris* and are tentatively identified in table 3.3. 12.72% of the compounds were monoterpene hydrocarbons and 37.34% of the compounds were sesquiterpenes hydrocarbons. The major compounds present in the volatile component of *Warburgia salutaris* were limonene (6.66%), α-copaene (5.98%), and β-caryophylline (6.96%) (figure 3.3).



β-caryophylline



Limonene



α-copaene

Figure 3.3: The major compound present in the volatile component of *Warburgia salutaris.*

Table 3.3: Volatile components of *Warburgia salutaris* extract

RRI	Compound	Quality %	Peak area %
907	Trimethylphosphine	33	1.37
1014	3,4,4-trimethyl-1,2,6-oxadiazine N-oxide	25	2.01
1020	Myrcene	95	3.68
1038	Dimethylphosphorylmethane	9	8.54
1051	**L-limonene**	**98**	**6.66**
1096	Trans-β-ocimene	96	1.03
1177	α-terpinolene	96	0.28
1188	Thieno [2,3-b] pyridine 7-oxide	27	1.43
1192	N-Formylnorisosalutaridine	43	0.65
1207	α-cubebene	99	1.73
1212	**α-copaene**	**99**	**5.98**
1214	β-bourbonene	90	1.72
1219	Camphene	91	1.06
1226	Urocanic acid	53	0.41
1231	Germacrene D	93	0.80
1233	β-cubebene	91	1.80
1242	**β-caryophyllene**	**99**	**6.96**
1245	Trans-β-farnesene	96	5.00
1257	Humulene	99	6.48
1267	α-cadinene	96	1.90
1269	α-cadinene	99	1.63
1273	Neryl Acetone	11	0.44
1297	δ-cadinene	99	2.10
1310	l-calamanene	98	0.43
1330	Farnesol	81	0.49
1367	Isopropyl laurate	72	0.61
1374	1,Z-5,E-7-Dodecatriene	60	0.92
-	Tetradecane	91	0.33
-	Trans-3,4-Dimethyl-1-cyclohexenecarboxaldehyde	58	0.49
-	9-Isopropylenyl-biclyclonanane	49	0.39

RRI = Relative Retention Index

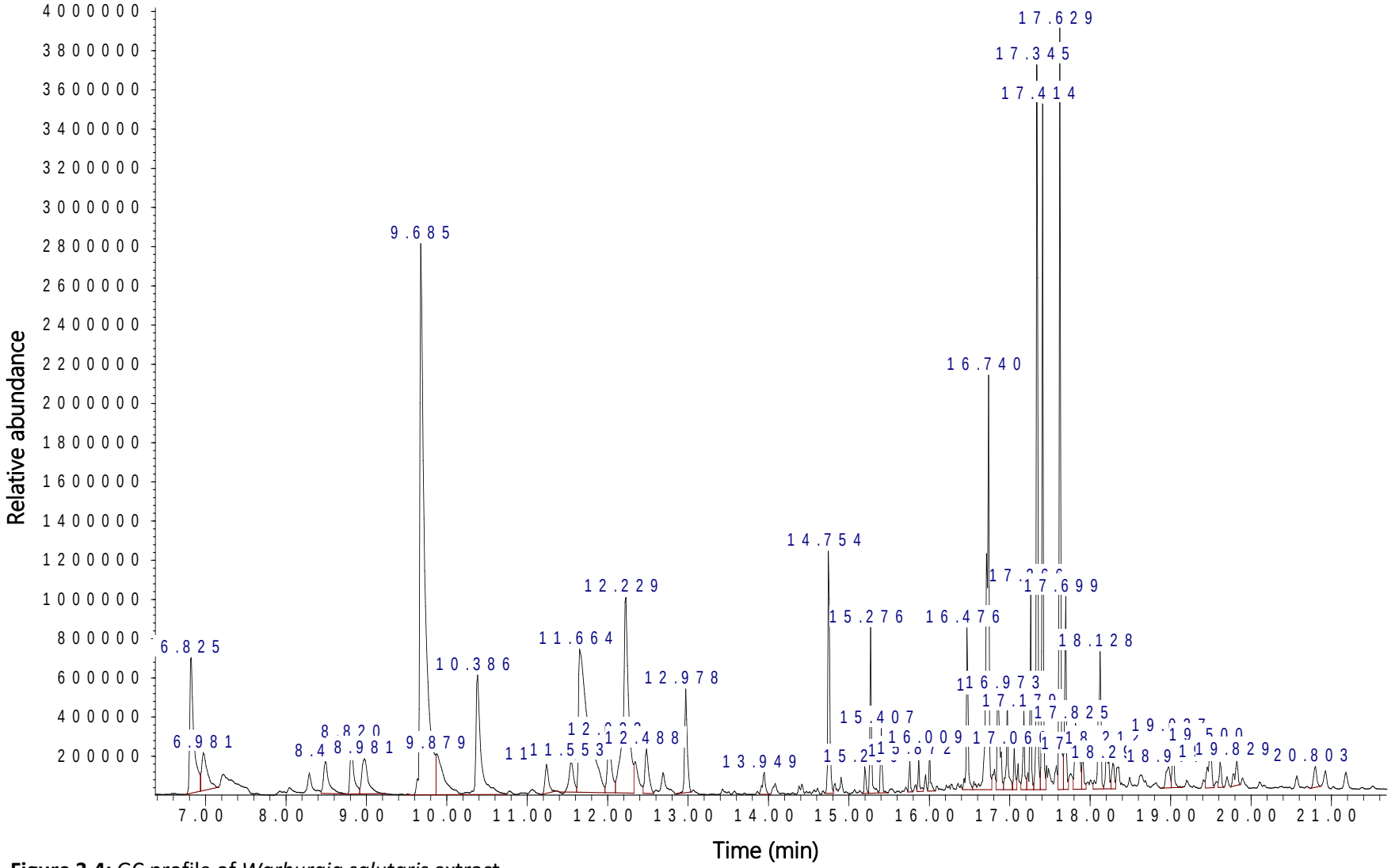


Figure 3.4: GC profile of *Warburgia salutaris* extract.

3.2.3 Major components in Buchu

The GC profile of the solvent extract of Buchu is shown in figure 3.6. 49 compounds were revealed in the volatile component and are tentatively identified in table 3.4. The majority of compounds (94.78%) were monoterpene hydrocarbons. The remaining 1.15% of compounds were sesquiterpenes hydrocarbons and a few alkaloids. The major compounds present in the volatile component of Buchu include l-limonene (45.10%), menthone (17.42%), pulegone (7.97%), and myrcene (7.04%) (figure 3.5).

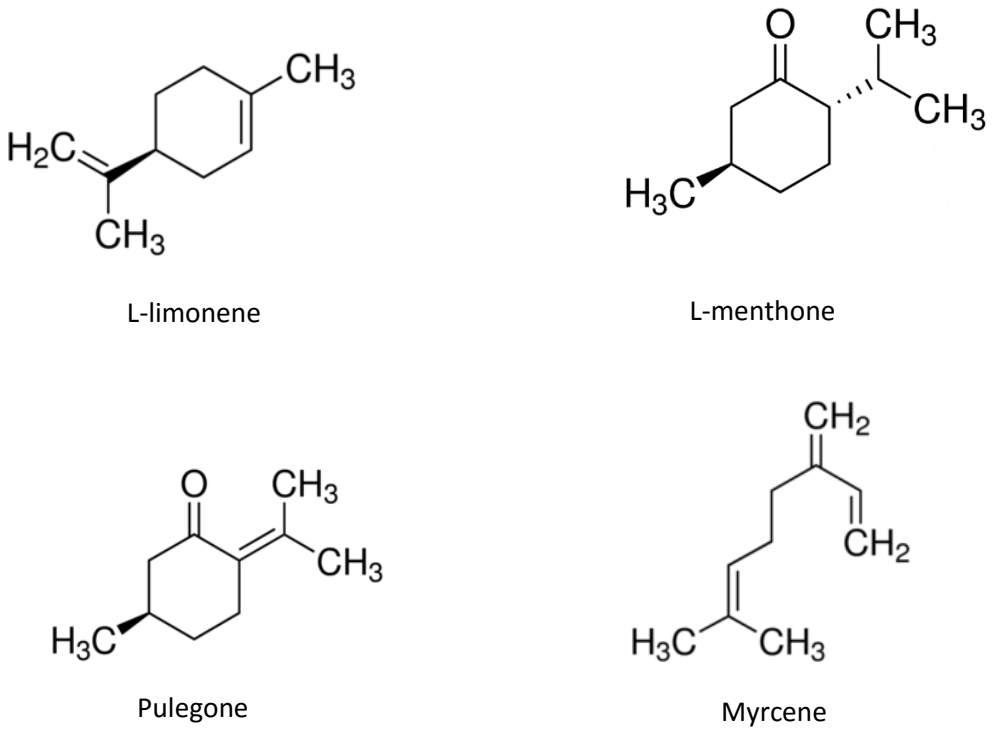


Figure 3.5: The major compounds present in the volatile component of Buchu.

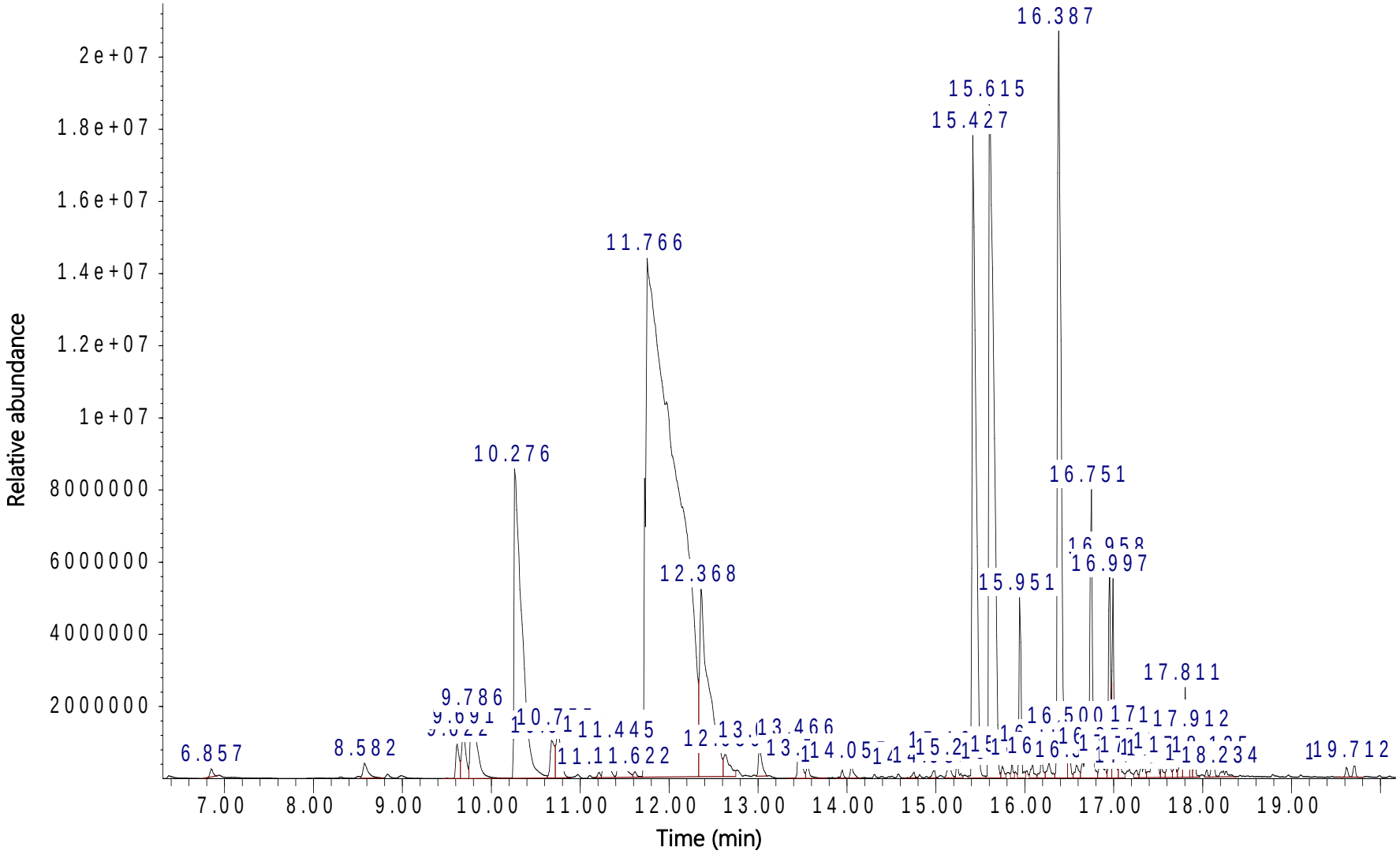


Figure 3.6: GC profile of Buchu capsule extract.

Table 3.4: Volatile components of Buchu extract

RRI	Compound	Quality %	Peak area %
961	α-phellandrene	91	0.23
997	α-pinene	97	0.32
1003	α-pinene	97	1.33
1018	**Myrcene**	**97**	**7.04**
1030	Sabinene	96	0.44
1032	Sabinene	96	0.74
1046	β-pinene	96	0.04
1048	β-pinene	97	0.52
1053	3,3-Dimethyl-1-phenyltriazene	9	0.52
1059	Camphene	72	0.05
1063	**L-limonene**	**98**	**45.10**
1081	Trans-β-ocimene	90	5.24
1089	β-phellandrene	86	0.42
1102	γ-terpinene	96	0.29
1128	Eucalyptol	99	0.41
1132	Terpinolene	97	0.15
1154	(E)-4,8-Dimethyl-1,3,7-nonatriene	53	0.06
1161	p-cymene	95	0.12
1221	2-(2-Nitro-2-propenyl)cyclohexanone	59	0.07
1237	Linalool	97	0.18
1244	Cryogenine	27	0.13
1261	**l-menthone**	**99**	**7.23**
1278	**l-menthone**	**98**	**10.19**
1324	Trans-isopulegone	96	1.06
1356	Terpinen-4-ol	92	0.13
1379	Trans-dihydrocarvone	97	0.19
1349	Exo-2-Methyl-4-homobrendane	64	0.19
1361	**Pulegone**	**96**	**7.97**
1376	1-Ethyl-2-propylcyclohexane	30	0.33
1386	Citronellene	53	0.15
1405	Diosphenol (Buchu camphor)	89	2.53
1419	Anabasine	40	0.29
1434	Diosphenol (Buchu camphor)	95	1.24
1439	Myrtanyl acetate	38	1.11
1451	1,6-Dimethyl-3-piperidinone	53	0.17
1476	1-(4'-pyridyl)-1-methylcyclopentane	64	0.07
1483	8-Hydroxy-δ-4(5)-p-menthen-3-one	59	0.17
1500	Myrtanyl acetate	90	0.58
-	10,10-dimethyl-1,11-dehydro-bicyclo [6.3.0(1,8).0(2,6)] undecanone-3	64	0.09
-	(5Z)-5-ethylidene-1-methylcycloheptene;	78	0.17
-	γ-muurolene	94	0.14
-	8-mercaptomenthone	97	0.28
-	8-mercaptomenthone	95	0.62

-	β-selinene	89	0.09
-	Bicyclogermacrene	98	0.24
-	Trans-γ-bisabolene	93	0.05
-	δ-cadinene	99	0.15
-	Spathulenol	81	0.07
-	(2'-nitro)-1-isopropyl-4-methyl-cyclohexanone	72	0.09

RRI = Relative Retention Index

3.3 Inhibition assays

The potential of the selected herbal products to inhibit the enzyme activity of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 was investigated. Initially, reversible and irreversible inhibition was conducted on the specific recombinant CYP isoforms. CYP1A2, CYP2C9, and CYP2D6 resulted in a low signal to noise ratio (< 5.0) and were therefore excluded from IC_{50} determination to avoid analytical errors. In 1% of the total assay volume, methanol decreased CYP3A4 activity by 8%, and CYP2C19 activity by 2%.

3.3.1 Two - point screening

Extracts of the herbal products were screened at two concentrations (10 µg/ml and 100 µg/ml) and the observed activity was expressed as percentages of the negative control (no inhibitor). All herbal products inhibited CYP2C19 in a concentration dependent manner (figure 3.7). *Warburgia salutaris* showed the strongest inhibition, reducing CYP2C19 enzyme activity to less than 20% at high concentrations and less than 50% at low concentrations. CYP3A4 activity was also reduced to less than 50% at low concentration by *Warburgia salutaris* and less than 30% at low concentrations by African ginger, indicating strong inhibition (figure 3.7). The positive controls, ketoconazole (CYP3A4) and miconazole (CYP2C19), both reduced enzyme activity to less than 25%. The observed inhibition was not concentration dependent.

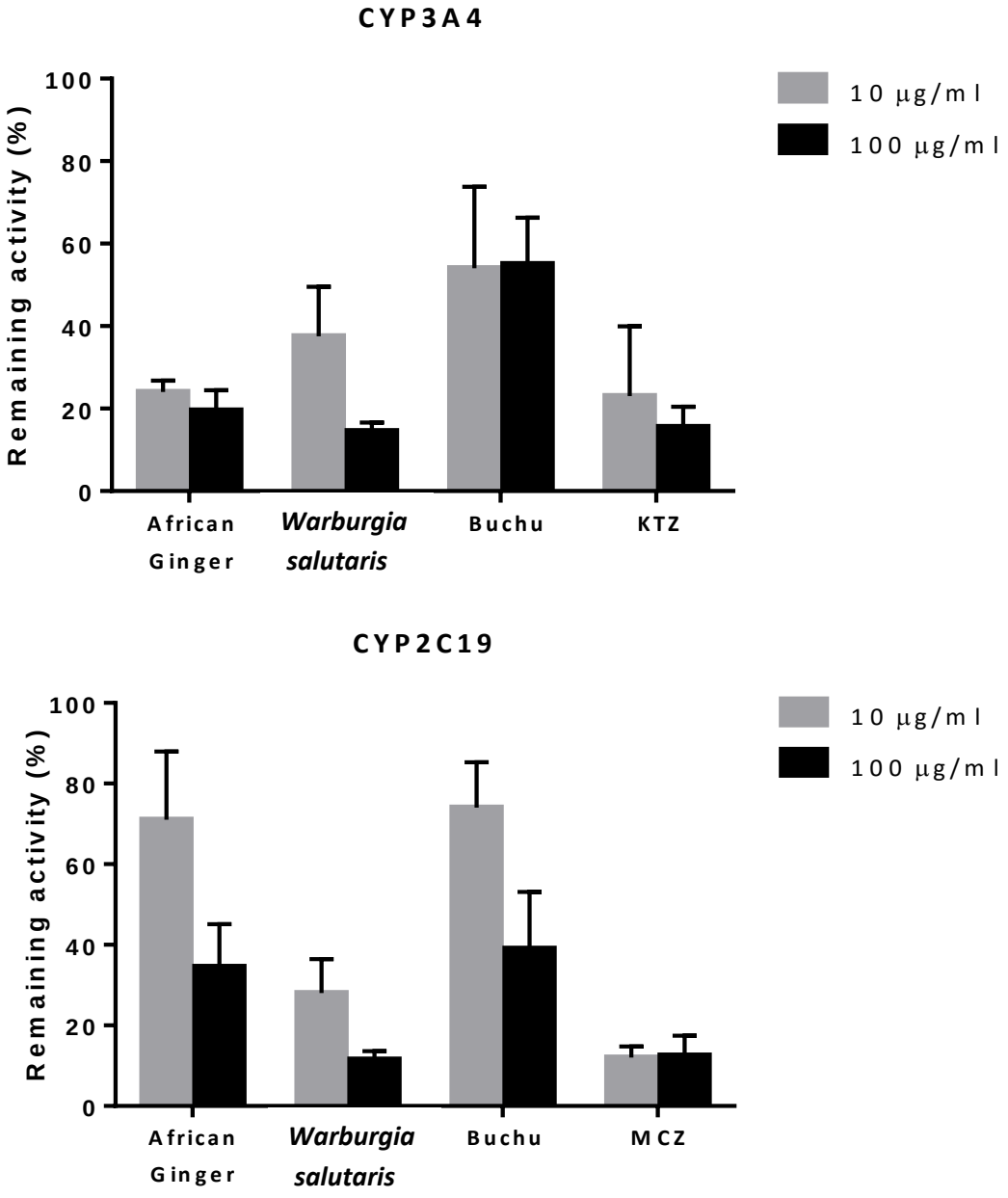


Figure 3.7: Two - point screening of herbal products at 10 µg/ml and 100 µg/ml. Miconazole (MCZ, 3.33 and 30 µM) and ketoconazole (KTZ, 0.06 and 0.05 µM) and were used as positive controls for CYP2C19 and CYP3A4, respectively.

3.3.2 IC_{50} determination

Warburgia salutaris, African ginger, and Buchu inhibited CYP2C19 with IC_{50} values of 5.88 µg/ml, 32.38 µg/ml, and 53.52 µg/ml, respectively. Likewise, CYP3A4 was inhibited by *Warburgia salutaris,* African ginger, and Buchu with IC_{50} values of 5.64 µg/ml, 1.09 µg/ml, and > 100 µg/ml, respectively. The dose-response curves from which the IC_{50} values were calculated are shown in figures 3.8 and 3.9.

The IC_{50} values for the known inhibitors, 0.075 ± 0.036 µM (miconazole, CYP2C19) and 0.028 ± 0.011 µM (ketoconazole, CYP3A4) were in agreement with literature - reported values (table 3.5) and confirm the validity of the assay.

Table 3.5: Literature reported IC_{50} values of positive controls

Positive control	Determined IC_{50} values	Reported IC_{50} values	Literature reference
Ketoconazole (CYP 3A4)	0.028 ± 0.011 µM	0.018 ± 0.003 µM[1]	Cohen *et al.,* 2003
		0.1 µM	Zambon *et al.,* 2015
		0.083 ± 0.025 µM[2]	Crespi *et al.,* 1997
		0.22 µM	Wu *et al.,* 2003
Miconazole (CYP2C19)	0.075 ± 0.036 µM	0.33 µM	Niwa *et al.,* 2005
		0.07 µM	Zambon *et al.,* 2015
		0.04 µM	McGinnity *et al.,* 2005
		0.02 – 0.5 µM	Peet *et al.,* 2011

[1] Mean ± SD
[2] Mean ± SEM

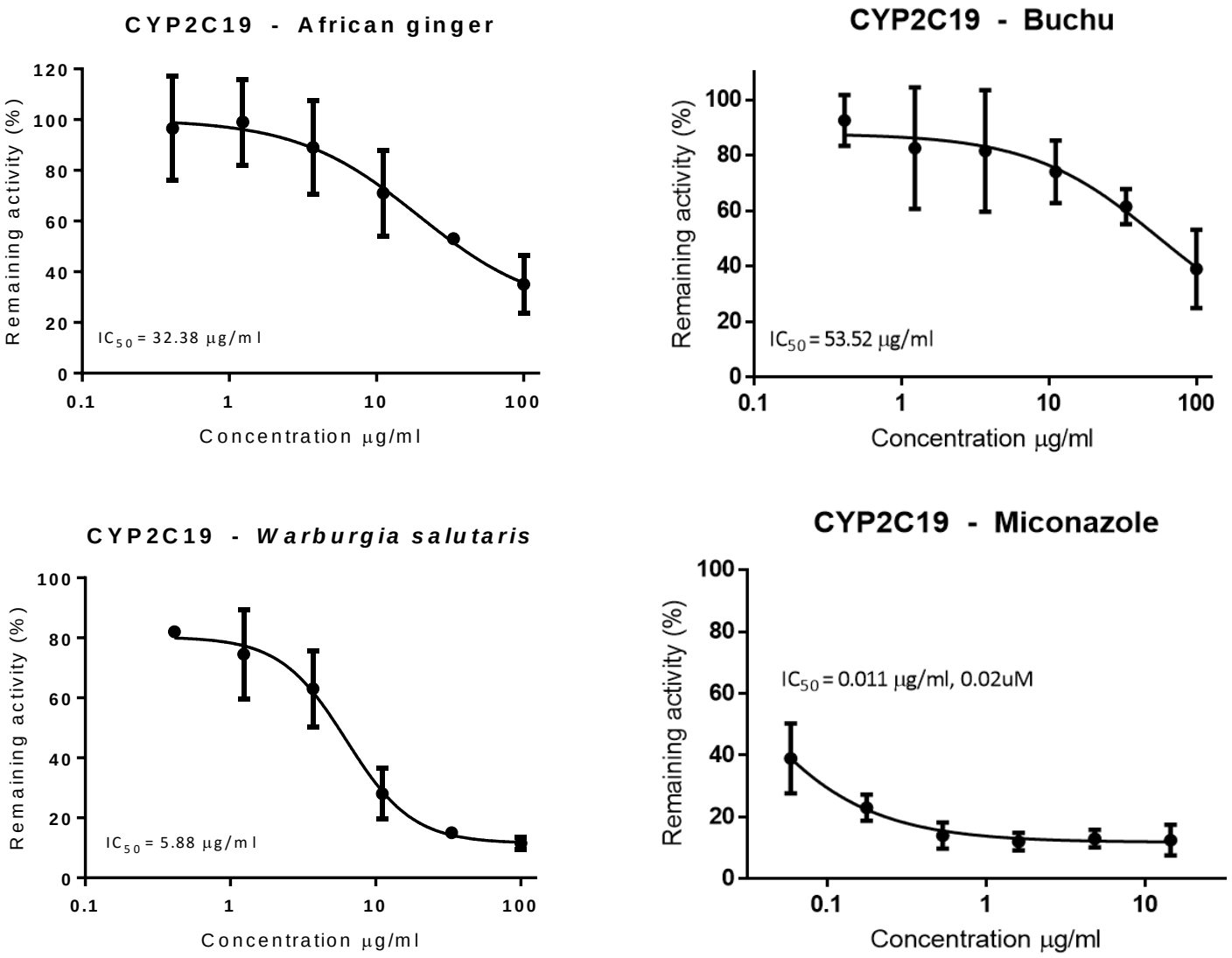


Figure 3.8: The profile of CYP2C19 enzyme activity in the presence of African ginger, Buchu, *Warburgia salutaris,* and the positive control, miconazole. Data is expressed as Mean ± SD, n = 2.

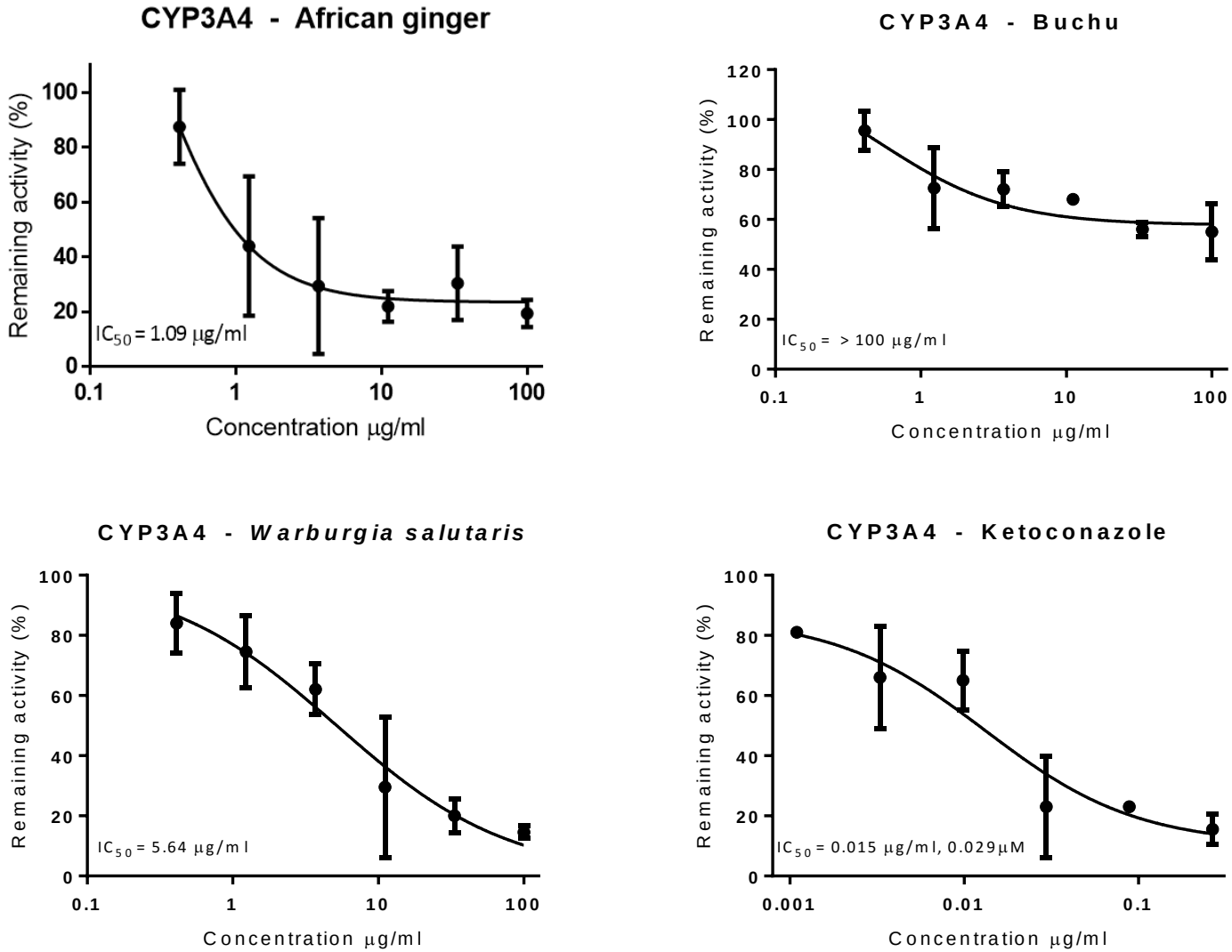


Figure 3.9: The profile of CYP3A4 enzyme activity in the presence of African ginger, Buchu, *Warburgia salutaris,* and the positive control, miconazole. Data is expressed as Mean ± SD, n = 2.

3.4 TDI by normalized ratio

Time - dependent inhibition was investigated for CYP1A2 and CYP3A4. By applying the normalized ratio, the various herbal products were categorized into clear TDI (≤0.70), intermediate TDI (0.7 - 0.9), and non-TDI (> 0.9) (figure 3.10A and 3.10B). African ginger showed clear TDI of CYP1A2 that was not concentration dependent. *Warburgia salutaris* showed clear TDI at 100 µg/ml and no TDI of CYP1A2 at 10 µg/ml and Buchu showed intermediate TDI that was not concentration dependent. The negative control for CYP1A2, α -naphthoflavone (αNP), showed no TDI potential as it is not a TDI inhibitor. An established TDI inhibitor for CYP1A2, furafylline (FFL), served as the positive control and showed clear TDI that was not concentration dependent.

African ginger and *Warburgia salutaris* both showed clear TDI of CYP3A4 at 10 µg/ml, but not at 100 µg/ml. Buchu showed TDI of CYP3A4 that was not concentration dependent. Ketoconazole (KTZ), used as negative control, showed no TDI, and erythromycin (EMC), used as positive control, showed intermediate TDI.

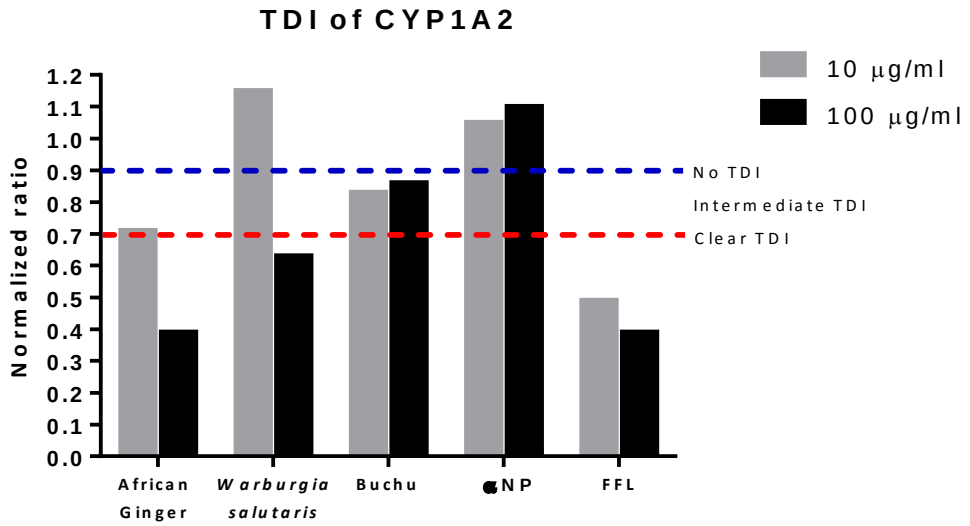


Figure 3.10A: The TDI classification of herbal products. Furafylline (FFL, 12.5 µM and 25 µM) served as a positive control. α-naphthoflavone (αNP, 12.5 µM and 25 µM) served as a negative control.

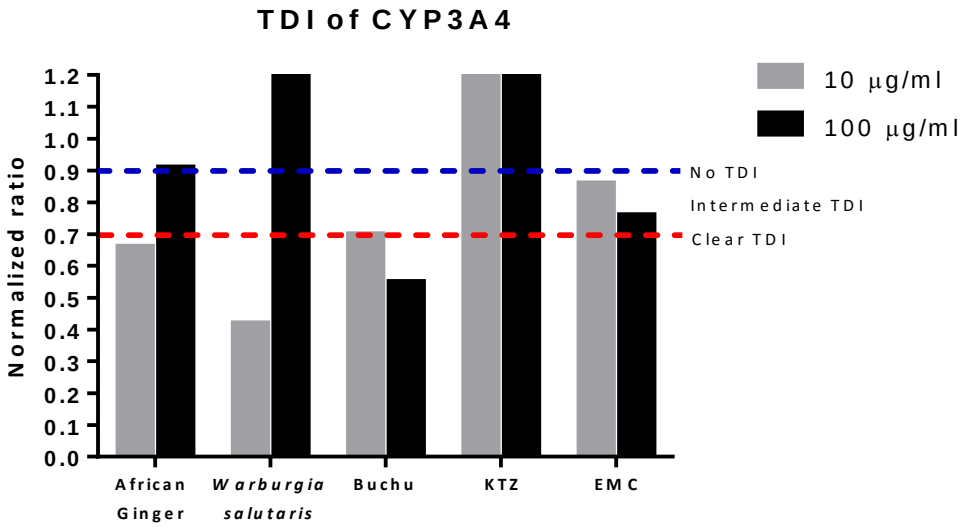


Figure 3.10B**:** The TDI classification of herbal products. Erythromycin (EMC, 12.5 µM and 25 µM) served as a positive control. Ketoconazole (KTZ, 12.5 µM and 25 µM) served as a negative control.

3.5 $NADP^+$ - dependent inhibition

To determine whether the observed inhibition was $NADP^+$ - dependent, the enzyme activity of CYP1A2 and CYP3A4 was determined in the presence and absence of $NADP^+$. At 100 µg/ml, African ginger and the positive control, furafylline displayed $NADP^+$ - dependent inhibition (figure 3.11A). No other extracts, including those at 10 µg/ml, inhibited CYP1A2 in an $NADP^+$ - dependent manner. CYP3A4 was inhibited by Buchu in an $NADP^+$ dependent manner. The inhibition observed by *Warburgia salutaris* dependent on concentration but not $NADP^+$ (figure 3.11B).

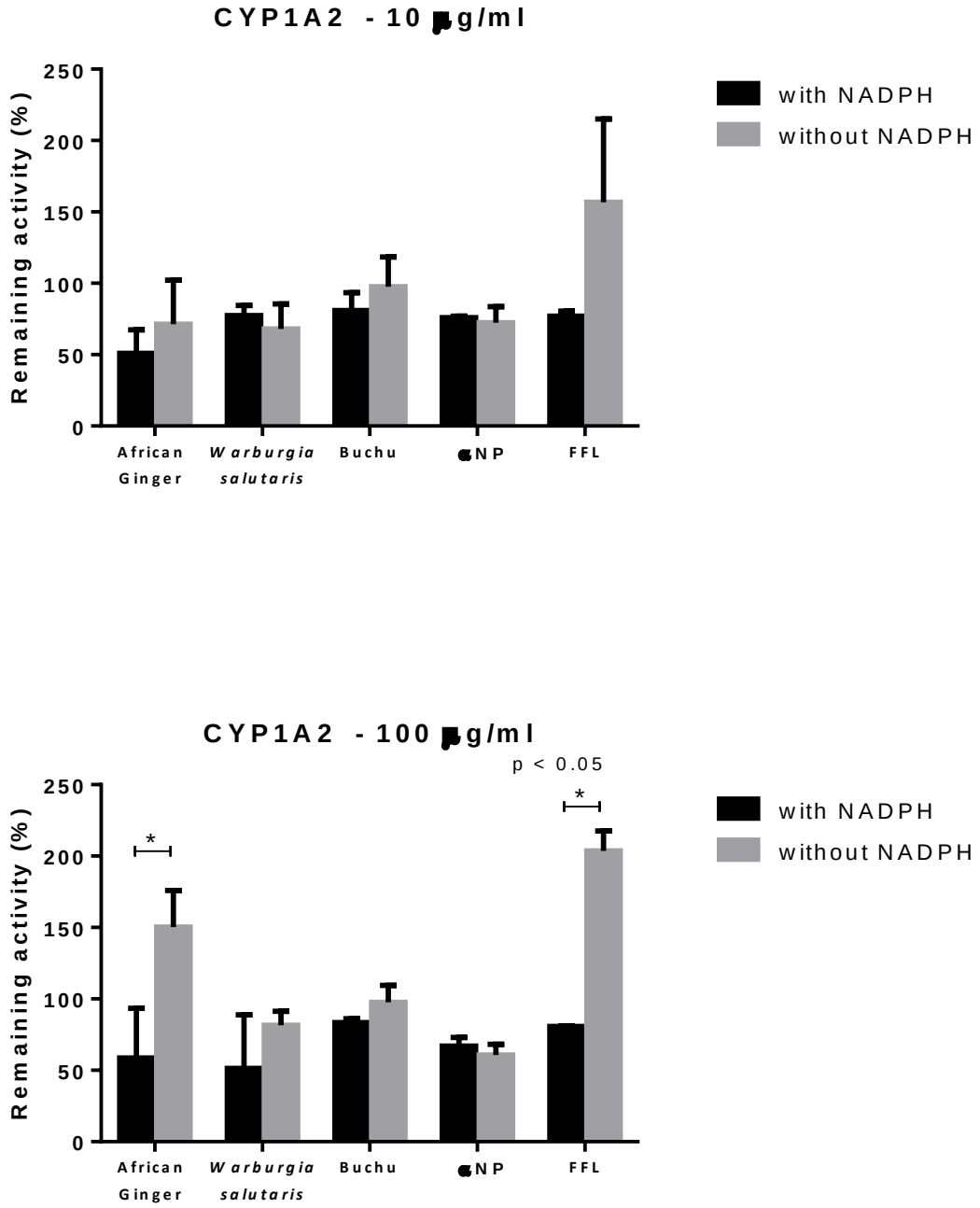


Figure 3.11A: The inhibitory effects of herbal extracts on CYP1A2 after a 30-minute pre-incubation in the presence or absence of $NADP^+$. Data is expressed as Mean ± SD, n = 3. An un-paired t-test was used to compare the percentage remaining activity for each herbal product, positive (furafylline, FFL), or negative control (α-naphthoflavone, αNP), where $p < 0.05$ was considered significant.

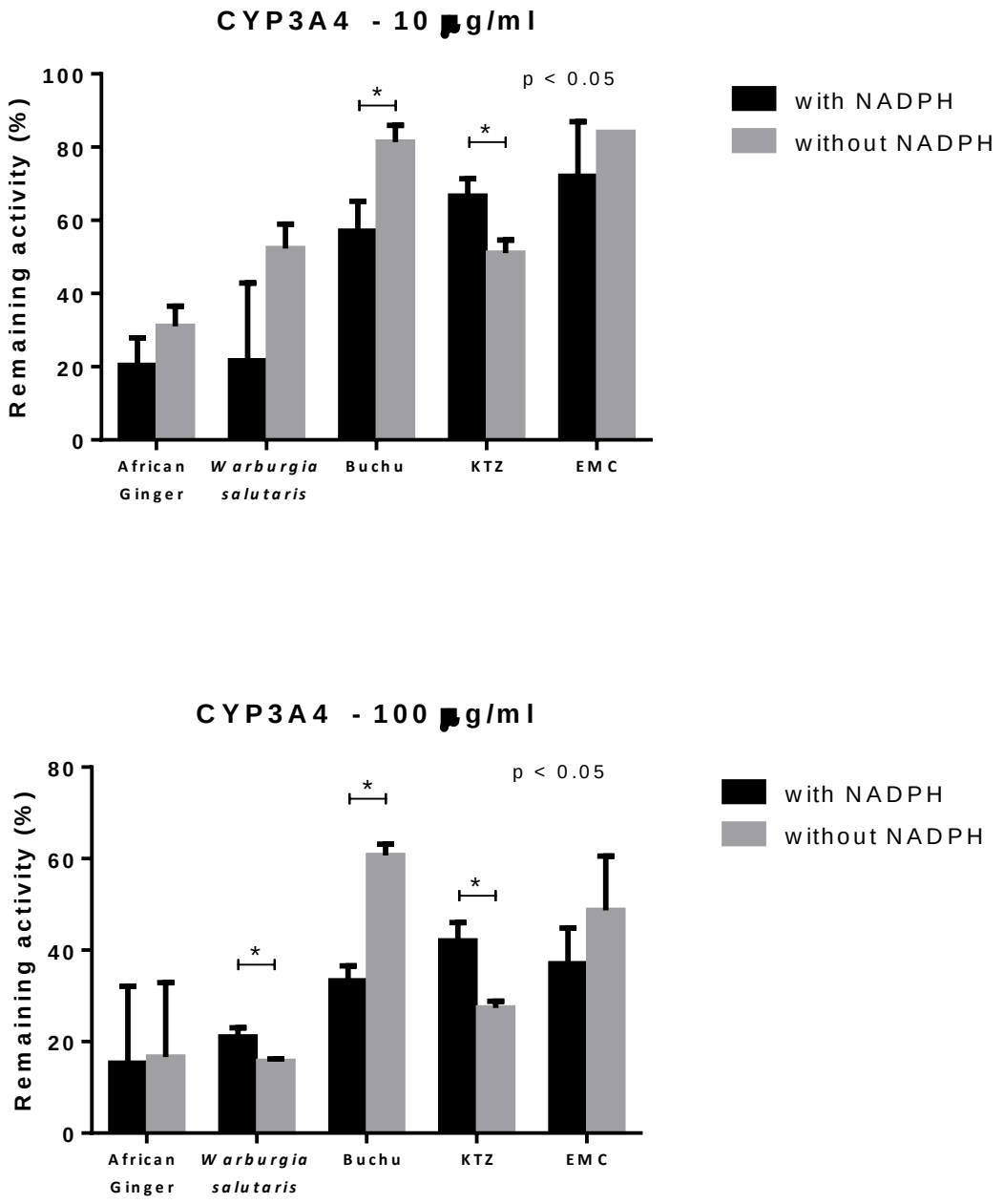


Figure 3.11B: The inhibitory effects of herbal extracts on CYP3A4 after a 30-minute pre-incubation in the presence or absence of $NADP^+$. Data is expressed as Mean ± SD, n = 3. An unpaired t-test was used to compare the percentage remaining activity for each herbal product, positive (erythromycin, EMC), or negative control (ketoconazole, KTZ) where $p < 0.05$ was considered significant.

3.6 Kinetic time-dependent inhibition

The inhibition of CYP3A4 by African ginger and by the positive control, erythromycin, appears to be time dependent, as the IC_{50} values of both inhibitors decreased over the 30 - minute incubation time (Figures 3.12A and 3.12E). The IC_{50} values of Buchu, *Warburgia salutaris,* and ketoconazole, the negative control, increased over time, indicating that the inhibition caused by these inhibitors is not time dependent (Figures 3.12B, 3.12C, and 3.12D). Figures 3.12A, 3.12B, 3.12C, 3.12D, and 3.12E, show the concentration-dependency of the inhibition obtained over a 30-minute period and figures 3.13A, 3.13B, 3.13C, 3.13D and 3.13E, show the IC_{50} values obtained at various incubation times.

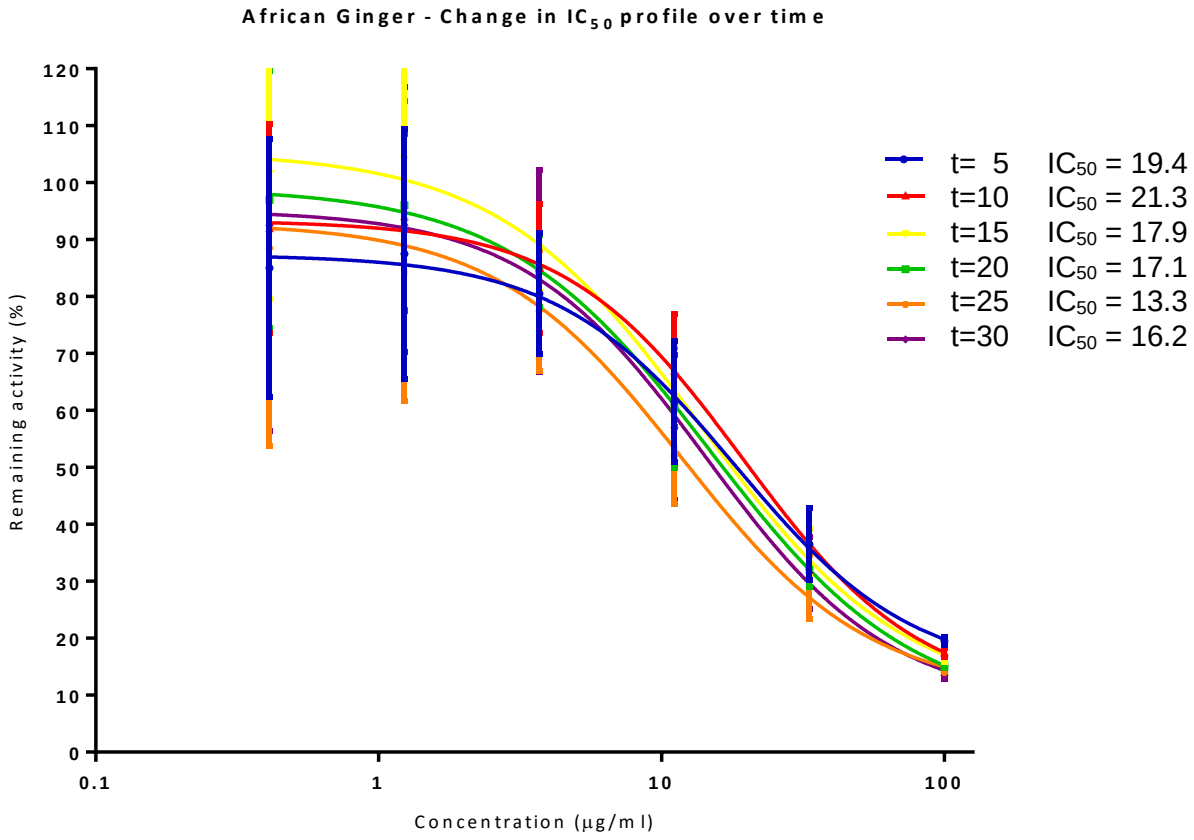


Figure 3.12A: The concentration-dependency of the inhibition of CYP3A4 by African ginger obtained at various incubation times. Data is expressed as Mean ± SD, n = 2.

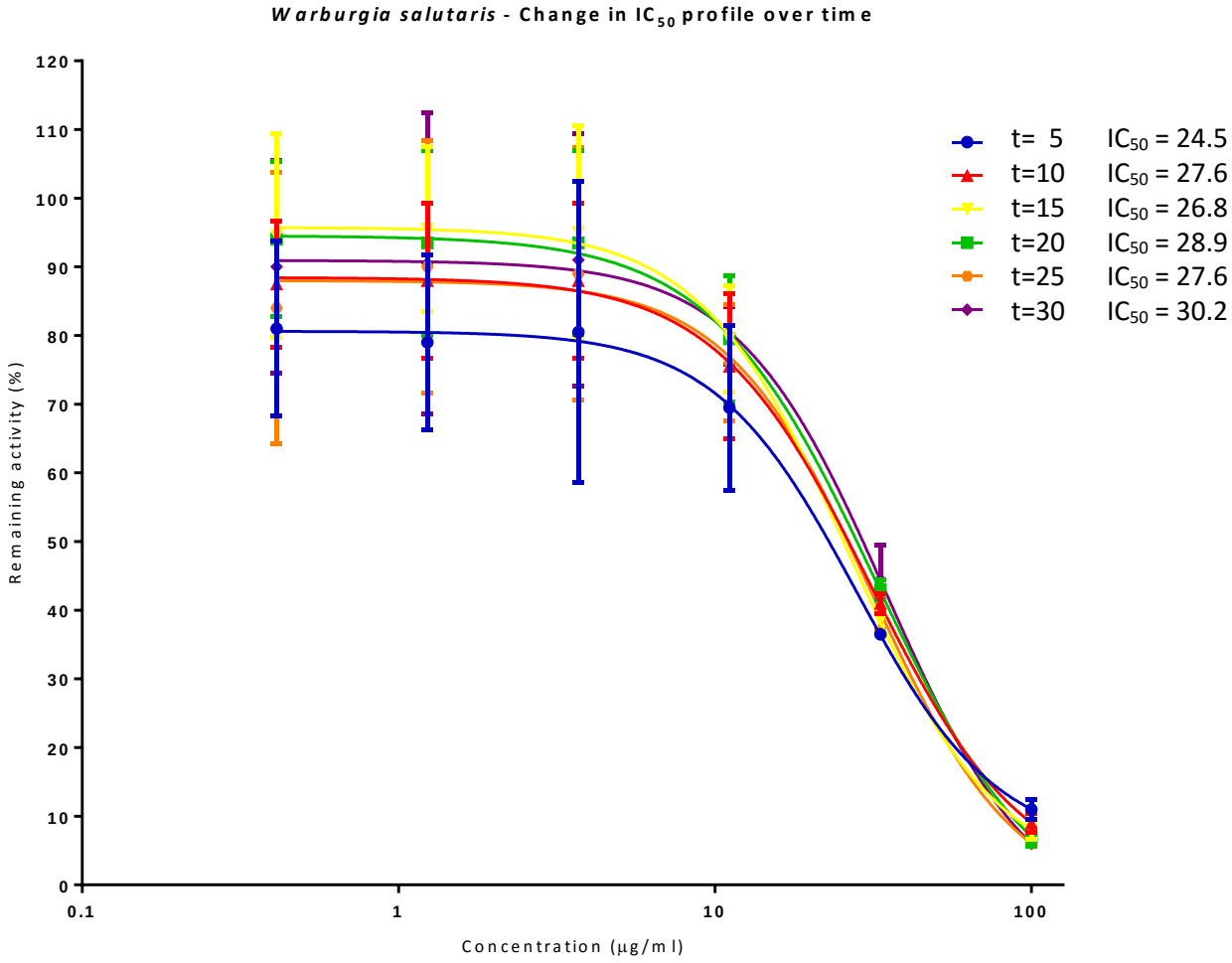


Figure 3.11B: The concentration-dependency of the inhibition of CYP3A4 by *Warburgia salutaris* obtained at various incubation times. Data is expressed as Mean ± SD, n = 2.

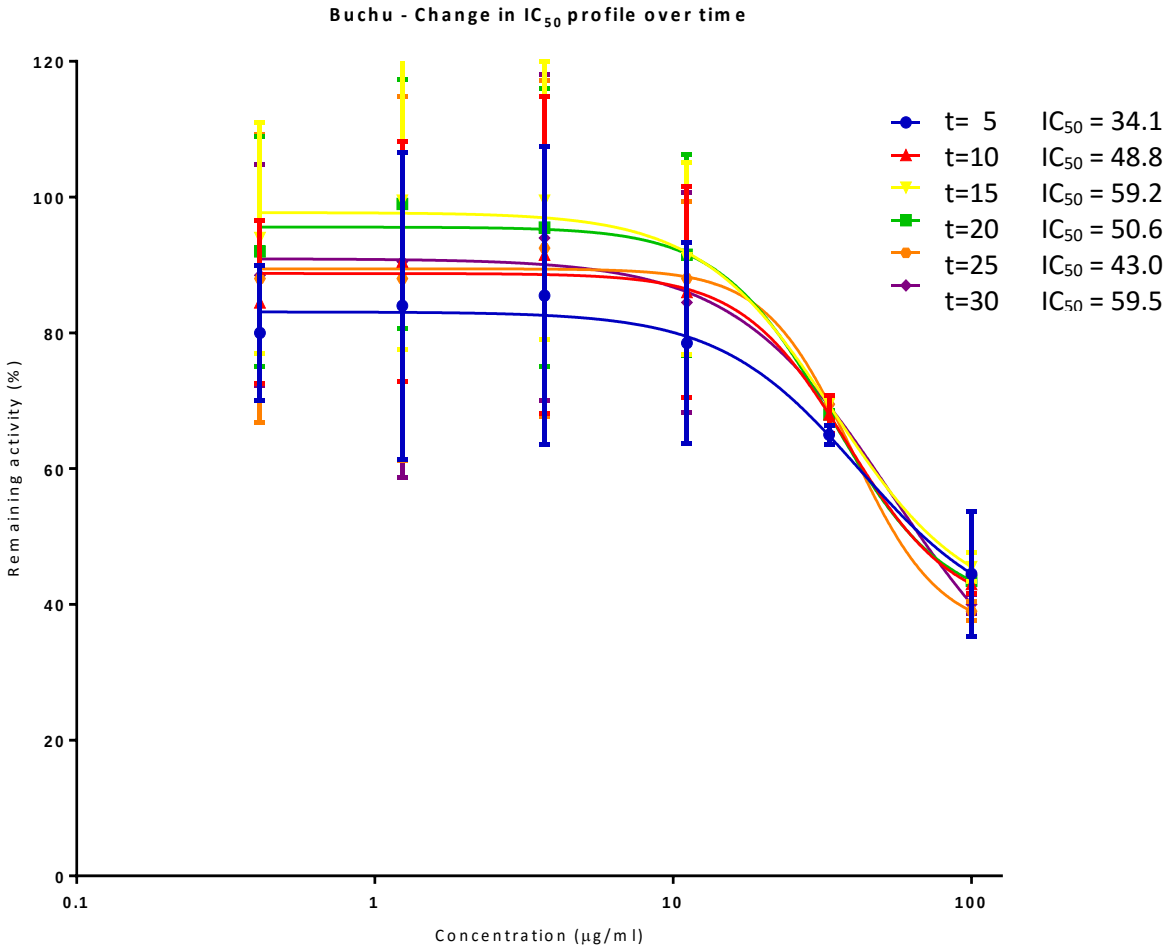


Figure 3.11C: The concentration-dependency of the inhibition of CYP3A4 by Buchu obtained at various incubation times. Data is expressed as Mean ± SD, n = 2.

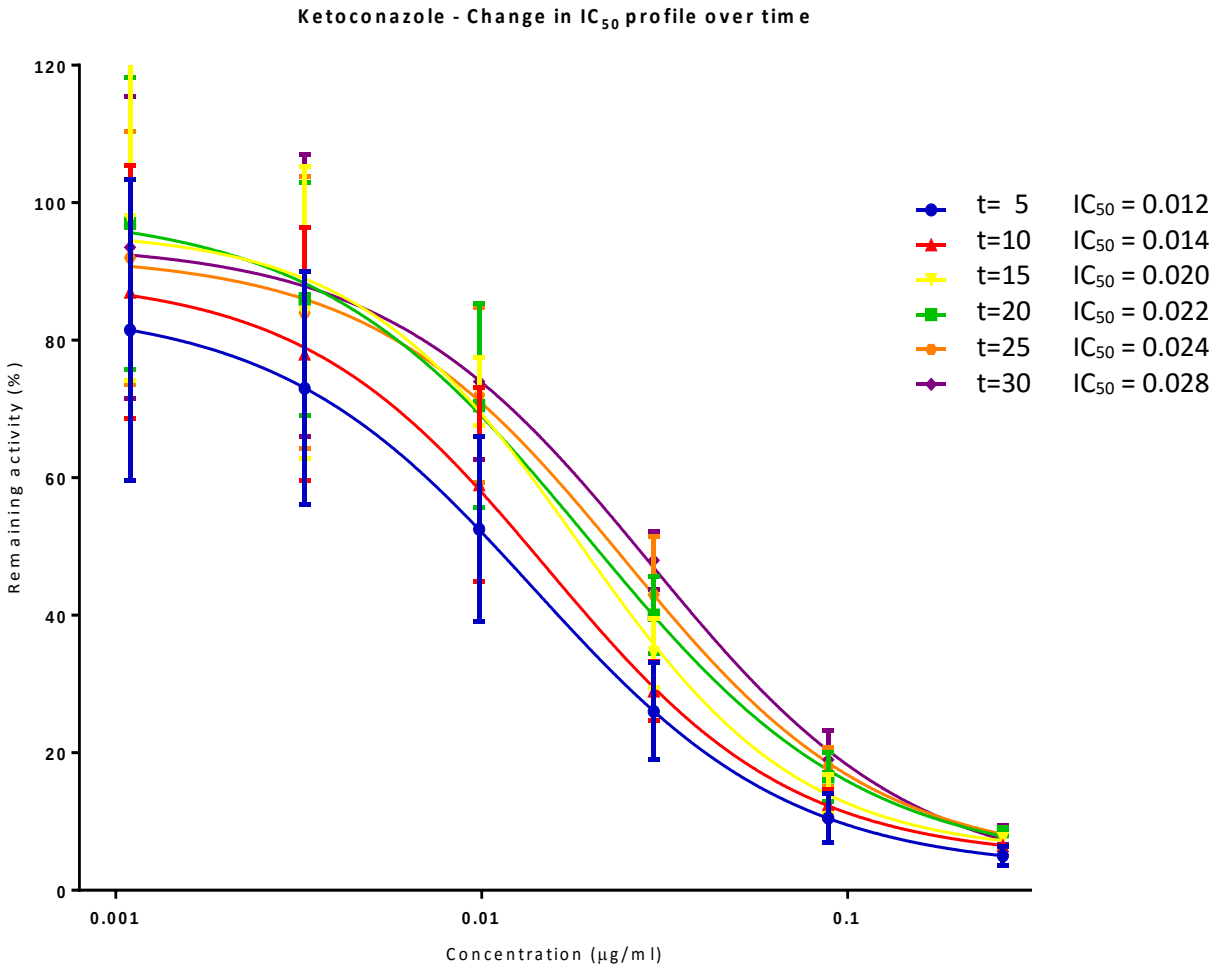


Figure 3.11D: The concentration-dependency of the inhibition of CYP3A4 by the negative control, ketoconazole obtained at various incubation times. Data is expressed as Mean ± SD, n = 2.

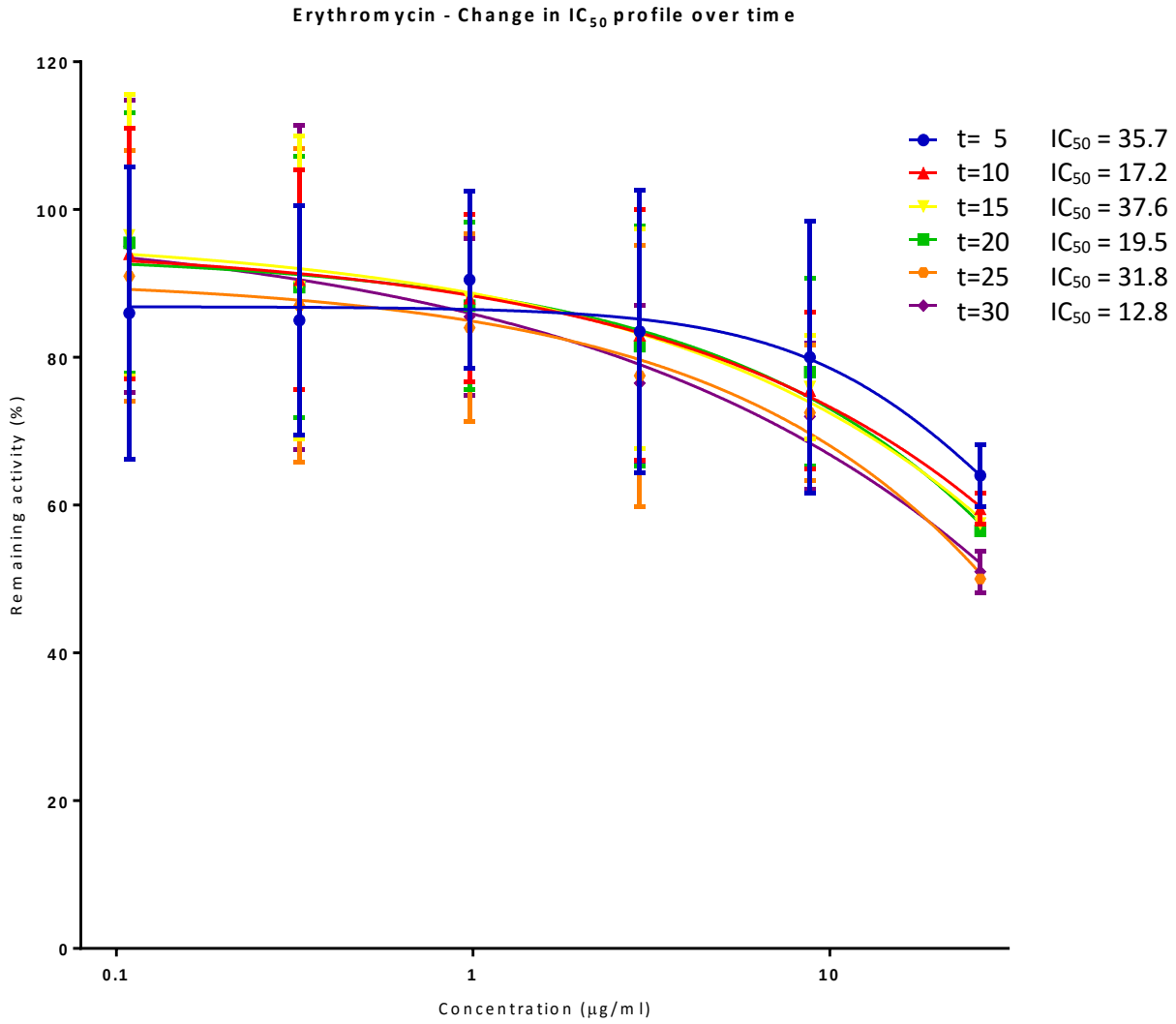


Figure 3.11E: The concentration-dependency of the inhibition of CYP3A4 by the positive control, erythromycin obtained at various incubation times. Data is expressed as Mean ± SD, n = 2

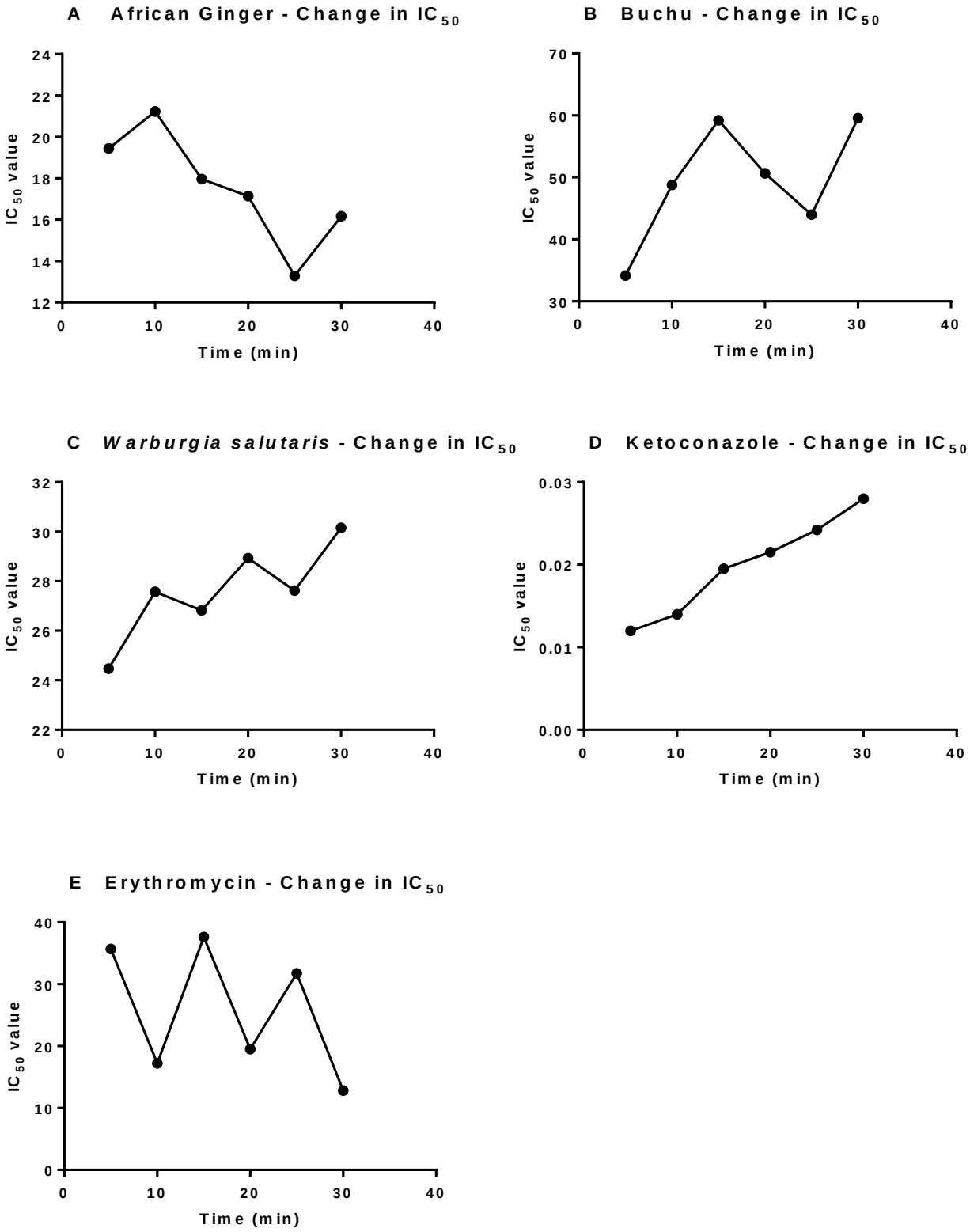


Figure 3.13: IC50 values obtained at various incubation times.

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

4.1 Phytochemical constituents

Medicinal herbs are usually prescribed as decoctions prepared by heating or boiling in water, such as teas or extracts, or as whole herbs, either fresh, in dried form, or as fresh juice (Watt & Breyer-Brandwijk, 1962; van Wyk & Gericke, 2000). Some medicinal herbs are also administered in form of tablets or capsules containing dried herbs. Depending on the mode of administration, different constituents contribute to the therapeutic and other effects of the herbal product. For the present study an extraction method using 60% methanol/ water seemed appropriate to investigate the potential effects of the herbs on drug metabolism, since this method extracts both water-soluble and water insoluble phytochemicals. In addition, medicinal herbs contain volatile phytochemicals when given as whole herbs. Since these volatile components evaporate during the extraction and drying process, they were unlikely to have been included in the *in vitro* inhibition assays and are therefore unlikely to have caused the effects observed in this study. Therefore, the extracts used in the assays do not reflect the full potential activity of the investigated herbs. Volatile compounds do, however, possess potential inhibitory activity, and it is therefore useful to identify these components.

Various phytochemicals contained in the methanolic extract may be responsible for the observed inhibition of drug metabolism. These phytochemicals can be classified as alkaloids, carotenoids, anthocyanins, coumarins, flavonoids, terpenes, sterols, phenols, tannins, saponins, and glycosides (Raaman, 2006).

Phytochemicals are known to modulate CYP enzyme activity. Some flavonoids are potent inhibitors of CYP1A1, CYP1A2, CYP1B1, CYP3A4, CYP3A6 and CYP19, while others are activators of CYP3A4 and CYP1A2 activity (Boek-Dohalska *et al.,* 2001; Tsyrlov *et al.,* 1994; Ueng *et al.,* 1997). Various studies have shown that the differences in the inhibitory activity of flavonoids are due to their chemical functional groups. The amount of hydroxyl groups is generally related to the degree of inhibition of drug metabolism. The greater the amount of hydroxyl groups, the stronger the inhibitory activity. In contrast, flavonoids lacking hydroxyl groups stimulate enzyme activity (Boek-Dohalska *et al*., 2001; Ueng *et al*., 1997; Lee *et al.*, 1994).

There are functional groups that are responsible for mechanism-based inhibition (MBI) of CYP450s. This may be because metabolism of these compounds yield highly reactive oxygenated intermediates (Riley & Weaver, 2007). The chemical motifs implicated in MBI include alkenes (Wen *et al.,* 2001), alkynes (Lin *et al.,* 2002; Guengerich, 1990), furans (Koenigs & Trager, 1998), thiophenes (Koenigs *et*

al., 1999; Jean *et al.,* 1996; Ha-Duong *et al.,* 2001), alkylamines (Yamazaki & Shimada, 1998), dihydropyridines (Fontana *et al.,* 2005), and benzo-1,3-dioxole (Heydari *et al.,* 2004; Delaforge *et al.,* 1999).

The tentative identification of compounds by GC-MS analysis is done by comparing individual masses, the full mass spectrum, and the relative retention indices of the unknown compounds to those in a library of known compounds. Definitive identification then relies on isolation of each compound and structural determination, for example by nuclear magnetic resonance (NMR). The results obtained by GC-MS in this study support previous studies done on Buchu, African ginger, and *Warburgia salutaris* (Fluck *et al.,* 1961; Viljoen *et al.,* 2006; Holzapfel *et al.,* 2002; Igoli & Obanu, 2011; Viljoen *et al.,* 2001; Mohanlall & Odhav, 2009).

The GC-MS analysis of the respective volatile components showed that each product contained alkenes, as wells as other compounds that may cause inhibition of CYP450s. *Warburgia salutaris* and Buchu contain pyridines, and *Warburgia salutaris* and African ginger contain furans (Mohanlall & Odhav, 2009; Holzapfel *et al.,* 2001). Although none of these compounds would cause the inhibitory effect *in vitro* (due to loss of the volatile component mentioned previously), they are present in some commercial formulations and therefore may possess an inhibitory effect *in vivo.*

A detailed phytochemical analysis of the extract, such as LC-MS fingerprinting, was not performed in this study, and there is little published information about the phytochemical composition of the selected medicinal plants. In addition to the known mono- and sesquiterpenes, Buchu contains flavonoids, thiols (Moolla, 2005; Scott & Springfield, 2004), and phenols (Fluck, 1961), African ginger contains phenols, proanthocyanidin, flavonoids, and a small quantity of thiols (Nethengwe *et al.,* 2012), and *Warburgia salutaris* contains a high content of phenols and flavonoids (Steenkamp *et al.,* 2012). As discussed above, each of these constituents have an inhibitory potential on CYP450.

4.2 Inhibition

The initial two-point screening of the herbal products was a preliminary screening assay conducted to determine the potential inhibitory effects of each extract. The method serves to identify the herbal products that show inhibitory activity and eliminate the need for further analysis of the products that do no exhibit inhibitory activity. All three herbal products displayed an inhibitory effect on both CYP2C19 and CYP3A4 (Figures 3.8 and 3.9). *Warburgia salutaris* was a strong inhibitor of both isoforms, while African ginger displayed a more pronounced inhibitory effect on CYP3A4 than on

CYP2C19. As CYP3A4 metabolizes approximately 50% (Guengerich, 1999) and CYP2C19 approximately 15% (Faber *et al.,* 2005) of known drugs, inhibition of these enzymes may be clinically important.

The inhibition profiles obtained from the concentration - response curves give a better indication of the inhibitory potential of the herbal extract. The IC_{50} values for the effect of *Warburgia salutaris* on CYP2C19 and CYP3A4 are 5.88 µg/ml and 5.64 µg/ml, respectively, indicating very strong inhibition. African ginger displayed potent inhibition of CYP3A4 with an IC_{50} value of 1.09 µg/ml and mild inhibition of CYP2C19 with an IC_{50} value of 32.28 µg/ml. Buchu showed weak inhibition of CYP2C19 and very weak inhibition of CYP3A4 with IC_{50} values of 53.52 µg/ml and >100 µg/ml, respectively. It should be noted that this classification of inhibition strength is relative based on the *in vitro* results obtained; a low IC_{50} value indicates that a low concentration of herbal extract is needed to cause inhibition, whereas a high IC_{50} value indicates that a high concentration of herbal extract is needed to cause inhibition. The likelihood of inhibition observed *in vivo* would dependent on the amount of extract reaching the gastrointestinal mucosa and / or the liver which are the major sites of drug metabolism via CYP enzymes (Meyer, 1996).

4.3. TDI normalized ratio and $NADP^+$ dependent inhibition

Time -, concentration -, and $NADP^+$ - dependent inhibition of an enzyme are characteristics of mechanism - based inhibitors (Zhou *et al.,* 2005). These inhibitors are metabolized to form reactive metabolites that can inactivation the enzyme at which point *de novo* synthesis is required to restore enzyme activity (Halpert, 1995). As the majority of herbal products taken as supplements are used on a daily basis, the observed inhibition may increase over time. The herbal products were therefore screened to assess the time - and $NADP^+$ - dependent inhibitory effects on CYP3A4 and CYP1A2 using the normalized ratio.

Warburgia salutaris displays $NADP^+$ - and concentration -dependent inhibition of CYP3A4 as well as clear TDI at 10 µg/ml, but not at 100 µg/ml. Therefore, the inhibition of the herb increases with time at low concentrations, but not at high concentrations. A possible reason for this observed inhibition profile is that at higher concentrations, a greater concentration of reactive metabolites is formed. These metabolites may result in the uncoupling of the active constituent from the binding site, reducing the time-dependent effect of the active constituent (Blobaum *et al.,* 2004). At lower concentrations, there are less reactive metabolites that could cause this uncoupling/inhibition, which may explain the estimated TDI at low, but not at high concentrations.

African ginger caused inhibition of CYP3A4 that was time - and concentration - dependent, but not $NADP^+$ - dependent. Like *Warburgia salutaris,* the herb displayed clear TDI of CYP3A4 at 10 µg/ml, but not at 100 µg/ml, which may be attributed to the greater formation reactive metabolites formed. These metabolites may result in the uncoupling of the active constituent from the binding site. Therefore, higher inhibitory concentration may have less of a time - dependent effect.

Buchu displayed clear TDI of CYP3A4 that was both concentration - and $NADP^+$ - dependent at both high and low concentrations, indicating possible MBI. Buchu displayed an intermediate TDI on CYP1A2, which was not concentration - or $NADP^+$- dependent. Furthermore, *Warburgia salutaris* displayed time - and concentration - dependent inhibition of CYP1A2, and African ginger inhibited CYP1A2 in a time -, concentration -, and $NADP^+$ - dependent manner, possibly indicating MBI.

Time-dependent inhibitors have a prolonged interaction with the metabolism of drugs used in the treatment of chronic illnesses such as HIV/AIDS, diabetes, tuberculosis, asthma, and clotting disorders. Therefore, the TDI effects of each herbal product were further investigated in the kinetic time dependent inhibition assay.

4.4 Kinetic time - dependent inhibition

The time - and concentration - dependent inhibition of the herbal products were further explored in a kinetic time - dependent inhibition assay. In this assay, the effects of the herbal products are measured continuously rather at an endpoint after incubation, which gives a better indication of inhibitor activity over time. This method also distinguishes reversible and irreversible inhibitors based on the change in IC_{50} over time; irreversible inhibitors display a decrease in IC_{50} through the reaction (Naritomi *et al.,* 2004) and reversible inhibitors display a constant IC_{50} (Naritomi *et al.,* 2004) or an increase in IC_{50} through the reaction (Wu *et al.,* 2003).

The IC_{50} value of African ginger decreased throughout the reaction, indicating that it may be an irreversible inhibitor of CYP3A4. The IC_{50} value of Buchu increased in the first 15 minutes of incubation, indicating reversible inhibition. For the following 10 minutes, the IC_{50} value decreased, indicating possible irreversible inhibition, and finally increased again in the last stage of the procedure. Although it cannot be excluded that the observed changes in IC_{50} values were due to variability in assay performance, the pattern may be explained by the formation of reactive intermediates during the first 15 minutes of the reaction, leading to the apparent initial decrease in IC_{50} value. Thereafter, the reactive intermediates may have uncoupled from the enzyme, recovering the relative enzyme activity.

Warburgia salutaris displayed an increase in IC_{50} value during the reaction, indicating that the observed inhibition is most likely reversible (Naritomi *et al.,* 2004). Likewise, the IC_{50} value of ketoconazole increased during the reaction time, indicating that the inhibition is reversible. This is consistent with the known reversible inhibition of CYP3A4 by ketoconazole (Wu *et al.,* 2003).

The IC_{50} value of erythromycin increased and decreased throughout the 30-minute incubation period. However, the overall trend shows an eventual decrease in IC_{50} value, indicating irreversible inhibition, which is expected since erythromycin is known to be an irreversible inhibitor of CYP3A4. A possible explanation for the variable IC_{50} values is the fact that the inhibition activity of erythromycin is sensitive to changes in temperature. Fluorescent reading could not be conducted at 37 °C and consequently, the microplates containing the assay incubate had to be moved been the incubator and the microplate reader, which may have affected the results obtained.

The method of TDI inhibition in this study did not use different pre-incubation times as suggested by some authors (Burt *et al.,* 2010). Therefore it was not possible to estimate the kinetic parameters of inactivation, such as K_I and K_{inact}, the inhibition constant and the maximum rate of enzyme inactivation, respectively.

4.5 Herb-drug interactions

Currently, there is insufficient published information regarding the potential interactions of *Warburgia salutaris*, African ginger, and Buchu with clinically prescribed medications. As all three herbal extracts have shown inhibitory activity on at least two CYPs in our study, there is a high risk of HDI if used in combination with conventional medicines.

Warburgia salutaris is traditionally used in the treatment of stomach ulcers, rheumatism, abdominal pain, backache, inflammations, malaria, protozoal infections, candida, fungal infections, microbial infection, and blood disorders (Van Wyk & Gericke, 2000). Consumers might use *Warburgia salutaris* as a complementary medicine for the treatment of these health concerns in conjunction with their standard medication such as proton pump inhibitors, non-steroidal anti-inflammatory drugs (NSAIDS), analgesics, macrolide antibiotics, and schizonticides. As *Warburgia salutaris* inhibits CYP2C19 and CYP3A4, drugs taken concomitantly with this herb may not be metabolized effectively, resulting in increased plasma concentration of those drugs. This would result in HDI that may lead to potential toxicity, especially with repeated usage of the herb. This inhibition may also be observed for other drugs that are substrates of CYP2C19 and CYP3A4. These substrates may be affected by *Warburgia salutaris* are listed in tables 4.1 and 4.2, respectively.

Likewise, Buchu is traditionally used for its antispasmodic, antiseptic, diuretic, antipyretic, antitussive, and antiseptic properties. Other uses include the treatment of coughs, colds and flu, kidney and urinary tract infections, haematuria, prostatitis, stomach ailments, and rheumatism (Watt & Breyer-Brandwijk, 1962; Van Wyk & Gericke, 2000). Conventional medicines used to treat these health concerns include NSAIDs, analgesics, and antibiotics. If consumers use Buchu in conjunction with their current medicines used in the treatment of those specified illnesses, the inhibition caused by the herb may result in potential drug toxicity. Buchu may also be a MBI of CYP3A4, which will inactive enzyme with prolonged use. *De novo* synthesis will then be required to resume normal enzyme activity (Halpert, 1995). Consumers should therefore exercise caution when using this herb. Other drugs that may be inhibited by Buchu are listed in tables 4.1 and 4.2.

Traditionally, African ginger is used in the treatment of cold and flu, asthma, sinusitis, sore throats, dysmenorrhea, candida, pain, hysteria and malaria (Watt & Breyer- Brandwijk, 1962; Van Wyk & Gericke, 2000). Individuals using conventional medicine to treat those health problems may use drugs such as antibiotics, benzodiazepines, bronchodilators, NSAIDS, schizonticides, and analgesics. The use of African ginger as a complementary medicine for the treatment of these health problems may cause HDI, as African ginger strongly inhibits CYP2C19 and CYP3A4. In addition, African ginger showed a strong increase in inhibitory effect over time. Repeated administration of this herb may result in adverse effects. Drugs that are not used in the treatment of the specified health problems are also at risk for HDI. These drugs are listed in tables 4.1, 4.2, and 4.3.

Although an IC_{50} profile of CYP1A2 activity was not possible in this study, the TDI assay performed indicated that at a concentration of 100 µg/ml, *Warburgia salutaris* and African ginger displayed a TDI effect, and Buchu displayed an intermediate TDI effect. Therefore, drugs that are substrates for CYP1A2 may also be affected by these herbs (table 4.3).

Table 4.1: Examples of CYP2C19 substrates (adapted from Flockhart, 2007)

Drug	Drug class
Amitriptyline	Tricyclic antidepressant
Carisoprodol	Muscle relaxant
Chloramphenicol	Antibiotic
Citalopram	SSRI
Clomipramine	Tricyclic antidepressant
Cyclophosphamide	Alkylating agent
Diazepam	Benzodiazepine
Hexobarbital	Hypnotic
Imipramine	Tricyclic antidepressant
Indomethacin	NSAID
Lansoprazole	Proton pump inhibitor
Moclobemide	Antidepressant (Monoamine oxidase inhibitor)
Nelfinavir	Protease inhibitor
Nilutamide	Antiandrogen
Omeprazole	Proton pump inhibitor
Pantoprazole	Proton pump inhibitor
Phenobarbitone	Antiepileptic
Phenytoin	Antiepileptic
Primidone	Anticonvulsant
Progesterone	Steroid (contraceptives)
Proguanil	Schizonticide
Propranolol	β-blocker
Rabeprazole	Proton pump inhibitor
R-mephobarbital	Barbiturate
R-warfarin	Oral anticoagulant
S-mephenytoin	Antiepileptic
Teniposide	Chemotherapeutic

SSRI = selective serotonin reuptake inhibitor; NSAID = Non-steroidal anti-inflammatory drug

Table 4.2: Examples of CYP3A4 substrates (adapted from Flockhart, 2007)

Drug	Drug class
Clarithromycin	Macrolide antibiotic
Telithromycin	Macrolide antibiotic
Quinidine	Anti-arrhythmic
Alprazolam	Benzodiazepine
Diazepam	Benzodiazepine
Nidazolam	Benzodiazepine
Triazolam	Benzodiazepine
Cyclosporin	Calcineurin inhibitor
Tacrolimus	Calcineurin inhibitor
Indinavir	Protease inhibitor
Nelfinavir	Protease inhibitor
Ritonavir	Protease inhibitor
Saquinavir	Protease inhibitor
Cisapride	Gastroprokinetic
Astemizole	Antihistamine
Chlorpheniramine	Antihistamine
Terfenadine	Antihistamine
Amlodipine	Calcium channel blocker
Diltiazem	Calcium channel blocker
Felodipine	Calcium channel blocker
Lercanidipine	Calcium channel blocker
Nifedipine	Calcium channel blocker
Nisoldipine	Calcium channel blocker
Nitrendipine	Calcium channel blocker
Verapamil	Calcium channel blocker
Atorvastatin	HMG CoA Inhibitor
Cerivastatin	HMG CoA Inhibitor
Lovastatin	HMG CoA Inhibitor
Simvastatin	HMG CoA Inhibitor
Estradiol	Steroid (oral contraceptives)
Hydrocortisone	Glucocorticoid
Progesterone	Steroid (contraceptive)
Testosterone	Steroid

Table 4.2 continued…

Alfentanyl	Analgesic
Aprepitant	Antiemetic
Aripiprazole	Atypical antipsychotic
Buspirone	Anxiolytic
Caffeine	Stimulant
Cilostazol	Phosphodiesterase inhibitor
Cocaine	Stimulant
Codeine	Opioid analgesic
Dapsone	Schizonticide
Dexamethasone	Glucocorticoid
Dextromethorphan	Antitussive
Docetaxel	Taxane
Domperidone	Dopamine agonist
Eplerenone	Aldosterone antagonist
Fentanyl	Opioid analgesic
Finasteride	
Imatinib	Monoclonal antibody
Haloperidol	Antipsychotic
Irinotecan	Anticancer
levo-α-acetylmethadol	Used in opioid dependence
Lidocaine	Local anaesthetic
Methadone	Opioid analgesic
Nateglinide	Oral antidiabetic
Ondansetron	5HT3 antagonist
Pimozide	Antipsychotic
Propranolol	β-blocker
Quetiapine	Atypical antipsychotic
Quinine	Schizonticide
Risperidone	Atypical antipsychotic
Salmeterol	Bronchodilator
Sildenafil	Selective phosphodiesterase - 6 inhibitor
Sirolimus	Macrolide antibiotic
Tamoxifen	Anti-estrogen

Table 4.2 continued…

Taxol	Taxane
Trazodone	Antidepressant
Vincristine	Anticancer drug
Zaleplon	Hypnotic
Ziprasidone	Atypical antipsychotic
Zolpidem	Hypnotic

HMG CoA = 3-hydroxy-3-methylglutaryl coenzyme A

Table 4.3: Examples of CYP1A2 substrates (adapted from Flockhart, 2007)

Drug	Drug class
Acetaminophen	Analgesic
Amitriptyline	Tricyclic antidepressant
Caffeine	Stimulant
Clomipramine	Tricyclic antidepressant
Clozapine	Antipsychotic
Cyclobenzaprine	Tricyclic antidepressant
Estradiol	Steroid (oral contraceptives)
Fluvoxamine	SSRI
Haloperidol	Antipsychotic
Imipramine	Tricyclic antidepressant
Mexiletine	Antiarrhythmic
Naproxen	NSAID
Olanzapine	Antipsychotic drug
Ondansetron	Selective serotonin 5-HT3 receptor antagonist
Phenacetin	Analgesic (CYP1A2 probe substrate)
Propranolol	β - blocker
Riluzole	Non-ergoline dopamine agonist
Ropivacaine	Local anaesthetic
Tacrine	Centrally acting cholinesterase inhibitor
Theophylline	Bronchodilator
Tizanidine	Muscle relaxant
Verapamil	Calcium channel blocker
(R)warfarin	Oral anticoagulant
Zileuton	5-lipooxygenase inhibitor
Zolmitriptan	Selective agonists of 5-HT3 receptor

SSRI = selective serotonin reuptake inhibitor; NSAID = Non-steroidal anti-inflammatory drug; 5-HT3 = 5-hydroxytryptamine

4.6 Estimated concentration of extract in the gut

The putative GIT concentration of each of the investigated herbal products was estimated using the percentage yield and an estimated 250 ml available GIT fluid. The calculations are based on a single dose of each herbal product. However their labelling recommends that two to three Buchu capsules should be taken twice daily (Flora Force, Cape Town), one African ginger tablet may be taken three times a day, and one *Warburgia salutaris* tablet may be taken every eight hours for ten days, or in the case of severe infections, two tablets every eight hours for five days (Bioharmony, Durban). It is therefore difficult to estimate the concentration of herbal extract in the GIT. In addition, the putative GIT concentrations are based on the assumption that the whole soluble extract is available for interaction with intestinal enzymes and transporters. The amount of phytoconstituents present in the GIT depends on various factors such as intestinal fluid composition, GIT transit time, and disease state (Fasinu, 2013). Also, not all compounds, possibly including the active phytoconstituent, are able to pass through the intestinal membrane. The estimates are therefore only presented as a benchmark for a preliminary determination of a potential *in vivo* effect. Further clinical studies are required to determine the actual *in vivo* effects on drug metabolism.

Table 4.4: Calculation of estimated concentration of the herbal extract in the gastrointestinal fluid

Herbal products	**Yield (% $^{w}/_{w}$)**	**Usual human dose (single; mg)**	**Estimated extract per dose (mg)**	**Putative GIT concentration (µg/ml)**
Buchu	12.84%	370	47.508	190.03
African ginger	0.775%*	100	0.755	0.31
Warburgia salutaris	2.235%*	100	2.235	8.94

Estimated extract per dose = Yield (% $^{w}/_{w}$) x single dose recommended in human.

Putative GIT concentration = Estimated extract per dose/volume of GIT fluid (250 ml).

* The percentage yield of African ginger and *Warburgia salutaris* have been adjusted (yield/2) to reflect the yield from a single tablet (approximately 500 mg), rather than the 1 g used for the inhibition assays.

Based on the above, it is not likely that African ginger will cause any clinically relevant inhibition of CYP3A4 after a single dose, as the estimated putative GIT concentration is lower than the concentration needed to cause the observed inhibition. However, this herb displays TDI and may cause clinically relevant inhibition with repeated administration. It is possible that inhibition of CYP450 by *Warburgia salutaris* occurs under clinical conditions, as the putative GIT concentration is higher than the observed IC_{50} value. The IC_{50} values for CYP2C19 and CYP3A4 in the presence of Buchu were 53.52 µg/ml and > 100 µg/ml, respectively. Although a large concentration of this herb is needed to cause inhibition of the two CYPs, the concentration of Buchu present in the GIT may be sufficient to cause clinically relevant drug interactions.

4.7 Study Limitations

The GC-MS analysis performed in this study revealed the volatile component composition of each herbal extract. However, this assay method does not give an indication of the phytochemical composition of the methanolic extract used in the *in vitro* inhibition studies. Other techniques, such as LC-MS fingerprinting and bioassay - guided fractionation would be necessary to determine the class of compounds present in the extract and to isolate the compounds responsible for inhibition.

The contents of each Buchu capsule of does not correlate with the amount stated on the packaging. Each capsule is stated to contain 370 mg of dried leaf material, where only 360 mg, 350 mg, and 330 mg were weighed during experimental preparation. Although the contents of the capsule are easily removed to determine the actual amount of plant material, this is not possible for the tablet products.

In addition, the composition of phytochemicals extracted from these herbal products will vary between batches and manufacturers, depending on the location, season, and environmental conditions of the harvested plant. Consequently, a different batch or product may not cause the CYP interactions observed in this study.

This study reports the effects of the selected herbal products on CYP2C19, CYP3A4, and to some extent, CYP1A2. The effects of the selected herbal products on other isoforms remain unknown. Further testing on other CYP isoforms and transporters should be conducted to determine whether the herbal products have any additional effects on metabolism and pharmacokinetics of medicines.

4.8 Conclusion

This study has shown the effects of African ginger, *Warburgia salutaris,* and Buchu on CYP3A4 and CYP2C19 and to some extent, CYP1A2. All three herbal extracts displayed an inhibitory effect on the

investigated CYPs. For African ginger, the inhibitory effect increased with time, which may result in a prolonged and pronounced inhibition with repeated usage of the product. Use of these herbs may therefore have clinically relevant implications. In addition, various compounds in the volatile component may have an inhibitory effect *in vivo.* Further *in vivo* human studies are needed to determine the effects of short- and long - term administration of these herbs, in order to devise recommendations for healthcare professionals and consumers regarding the safety of herbs when used concomitantly with conventional medicines.

CHAPTER FIVE

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APPENDIX 1

Decision by Stellenbosch University Health Research Ethics Committee

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Ethics Letter

06-Oct-2014

Ethics Reference #: X14/07/014
Clinical Trial Reference #:
Title: Inhibitory Effect of Selected Herbal Supplements on CYP450 - Mediated Metabolism - An in vitro Approach.

Dear Miss Charlize Nieuwenhuizen,

Thank you for your application. The application is for laboratory research using commercially purchased microsomes therefore the Health Research Ethics Committee has considered this proposal to be exempt from ethical review.

This letter confirms that this project is now registered and you can proceed with the work.

If you have any queries or need further assistance, please contact the HREC Office 0219389657.

Sincerely,

REC Coordinator
Franklin Weber
Health Research Ethics Committee 1